INTRODUCTION

Dear Reader,

We are pleased to present you this CD-ROM with the compilation of the mini-papers of the poster contributions and of the extended abstracts of the oral papers presented at the 5th International Symposium on Fish and Shellfish Larviculture 'larvi 2009' organized at Ghent University, Belgium September 7-10, 2009. This CD-ROM is published as Special Publication N° 38 of the European Aquaculture Society.

A "content table" (with direct link to the respective articles) and a "search engine" (searching in the titles as well as in the text) should allow you to quickly retrieve the information you are interested in.

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Yours very sincerely,

Patrick Sorgeloos

larvi 2009 Conference Chairman

EDITORIAL

This CD-ROM contains the mini-papers of the poster contributions and the extended abstracts of the oral papers, presented at the occasion of larvi 2009, the fifth symposium on fish & shellfish larviculture, organized on September 7-10, 2009 at Ghent University, Belgium. In line with present evolutions within our information society, the scientific committee has chosen not to publish a classical book, but to present this information uniquely as a CD-ROM.

As in the previous larvi conferences, this CD-ROM primarily provides the participants with detailed information on the scientific contents of the meeting, especially of the poster displays. Additionally, and in a broader sense, it can be used, both by participants and others, as a publication reflecting – in a condensed form – the present state of fish and shellfish larviculture.

The papers, included on this CD-ROM, have been retained by the scientific committee in function of their relevance within the scope of the conference. Though not peer-reviewed, they have passed through a limited editing process in order to improve, where needed, compliance with the editors' scientific and technical guidelines and uniformity of formatting.

A lot of support was received from the members of the scientific committee:

Dominique Adriaens, Peter Bossier, Peter Britz, Ronaldo Cavalli, Luis Conceição, Hiroshi Fushimi, Mai Kangsen, Sachi Kaushik, Patrick Kestemont, Elin Kjørsvik, Giorgos Koumoundouros, Yngvar Olsen, Karin Pittman, Amos Tandler, Olav Vadstein and Wim Van Den Broeck

We would like to express our sincere thanks to them.

Finally we would like to acknowledge the secretarial staff of the Laboratory of Aquaculture & Artemia Reference Center, Ghent University for their continuous help: without Magda Vanhooren and Marc Verschraeghen the realization of this book would not have been possible.

Gent, August 6, 2009

The Editors

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EFFECT OF DIETARY PHOSPHATIDYLCHOLINE ON GROWTH, SURVIVAL AND DIGESTIVE ENZYME ACTIVITY OF CASPIAN BROWN TROUT (*SALMO TRUTTA CASPIUS*) ALEVIN

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Introduction

The beneficial effect of dietary phospholipids (PL) on optimal growth, prevention of skeletal deformities, stress resistance, and survival of larval and juvenile stages of many species of marine fish has been demonstrated (Tocher et al., 2008). This beneficial effect of PL may then be explained by numerous roles, including increase digestion and absorption of neutral lipid (Koven et al., 1993), or more possibly fish had limited ability to transport dietary lipids away from the intestine possibly through limitations in lipoprotein synthesis (Tocher et al., 2008). The difference in nutritional efficacy among PL – with phosphatidylcholine (PC) generally being the most active – may then be explained by the specific role of PC as the major constituent of polar lipids in membranes as well as specific function of PC for the synthesis and secretion of lipoproteins.

The Caspian brown trout is one of the nine subspecies of brown trout in the world (Quillet et al., 1992) considered a critically endangered anadromous fish by a biological conservation program in the southern part of the Caspian Sea (Kiabi et al., 1999).

The aim of the present study was to examine the effect of dietary PC on the incorporation of digested lipid to improve growth, survival, and some digestive enzyme activity of Caspian brown trout.

Materials and methods

A feeding experiment was conducted to using four practical semi-purified fishmeal-based diets. Purified soybean phosphatidylcholine (90% PC, AppliChem Co, Germany) was added at four levels (0, 20, 40, and 60g.kg^{-1}) by reducing soybean oil. Assayed composition of the diets was (% of dry matter diet): crude protein 57.5±0.7; Crude fat 17.1±0.2; Ash 8.6±0.2; Fiber 8.5±0.8; and moisture

8.4±0.3. The diets were fed to triplicate tanks (2 alevins.l^{-1}) for a period of 5 weeks. The alevins were reared in closed water systems with approximately 20% new water exchange daily. Dissolved oxygen was maintained above 6.2mg.l^{-1}. The concentration of ammonia-N and nitrite-N were 0.09 and 0.16mg.l^{-1} (respectively), and pH was maintained 7.6.

At 5 weeks (after 2d starvation) the alevins were collected for growth analysis and enzyme assay. Five alevins per replicate were dissected as described by Cahu and Zambonino (1994). On a glass maintained on ice (0°C), samples of whole digestive tract were homogenized in 35mg.ml^{-1} cold 10mM PMSF and 0.1M phosphate buffers (pH 7.5), followed by centrifugation (13 500G; 30min at 4°C). Lipase specific activity was assayed according to Mongklthanaruk and Dharmosthiti (2002) using *p*-NPP as substrate, Protease activity was measured with Casein as the substrate according to the method Anson (1938) slightly modified by Mongklthanaruk and Dharmosthiti (2002). Amylase activity was assayed using starch as a substrate (Bernfeld, 1955), and phospholipase A2 was measured with phosphatidylcholine as the substrate according to Price Ш (2007). Protein was determined using the Bradford (1976) procedure. Enzyme specific activities are expressed as specific activities (μM.μg $protein^{-1}$.min).

Result analyzed by one-way ANOVA and significant differences determined by Duncan test. All statistical analysis was performed using the software SPSS 15.0 for Windows.

Results and discussion

At the end of the experiment the survival of alevins was not significantly affected by the dietary PC levels ($P>0.05$), Final weight and specific growth rate were significantly higher in larvae fed the PC4 and PC6 diets than the PC0 and PC2 diets ($P<0.05$) (Table I). Specific activity of amylase and protease enzymes had not significant difference between treatments, although protease specific activity decreased significantly in all groups. Significant higher levels of Lipase specific activity were observed in larvae fed the PC incorporated diets. The increase in dietary PC up to 4% led to significant increase in Phospholipase A2 activity.

The increase in dietary PC up to 4% led to increase in alevin final weight. The inclusion of PC may increase growth by supplying preformed PC to the fish, thereby reducing energy normally expended in biosynthesis of PC. Growth rates were probably reduced at PC6 due to the reduced level ratio of n-3:n-6 fatty acids in these diets. In this study survival was not effected by PC, which is in agreement with a similar previous experiment (Hamza et al., 2008). In this study, we observed that lipolytic enzyme specific activity increased with the dietary supplementation of PC. Liddle (2000) concluded that the most potent stimu-

lants of cholecystokinin (CCK) secretion (that releases pancreatic digestive enzymes) are the partial digestion products of fat, including hydrolysis of triglycerides to fatty acids. Therefore PC may increase enzyme activity by lipid emulsification and digestion in the intestine of the fish.

Table I. Final results of growth parameters, survival, and enzyme activity in Caspian brown trout after 5-week experiment. Enzymes were assayed using whole digestive tract.

Variable	Initial	PC levels (% of diet)			
		PC0	PC2	PC4	PC6
Growth(g)	0.8 ± 0.2^{c}	2.87 ± 0.06^{b}	2.85 ± 0.10^{b}	3.18 ± 0.12^{a}	3.19 ± 0.09^{a}
Specific growth rate	1.67 ± 0.1^{b}	1.68 ± 0.2^{b}	1.78 ± 0.3^{a}	1.78 ± 0.2^{a}	
Condition factor	1.12 ± 0.06^{a}	1.14 ± 0.1^{a}	1.11 ± 0.09^{a}	1.18 ± 0.1^{a}	
Survival	99.3^{a}	99.55^{a}	100^{a}	98.66^{a}	
		Enzyme Specific Activity			
Phospholipase A2[1]	19.6 ± 2.80^{c}	22.3 ± 1.37^{bc}	26.5 ± 1.39^{b}	32.9 ± 0.91^{a}	32.3 ± 1.99^{a}
Amylase[2]	0.37 ± 0.005^{b}	0.89 ± 0.01^{a}	0.77 ± 0.004^{a}	0.78 ± 0.01^{a}	0.75 ± 0.04^{a}
Protease[2]	0.26 ± 0.008^{a}	0.15 ± 0.01^{b}	0.15 ± 0.01^{b}	0.14 ± 0.01^{b}	0.14 ± 0.01^{b}
Lipase[2]	0.58 ± 0.01^{c}	0.73 ± 0.05^{b}	1.34 ± 0.25^{a}	1.07 ± 0.04^{a}	1.17 ± 0.03^{a}

Values are presented as mean±SE of the mean (n=5). Treatments having the same letter(s) are not significantly different ($P<0.05$). [1]- $\mu M.\mu g\ protein^{-1}.min$, [2]- $nM.\mu g\ protein^{-1}.min$

Conclusions

The present study showed that the addition of phosphatidylcholine in diets of Caspian brown trout had a growth promoting effect. It appears that a diet containing 4-6% PC can support good growth and had a positive effect on lipolytic enzyme activity.

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HISTOCHEMICAL DEVELOPMENT OF DIGESTIVE ENZYMES IN MARBLE GOBY *(OXYELEOTRIS MARMORATUS)* LARVAE

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Introduction

Marble goby is a freshwater fish that is successfully spawned and reared under artificial conditions (Abol-Munafi et al., 2002), however high mortality during the larval stages and the requirement for use of live feeds (Liem, 2001) are major problems in mass production. The ability of fish to utilize ingested nutrients depends on the activities of digestive enzymes present in the digestive tract. A better knowledge on the physiology of digestion during early growth and development appears essential for the understanding of larval marble goby nutritional needs. Information on what the larvae are capable of ingesting and digesting during ontogeny is important for the development of such diets and reduce production costs.

Materials and methods

The larvae of marble goby *(Oxyeleotris marmoratus)* were obtained from natural spawning of broodstock cultured in a polyculture system. The fertilized eggs were incubated in optimum condition. Green water was used to maintain water quality. At two days after hatching (DAH), larvae were fed freshwater rotifers (5-10ind.ml^{-1}) twice daily for the first 10DAH (Liem, 2001). Histochemical methods used were as reported in Drury and Wallington (1973) and Chayen (1973).

Results and discussion

At hatching, the marble goby larvae had only an undifferentiated straight-tube gut with yolk (Figs. 1 and 2). During the metamorphosis stage, the liver and pancreas formed early (Fig. 3) which constituted the sources of digestive enzymes in the intestine of the larvae. According to Abol-Munafi (1991) in the

yolk-sac stage, glycogen is secreted by the liver after carbohydrate was absorbed from the yolk sac, while the zymogen granule develops into pancreatic enzyme when suitably activated, and is used in digestion processes.

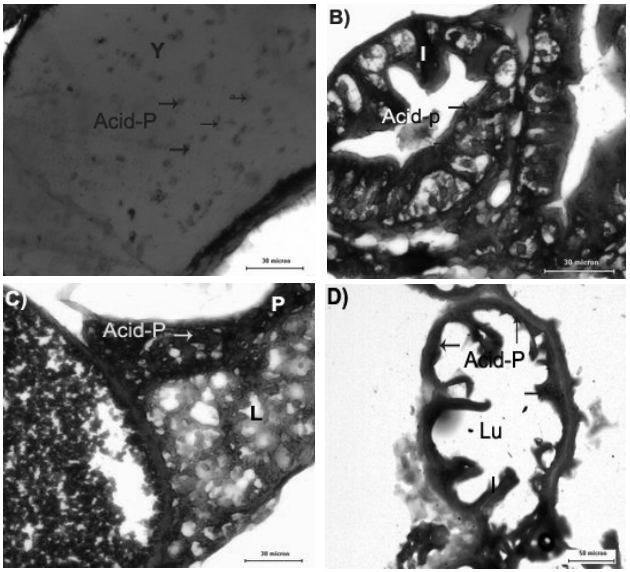

Fig. 1. Sagittal sections (8-12µm) of Marble goby larvae embedded in OTC. A) Yolk sac of 1-DAH larvae (×40; bar = 30µm). B) Intestine and rectum of 3-DAH larvae (×40; bar = 30µm). C) Pancreas and liver of 3-DAH larvae (×40; bar = 30µm). D) Intestine of 6-DAH larvae (×20; bar = 50µm). (Y: Yolk sac. I: Intestine. P: Pancreas. L: Liver. Lu: Lumen. Acid-P: Acid phosphatase).

In marble goby, both acid phosphatase and alkaline phosphatase were detected at the internal periphery of the yolk sac and the middle of the yolk mass at hatching (Figs. 1 and 2). The activity of these enzymes becomes more intense at the end of yolk absorption. In 3-DAH larvae Fig. 1B and1C), the acid phosphatase activity was found in the epithelium of the intestine, rectum, and pancreas which increased from 7DAH onwards.

In the present work we found that the lipase enzymes was detected at 3DAH in the intestinal and rectal epithelium, then in the liver and the pancreas of marble goby at 5DAH, and in the rectal epithelium at 7DAH (Fig. 3A and 3C). This enzyme activity increased in intensity until the end of the experiment, which suggests that an ontogenetic development of lipid digestion and metabolism may be important very early in life in marble goby larvae.

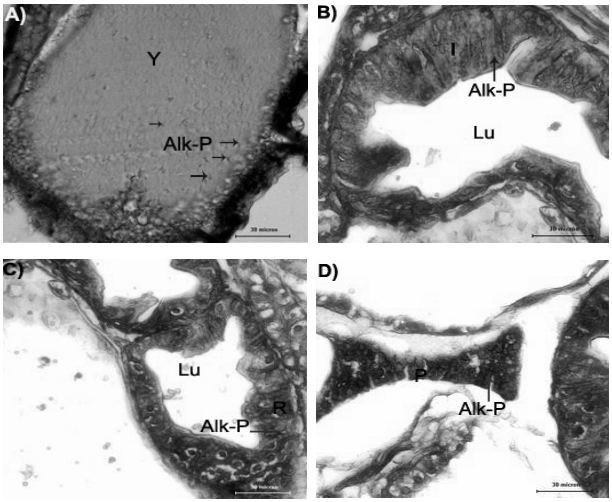

Fig. 2. Sagittal sections (8-12µm) of Marble goby larvae embedded in OTC. A) Yolk sac of 1-DAH larvae (×40; bar = 30µm). B) Intestine of 3-DAH larvae (×40; bar = 30µm). C) Rectum of 3-DAH larvae (×40; bar = 30µm). D) Pancreas of 6-DAH larvae (×40; bar = 30µm) (Y: Yolk sac. I: Intestine. P: Pancreas. R: Rectum. Alk-P: Alkaline phosphatase. Lu: Lumen).

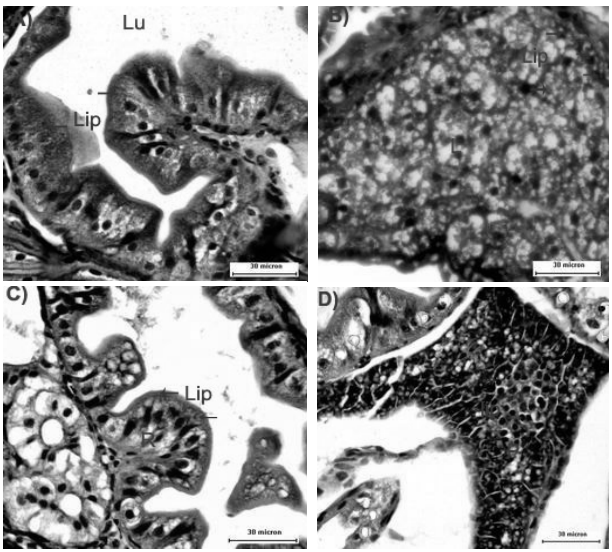

Fig. 3. Sagittal sections (8-12µm) of Marble goby larvae embedded in OTC, counter-stained in Haematoxylin. A) Intestine of 7-DAH larvae (×40; bar = 30µm). B) Liver of 8-DAH larvae (×40; bar = 30µm). C) Rectum of 7-DAH larvae (×40; bar = 30µm). D) Pancreas of 8-DAH larvae (×40; bar = 30µm) (I: Intestine. L: Liver. P: Pancreas. Lip: Lipase. Lu: Lumen).

Conclusion

The results of this study indicate that larvae of marble goby possess the ability for the digestion and absorption of exogenous nutrients at early larval period. This may be used as an indicator of larval development as well as a predictor of their future survival.

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COMBINATION OF LIVE FOOD AND COMMERCIAL FEED VERSUS LIVE FOOD IN EARLY FEEDING OF *HUSO HUSO* LARVAE

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Abstract

This paper presents the result of studies on the early feeding of *Huso huso* larvae and discusses about the possible methods of shifting from live food to formulated feed while maintaining the highest survival and growth rates. The research was conducted in six different feeding treatments in an open flow through system using underground fresh water throughout the experiments. The results proved that using a mixture of live food and commercial feed and gradual shifting to total commercial feed results in higher growth and survival compared to those fed only on live food during first few days followed by a sudden or gradual change to formulated feed.

Introduction

Economically sturgeons are among the most important fishes. Artificial fertilization and rearing of sturgeon larvae was first started by Russians in 1969, followed by considerable achievements in 1970s (Chebanov and Billard, 2001). Sturgeon farming in western countries began during the 1980s that has resulted in production of many tons of Caviar and fish meat during last decade (Williot et al., 2001). Larval rearing of sturgeon fish (Caspian Sea species) in Iran started by the Iranian Fishery Company in early 1970s mainly as a consequence of conservation efforts for threatened wild populations. But aquaculture of sturgeon in Iran began much later in year 2000 (Iranian Fishery Company, 2007).

Optimization of early feeding is very important to achieve better growth and higher survival of fish and crustacean larvae. *Artemia* nauplii have been used as a live starter food in larviculture of many fish and crustacean species since long (Sorgeloos et al., 2001). *Artemia* is considered as a complete food containing considerable quantities of protein, lipids (especially unsaturated fatty acids), minerals and vitamins. In this we report on optimal combination of *Artemia* nauplii and commercial feed in feeding *Huso huso* larvae.

Materials and methods

Huso huso yolk-sac larvae were obtained from Shahid Beheshti sturgeon hatchery and transported to Urmia in double-layered, 1000-l tanks equipped with oxygen gas pipe. After absorption of yolk sac, larvae were transferred to 40-liter polyethylene tanks containing 25 litres of UV-treated well water with flow rate of 1 $l.min^{-1}$. The well water was warmed up to 19-20°C by a central heating system and aerated continually by an air pump. The experiments were performed in a flow-through system. Each feed treatment included six replicates; the larvae were fed 30% body weight daily, with a feeding frequency of six times per day. The pH (7.30-7.50), temperature (19-20°C), and dissolved oxygen level (7-7.65$mg.l^{-1}$) of water in each tank was monitored twice a day.

Two feeding groups were tested. In the first group the fish larvae were fed on newly hatched *Artemia urmiana* nauplii for first 5 days followed with gradual replacement by commercial feed. The larvae in the second group were fed on different combinations of newly hatched *Artemia* nauplii and commercial feed from the onset of exogenous feeding (Table I). The experiment in both groups was continued for 20 days until all feeding treatments were totally converted to commercial feed. Survival was monitored every day and growth parameters were accessed at the end of the experiment.

Results and discussion

Results obtained from this study are briefly summarized in Tables I and II. Final dry weight was significantly higher in fish fed on 70% *Artemia* nauplii and 30% commercial food on the first day followed by 10% daily replacement with CF (treatment 5). SGR was also highest in treatment 5, but no statistical differences were observed among different feeding groups. FCR did not show any significant differences among groups. Highest survival was found in treatment 4 (larvae fed on 90% *Artemia* nauplii and 10% commercial food on the first day followed by 10% daily replacement with CF), significantly higher than all feeding groups except with that in treatment 5. It could be concluded from the results that feeding *H. huso* larvae with a combination of live food and commercial feed from the first day of exogenous feeding gives better results in terms of growth parameters and survival. It resulted in considerable reduction in consumable costs (including *Artemia* cysts), materials, personnel, and space needed for preparation of feed and feeding process. It is therefore advisable to the *Huso huso* sturgeon aquaculturists to combine the live food with commercial feed from the first day of feeding. However, it is necessary to perform similar experiments with other species of sturgeon fish.

Table I. Initial and final length, wet weight and dry weight of *Huso huso* larvae fed on different combinations of live food and commercial feed.

Treatments	Initial length (mm)	Initial wet wt. (mg)	Initial dry wt. (mg)	Final length (mm)	Final wet wt. (mg)	Final dry wt. (mg)
1*	25	59	10.3	65.9 ± 2^{a}	1566 ± 192.8^{a}	188.1 ± 33.6^{a}
2*	25	59	10.3	65.5 ± 2.5^{a}	1568.3 ± 109.3^{a}	194 ± 6.1^{a}
3*	25	59	10.3	68.3 ± 2.9^{a}	1645.6 ± 201.5^{a}	205.2 ± 25.1^{ab}
4*	25	59	10.3	68.9 ± 1.6^{a}	1702.3 ± 100.4^{a}	210.6 ± 11.7^{ab}
5*	25	59	10.3	69.4 ± 2.2^{a}	1814.5 ± 66.9^{a}	233.4 ± 13.3^{b}
6*	25	59	10.3	66.2 ± 1.4^{a}	1579.3 ± 115.8^{a}	192.5 ± 13.4^{a}

*(1) Fed on *Artemia* nauplii (N) for 5 days + 10% daily replacement of N with Commercial feed (CF) from day 6, (2) Fed on N for 5 days + 30% replacement of N with CF on day 6 and 10% daily increase in CF, (3) Fed on N for 5 days + 50% replacement of N with CF on day 6 and 10% daily increase in CF, (4) Fed on 90% N and 10% CF on first day + 10% daily replacement of N with CF, (5) Fed on 70% N and 30% CF on first day + 10% daily replacement of N with CF, (6) Fed on 50% N and 50% CF on first day + 10% daily replacement of N with CF.

Table II. Mean SGR, FCR, and survival of *Huso huso* larvae fed on different combinations of live food and commercial feed.

Treatments	SGR	FCR	Survival
1	16.37 ± 0.6^{a}	0.52 ± 0.07^{a}	53.7 ± 2.3^{a}
2	16.39 ± 0.3^{a}	0.52 ± 0.04^{a}	50.8 ± 2^{a}
3	16.62 ± 0.6^{a}	0.53 ± 0.07^{a}	47.7 ± 3^{a}
4	16.8 ± 0.3^{a}	0.55 ± 0.03^{a}	65.7 ± 6.8^{c}
5	17.13 ± 0.2^{a}	0.57 ± 0.02^{a}	59.7 ± 11^{bc}
6	16.62 ± 0.46^{a}	0.55 ± 0.05^{a}	52.7 ± 8.8^{ab}

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PROCESS MONITORING AND CONTROL IN THE NEXT GENERATION HATCHERY

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Process control techniques have been successfully employed in many industries, and can benefit marine fish hatcheries equally well. The automated hatchery of the future will have a control room from which all information about individual processes is available, and from which the entire hatchery is managed. This room need not be limited to a physical location, since networking offers wide possibilities for remote control. In this hatchery the operator's role is to oversee and direct the production process rather than directly performing all tasks. The control room relies on instrumentation and controllers covering the production line's processes in categories such as water treatment, live food cultivation and start feeding, many of which require specialized instrumentation and controllers.

In order to put the current status of hatcheries into perspective, we will give a classification of how control systems are typically employed in phases of increasing sophistication:

1. The manual phase, where process control is primarily based on manual (visual) observations and manual process intervention.
2. The instrumentation and mechanization phase, characterized by increased use of sensors and actuators, such as pumps, valves and motors, to facilitate common control tasks.
3. The open loop control phase, where preprogrammed sequential controls such as PLCs are incorporated to relieve the operator from routine operations.
4. The closed loop control phase, where automatic feedback control is utilized to control processes by use of computers, sensors and actuators. Automatic controllers more or less take the place of manual observation and control.
5. The advanced process control phase, where sophisticated engineering techniques such as optimal control algorithms and model based control are employed. The latter technique relies on mathematical process models in order to supplement measurements and predict those variables that are difficult to

measure or that cannot be measured often enough. In optimal control mathematical models and optimization criteria are used to compute optimal control inputs.

Current hatcheries can usually be placed at Level 2 or 3. The most advanced hatcheries employ instrumentation systems to monitor environmental parameters, and have automatic feeding systems for dry and live food. What is missing in most places is the closing of the loop, i.e. the utilization of sensor data in order to direct control inputs. The most significant benefit of closing the loop is that the controller can actively suppress deviations from the desired system state, and in this way compensate for errors. The end result is increased stability and predictability of the operation. Furthermore, one can extract information about the system state based on the way it reacts to control inputs.

There are many examples of hatchery processes that can be automated and optimized, for instance in live food production where counting, feeding, washing and distribution should be addressed. More basic processes must also be considered, such as the control of the water exchange rate of fish tanks and live food cultures – with water flow coordinated with feeding, the loss rate of food can potentially be reduced, especially in live feed cultures. Another interesting example is the feeding of marine fish larvae in the phase when live food is used. Measurements of live food concentrations can be made on-line, and this information used to direct feeding rates in order to achieve appetite dependent feeding. Such Level 4 feedback control has the benefit of providing sufficient food at all times without the risk of overfeeding. By utilizing mathematical models of rotifer and larval dynamics, the relation between feeding rate and measured food concentrations can be used to roughly estimate food ingestion rates and even larval mortality rates.

Most of the above examples have been demonstrated in the laboratory, and some are based on mathematical models that have been developed describing the dynamics of live food cultures, larval growth and of the first feeding scenario. Focusing on automation at Level 5, these models offer opportunities in longer term production coordination, where one can predict the demands each process puts on other processes – for instance, the first feeding process puts a predictable demand on future live food production, and this information can be used to optimize the control of live food cultures in order to meet demand.

It is a general trend in other industries that production is modularized with actors specializing on either production of a subset of the parts, or on the assembly of parts into end user products. For the hatchery business, the production of live food might in the future be handled by separate actors specializing on efficient large-scale production. The same is possible for egg production, making the future hatchery much more specialized than what is the case today.

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EFFECTS OF AMMONIA EXPOSURE IN SENEGALESE SOLE (*SOLEA SENEGALENSIS*) LARVAE

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Introduction

In larval rearing, increased ammonia concentrations are sometimes found in the water due to unconsumed feed and larval excretion, in combination with low water renewal which is often required for the rearing of these small and fragile animals. Ammonia exposure may be detrimental to larvae, affecting survival, feeding, and gill structure (Lease et al., 2003; Rodrigues et al., 2007). Moreover, in juvenile sole, ammonia exposure affects growth, amino acid metabolism, and stress response (Pinto et al., 2007). This work analysed the effects of ammonia exposure in survival, growth, protein retention, and feeding of Senegalese sole (*Solea senegalensis*) larvae.

Materials and methods

Newly settled Senegalese sole larvae (21 days after hatching) were exposed to different ammonia concentrations in water (0, 2.25, or 4.5mg.l^{-1} total ammonia nitrogen - TAN) during 8 days, using triplicate tanks (0.25m^2, 35 l, 3000 larvae.m^{-2}) for each concentration. TAN concentrations in water were maintained by pumping continuously ammonium chloride solutions to the inlet water flow, using a peristaltic pump. Besides this, larvae were reared using standard procedures (water temperature 20±1ºC, salinity 37‰, oxygen levels >90% saturation; nitrite levels <0.01ppm). Larvae were fed in excess with frozen *Artemia* metanauplii previously enriched with commercial products. At the end of the experiment, survival was assessed and larvae were sampled for individual dry weight determinations and cortisol analysis. The latter was done on whole larval homogenates after extraction with diethyl ether, followed by the procedures described by Rotlant et al. (2006).

At the end of the experiment, larvae from each treatment were transported from the rearing facilities to a hot lab, where they were kept overnight in small tanks with same TAN concentrations in water as before. Larvae were sampled for cortisol analysis immediately after transport and just before the feeding experiments (16h after transport). Larvae were fed during 30min with ^{14}C-enriched *Artemia* metanauplii (Morais et al., 2004) and then individually transferred to metabolic chambers connected to metabolic traps for $^{14}CO_2$-entrapment. After 8h of incubation, evacuated, catabolised, and body fractions were collected and counted (Rønnestad et al., 2001). Feed consumption was based on the total DPM in each larva and the DPM in each *Artemia*. Feeding ratio was considered as the percentage of larvae that ate radiolabelled preys.

Results and discussion

During the experiment, mortality was less than 1% in all treatments. Moreover, relative growth rate was also similar among treatments (RGR = 13.8±1.1, 13.7±2.0, 13.6±1.6% day^{-1}, respectively for 0, 2.25, and 4.5mg l^{-1} TAN) and larval dry weight tripled in all treatments (initial DW = 2.2±0.5mg; final mean DW = 6.1±0.7mg). Therefore, survival and growth of sole larvae were unaffected by 8 days of ammonia exposure, suggesting that larvae were able to cope with these TAN concentrations in the water. Senegalese sole larvae are less sensitive to ammonia exposure than for instance gilthead seabream, probably due to the greater amount of mucus on their skin (Parra and Yúfera, 1999).

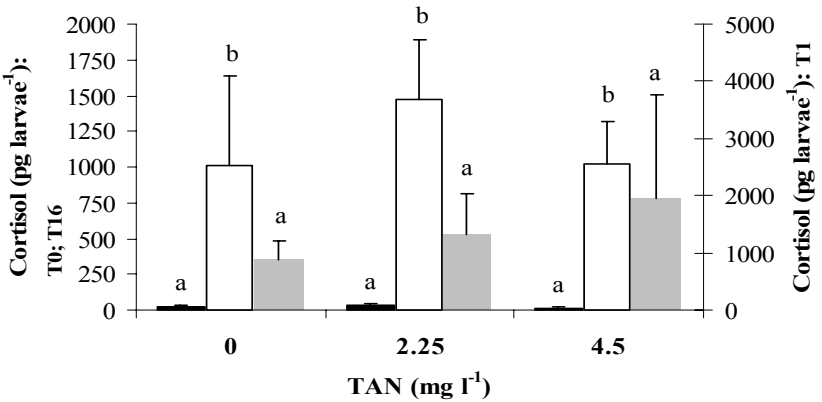

Fig. 1. Cortisol concentrations in *S. senegalensis* larvae from the different ammonia exposure treatments at the end of the rearing experiment (T0: black), immediately after transport (T1: white), and before the feed consumption experiment (T16: shaded). Means+standard deviation (n=15). Different letters represent significant differences among the same treatment (two-way ANOVA, $p<0.05$). Note the different y-axis scales.

Results show that after 8 days of ammonia exposure, cortisol levels were similar in larvae from all treatments (Fig. 1 – T0). Therefore, exposure to these TAN concentrations did not induce a stress response in sole larvae or these were able to cope with the stressful conditions within the course of the rearing experiment. However, transport significantly increased cortisol levels in all treatments (Fig. 1 – T1) and may be considered as a stress challenge to these larvae. Larvae from all treatments were able to recover from this stress situation before the beginning of the feeding experiments (Fig. 1 – T16), attaining cortisol levels not significantly different from the ones previous to the transport.

Table I. Descriptive statistics concerning feed consumption (*Artemia* per larvae) in *S. senegalensis* larvae from the different ammonia exposure treatments.

Treatments	m - M	Mean ± SD	Feeding Ratio (%)
0mg.l^{-1} TAN	0 – 72	9 ± 18	55
2.25mg.l^{-1} TAN	0 – 69	14 ± 21	60
4.5mg.l^{-1} TAN	0 – 69	25 ± 24	80

Minimum (m) and maximum (M) values of preys in the larval gut; SD = standard deviation; n=20.

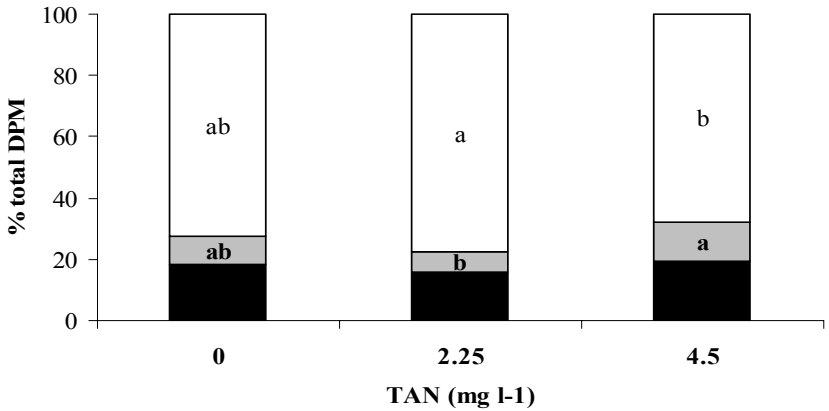

Fig. 2. Radiolabel evacuated (black), catabolised (shaded), or retained in the body (white) of *S. senegalensis* larvae from the different ammonia exposure treatments after feeding ^{14}C-labelled *Artemia*. Different letters within compartments indicate significant differences among treatments (one-way ANOVA, $p<0.05$, n=20).

Sole larvae reacted differently to the stress challenge caused by transport. As described in Table I, although minimum and maximum number of *Artemia* ingested by the larvae was similar among treatments, different tendencies were found. Mean values for *Artemia* consumption were inversely proportional to TAN concentrations in water. Feeding ratio was also higher in larvae exposed to 4.5mg.l^{-1} TAN than in the other treatments. Moreover, larvae exposed to 2.25mg.l^{-1} TAN catabolised a lower fraction of the ingested *Artemia* and re-

tained a higher fraction than larvae exposed to 4.5mg.l^{-1} TAN (Fig. 2). Hence, larvae previously exposed to ammonia seem to better react to a stress challenge, as feed consumption seems to be less affected.

Therefore, larvae exposed to different TAN concentrations in water may cope with this disturbance differently. While larvae exposed to 2.25mg.l^{-1} TAN retain a higher percentage of the ingested *Artemia* in their bodies, larvae exposed to 4.5mg.l^{-1} ingested more *Artemia*. Both strategies can contribute to maintain a similar growth in these larvae than in larvae not exposed to TAN in water.

Acknowledgements

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IMPORTANCE OF RELATIVE LEVELS OF DIETARY ARA AND EPA FOR CULTURE PERFORMANCE OF GILTHEAD SEA BREAM (*SPARUS AURATA*) LARVAE

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Introduction

The importance and effect of dietary ARA is being studied as a function of its relative value in relation to dietary EPA. The objective of the present study was to determine the effect of different ARA dietary contents at several dietary EPA levels to better define the importance of this fatty acid as a function of EPA.

Materials and methods

Sparus aurata larvae were obtained from natural spawnings. Larvae were previously fed enriched rotifers (DHA Protein Selco, INVE, Dendermonde, Belgium) until they reached 18 days old, Then they were randomly distributed into 24 tanks at a density of 1400 larvae.tank^{-1} and were fed one of the eight experimental diets (Table I) tested in triplicates for 14 days, at a water temperature of 19.2 to 21ºC, and light photoperiod was kept at 12h light:12h dark.

Table I. Lipid sources (% total ingredients) of the experimental diets

Diet (EPA/ARA)	EPA45[1]	ARA44[1]	DHA45[1]	Oleic acid[2]
0.3/0.1	0	0	0	14.9
2/0.6	0	0.9	9.2	4.8
2/1.2	0	2	9.2	3.7
3/0.3	2.2	0	8.7	4.0
3/1.2	1.9	2	8.6	2.4
4/0.3	4.5	0	8	2.4
4/0.6	4.5	0.7	8	1.7
4/1.2	4.3	2	7.9	0.7

[1] Polaris, Pleuven, France; [2] Merck, Darmstadt, Germany.

Final survival was calculated by individually counting all the live larvae at the beginning and at the end of the experiment. Growth was determined by measur-

ing dry body weight (105ºC, 24h) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish.tank^{-1}. Moisture, crude protein, and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982) separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo et al. (1990), and identified by comparison to previously characterized standards and GLC-MS.

All data were treated using one-way ANOVA and means were compared by Duncan's test ($P<0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Results and discussion

In the present study, when the dietary DHA was administrated with a sufficient amount (5%), dietary increase of ARA when EPA levels were high (3 and 4%) markedly increased survival (Fig. 1), being significantly different when EPA was kept at 4%. On the contrary, elevation of dietary ARA from 0.6 to 1.2 when EPA contents were low (2%) did not improve survival, denoting the importance not only of absolute ARA levels, but also of the relative ratios EPA/ARA.

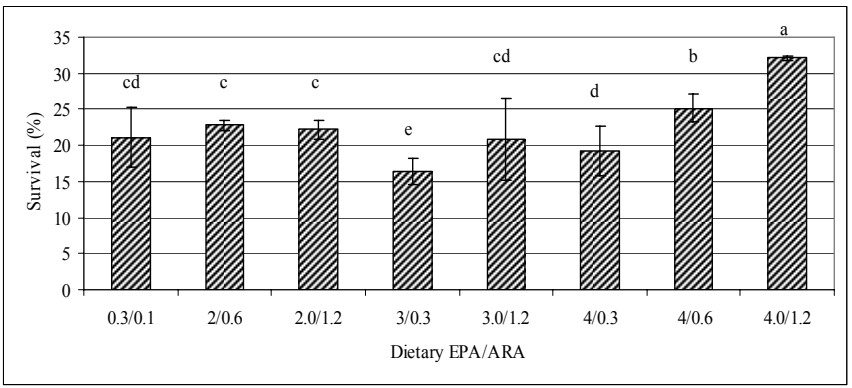

Fig. 1. The survival rate of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences ($P<0.05$) among larvae fed different diets).

At the end of the experiment, larvae fed the diet with the lowest EPA/ARA contents (Diet 0.3/0.1) showed the lowest whole body weight and standard length. Highest whole body weight was found in fish fed the highest EPA and ARA contents (Diet 4/1.2) even after only seven days of feeding. Increase of both EPA and ARA significantly improved growth and highest body weights were found in larvae fed diet 4/1.2. In agreement with Bessonart et al. (1999) who

showed that increased of ARA up to 1.8% improved growth and survival when both DHA and EPA were provided in the diet.

Fatty acid composition of total lipids from whole larval body lipids reflected the dietary fatty acid profiles, where increased dietary ARA levels were followed by increased ARA and reduced monounsaturated and n-9 fatty acids and 18:1n-9. Increased ARA in larval lipids was also followed by a reduction in EPA contents when dietary EPA was kept at higher levels (3 and 4%). Dietary ARA was more efficiently incorporated into larval tissues, Moreover, increased incorporation of ARA in larval lipids slightly reduced EPA incorporation when the later was high in the diet.

Conclusions

The results of this study have shown that when DHA is not a limiting factor in larval diets, only the increase in both ARA and EPA enhanced growth and survival, suggesting an optimum dietary ratio close to 4 for gilthead sea bream.

Acknowledgements

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USE OF CALRETININ (CR) AND PARVABUMIN (PV) AS MAUTHNER CELL MARKERS IN SEA BASS LARVAE (*DICENTRARCHUS LABRAX*)

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Introduction

Studies on Mauthner neurons have provided fundamental information on neural biochemistry, development, synaptic morphology and physiology, and control of behaviour as fast-escape motor response after the reception of unexpected vibrational and/or visual stimuli (Eaton and Bombardieri, 1978) that can be generalized to many central neurons throughout vertebrates (Faber and Korn, 1978a; Nissanov and Eaton, 1989; Korn et al., 1990). Different studies using immunohistochemistry have described the calretinin (CR) and parvalbumin (PV) positive Mauthner cells (Crespo et al., 1998), revealing that their presence indicates these neurons need complex calcium-buffering system. Description of Mauthner cells in fish larvae constitutes a powerful tool to study fish larval behaviour. In the present study, we analyze the presence of these antibodies in the Mauthner cells of European Sea Bass larvae (*Dicentrarchus labrax*).

Material and methods

European Sea Bass larvae were obtained from natural spawning from France (Ecloserie Marine de Gravelines, Nord-Pas-de-Calais). The experiment was carried out in the Grupo de Investigación en Acuicultura facilities (Las Palmas de Gran Canaria, Canary Islands, Spain). Larvae were distributed into 2 tanks ($2m^3$) and fed with rotifers enriched with EFA (Selco, DHA Protein Selco, INVE, Dendermonde, Belgium) until they reached 15dah. At 7dah, larvae were fed with *Artemia* enriched with EFA followed by a commercial microdiet until they reached 50dah. All tanks were supplied with filtered sea water ($34g.l^{-1}$ salinity). Water temperature and dissolved oxygen during the experimental period ranged between 16.5-21ºC and 5.04-8.32ppm, respectively.

Sixty larvae were collected daily and fixed in 10% buffered formalin, dehydrated through graded alcohols, xylene, and finally embedded in paraffin wax.

Paraffin-embedded complete larvae sections were serially cut on Leica microtome at 3µm, stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970), Nissl (Raimundo García del Moral, 1993), and histologically evaluated using immunohistochemistry techniques.

For immunohistochemistry, some slides for each paraffin sections were collected on Poly-L coated slides. Sea bass larvae were processed for the demonstration of calretinin (CR) and parvalbumin (PV) immunoreactivity. The slides were dewaxed in xylene, rehydrated through graded alcohols, and incubated with 3% hydrogen peroxidase in methanol for 30min on a moving platform to block endogenous peroxidase activity. Enzymatic treatment, protease, was applied according to the used primary antibody. The enzymatic treatment applied in both primary antibodies (Swant, Bellinzona, Switzerland; CR, 1:700; PV, 1:700) was pronase 0.1% in PBS for 3min at room temperature. After that, slices were covered with 10% goat serum (for both polyclonal antibodies, PV and CR) in PBS for 30min before incubation with the primary antibody for 18h at 4ºC. When primary polyclonal antibody was used, a biotinylated pig anti-rabbit immunoglobulin G diluted 1 in 250 in PBS was applied for 30min as secondary reagent. An avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) diluted 1 in 50 in PBS was applied for 1h at room temperature to detect the different substrates. Slides were then incubated with DAB (3,3-diaminobenzidina tetraclorhidrato), diluted in Tris 0.1M containing hydrogen peroxide 3%, and checked microscopically for adequate chromogen development. Finally, sections were rinsed in tap water, counterstained with Harris´ Hematoxylin, dehydrated and mounted. Negative controls were performed replacing each primary antibody by PBS.

Results and discussion

The Mauthner cells appeared dorsally to the medial reticular nucleus and lateral to medial longitudinal fascicle nucleus, coinciding with the emergence of the magnocellular nucleus of the *Area octavolateralis* in sea bass. Our recent work has shown that CR antibody was only detected in Mauthner axons that coursed within the dorsal part of the medial longitudinal fascicle in larvae with more 32dah (Fig. 1a) in agreement with the result found in tench (*Tinca tinca*) by Crespo et al. (1998). The specific distribution of CR in the axons suggests an involvement of CR-mediated calcium buffering mechanisms in functional aspects of the axonal physiology such as neurotransmitter release, or nervous signal transduction (Crespo et al., 1998). It has been demonstrated that the ultrastructural localization of calcium ions in the Mauthner cells changes under normal conditions and after prolonged stimulation (Moshkov et al., 1995).

Results showed that larvae at 6 and 10dah showed PV immunonegative in the Mauthner soma, dendrites and axon. By contrast, the first PV-reactive neurons

appeared in larvae from 13dah. PV immunolabeling was found in dendrites and axon (Fig. 1b). In larvae from 17dah, PV immunoreactive positive neurons were clearly distinguishable in all structures as dendrites, soma, and axons. In this study from 19dah, Mauthner soma, dendrites and axon were found to show a strong positive immunoreaction for a PV-like protein. In addition, there was still a marked PV immunonegative in the Mauthner soma membrane. The CR and PV activity found in agreement with previous reports in the Mauthner cell of lamprey (*Lampetra planeri*) (Schober et al., 1994), swordtail fish (*Xiphophorus helleri*) (Anken et al., 1996) and tench (Crespo et al., 1998), suggesting a role of nitric oxide in the circuits of fast escape responses. The presence of nitric oxide synthase in Mauthner cells could indicate an involvement of nitric oxide, as retrograde messenger, in long-term potentiation events taking place in these neurons (Anken et al., 1996).

The present study described a first report of the sea bass Mauthner cells development which will constitute an important tool in the understanding of the alternations in behavior found in fish larvae along development. Besides, it will further allow us to undertake tracing studies focusing the description of several sensorial systems.

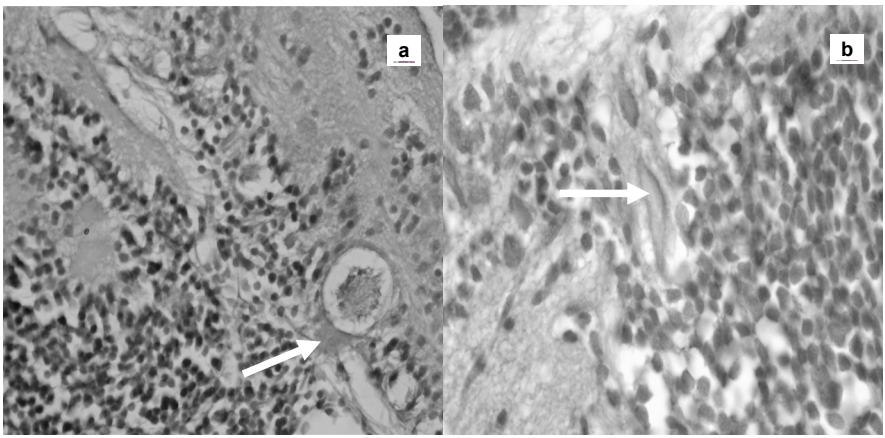

Fig. 1. (a) CR-immunositivity in Mauthner axon that coursed within the dorsal part of the medial longitudinal fascicle. (b) PV-immunopositivity in the Mauthner dendrites and axon and PV immunonegativity in soma. Scale bar: 100μm.

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EFFECTIVE TREATMENT OF BACTERIAL LOADS IN ROTIFER CULTURES

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Introduction

Bacteria are always associated with mass production of rotifers and may cause unexpected mortality or suppressed growth to rotifers. In some other cases, no harm is caused to the rotifers but infected rotifers cause a detrimental effect on fish larvae, resulting in poor survival and growth (Perez-Benavente and Gatesoupe, 1988; Gatesoupe, 1989). Although most bacteria are not pathogenic for rotifers, their proliferation must be avoided since a real risk of accumulation and transfer via the food chain can cause detrimental effects on the predator (Dhert et al, 2001). For brine shrimp Artemia suitable products can be applied (Gomezgil et al, 1994; Sorgeloos et al, 2001), however, for rotifers all tested products appear to be too toxic.

Materials and methods

All experiments were performed with *Brachionus* sp. Cayman strain. The batch culture system was performed following the culture procedure described in Sorgeloos and Lavens (1996). In this batch culture system rotifers were stocked at an initial density of 300 individuals per milliter in a 100-l tank, the culture water consisted of diluted seawater (25g.l^{-1} salinity), at 25±1°C.

In all experiments the commercial rotifer food S.Parkle (INVE Aquaculture NV, Belgium) was used. This dry food was suspended in 800 ml water and mixed vigorously with a kitchen blender. The suspension containing exactly the daily food ratio was kept in cold storage (4°C) for 24h. From the cold storage tanks the food was administered automatically by means of a peristaltic pump to the individual rotifer cultures

At the last day of the batch culture, just before harvesting and collecting the rotifers, the animals were treated in the culture tank with EBMIX017 (a proprietary natural product mix) . Without prior rinsing or replacing the culture water the effect of EBMIX017 on the microbial population associated with the rotifers (in the culture water and in the animal) was evaluated. Subsequently EBMIX017

was washed out by rinsing the rotifers with diluted seawater (25g.l^{-1} salinity) and the culture water and rotifers were analyzed again.

Microbial analysis of the culture water and rotifers were performed as follows: rotifers were separated from the culture water by means of a sterile 40µm filter and washed with 2×10-ml autoclaved artificial seawater (25g.l^{-1} salinity). The rotifers on the filter were transferred to a sterile receptacle and suspended in 20 mL autoclaved sterile seawater. The rotifers were then blended with a sterile POLY TONIC 21000 blender at 19 000 rpm to dislodge surface and intestinal bacteria. This suspension and the filtered culture water were serially diluted in sterile seawater and plated on marine agar for a total count of marine heterotrophic bacteria and on TCBS for *Vibrio* count. The inoculated plates were incubated for 24h at 29°C.

Results and discussion

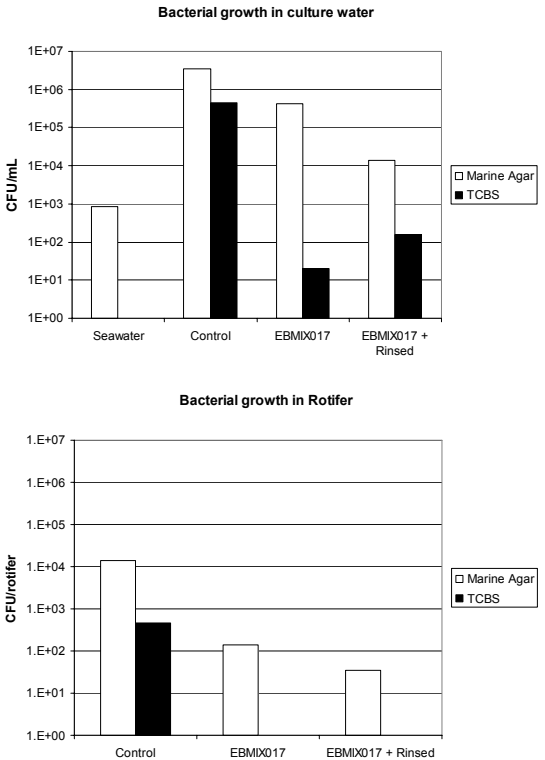

Fig. 1. The effect of EBMIX017 on the bacterial population associated with rotifers applied prior to harvesting. Top: the bacterial load in the culture water; Bottom: the bacterial load in the rotifers.

The results show that the total amount of heterotrophic bacteria present in the culture water of rotifers and associated with the rotifers can be significantly reduced by 2-3 log units by using EBMIX017 during 1h prior to rotifer harvesting. Moreover, the effect of EBMIX017 on the *Vibrio* population is even more pronounced as a significant reduction of the *Vibrio* population associated with the rotifers (in the culture water and in the animals itself) can be achieved.

Conclusions

Rotifers, the first food administered to vulnerable fish larvae, have been recognized as the major carriers of bacteria, including putative pathogens of fish larvae. For the first time it is possible to disinfect rotifers without negatively influencing the viability of the rotifers itself. Using EBMIX017, it is possible to feed rotifers to the fish larvae that are free of *Vibrio*-related bacteria.

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SCREENING TENTATIVE PROBIOTICS IN VIVO WITH COD LARVAE

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Abstract

The aim of this study was to screen different *Roseobacter* spp. and *Ruegeria* spp. for probiotic activity in vivo with cod, *Gadus morhua*, larvae. Screening for antagonistic activity in vitro was done according to Hjelm et al. (2004). Yolk sac larvae were bath challenged using a 24-well multidish system according to Sandlund and Bergh (2008). Each well contained 2ml sterile seawater and one cod egg. Final bacterial concentrations in the wells were 10^6CFU.ml^{-1}. Eggs and larvae not exposed to bacteria were used as negative controls. For in vivo screening of probiotic effects, the pathogens and tentative probiotics were added together. The method is validated for:

- Screening for adverse effects of the tentative probiotics towards the fish larvae
- Screening for probiotic effect in in vivo trials with a known pathogen

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POTENTIAL OF THREE NEW KRILL PRODUCTS FOR SEA BREAM (*SPARUS AURATA*) LARVAL PRODUCTION

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Introduction

Dietary phospholipids have been shown to improve culture performance in terms of growth, survival, and increased stress resistance, and reduce malformations in various freshwater and marine fish species at both larval and early juvenile stages (Coutteau et al., 1997). The main objective of this study was to evaluate three krill-derived products from AKER BIOMARINE ANTARCTICK as ingredients in microdiets for larval gilthead sea bream (*Sparus aurata),* as essential fatty acid, phospholipid and protein sources in substitution of fish oil, lecithin and fish meal.

Materials and methods

Gilthead sea bream larvae were fed enriched rotifers (DHA Protein Selco, INVE, Belgium) until 15 days old and then were randomly distributed into 16 tanks at a density of 1400 larvae per tank and were fed one of the five experimental diets (Table I) tested in triplicates. Larvae were fed manually each 45 minutes from 9:00 to 19:00.

Before the end of the experiment, an activity test was conducted by handling 20 larvae per tank out of the water in a scoop net for 1min and subsequently placing them in another tank supplied with clean seawater and aeration to determine survival after 24 hours. Final survival was calculated by individually counting all the live larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105ºC, 24h) and total length (Profile Projector V-12A Tokyo, Nikon) of 20 fish per tank at the beginning and at the end of the trial. All the remaining larvae in each tank were sampled for biochemical composition after 24 hours of starvation at the end of the trial. Thirty larvae from each tank were collected at the beginning, medium, and end of the feeding trial and fixed in 10% buffered formalin. Paraffin-embedded complete

larvae sections were cut at 5μm and stained with haematoxylin and eosin (H&E) for histopathological evaluation.

Table I. Ingredients and lipid and protein composition of the experimental diets.

Ingredients	Control	PLO	Krill Conc.	Krill Meal	PLO+SBL
Squid Powder	75.6	75.5	73.8	65.0	75.5
PLO	0.00	8.00	0.00	0.00	6.00
Krill Concentrate	0.00	0.00	6.00	0.00	0.00
Krill Meal	0.00	0.00	0.00	11.5	0.00
Soy lecithin	2.00	0.00	1.4	2.00	2.00
Sardine oil	5.9	0.00	2.30	5.00	0.00

Moisture and crude protein (A.O.A.C., 1995), and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982), separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) as per Izquierdo et al. (1990), and identified by comparison to previously characterized standards and GLC-MS.

All data were treated using one-way ANOVA and means were compared by Duncan's test ($P<0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Results and discussion

Increase in microdiet phospholipids from 2.74% (diet containing only krill phospholipids) up to 3.97% by addition of soybean lecithin did not affect either microdiet intake or larval growth and biochemical composition. As the minimum dietary phospholipids levels have been described for first feeding sea bream larvae as 9% of soybean lecithin (Seiliez et al., 2006), in the present study the lack of an effect on growth by the addition of soybean lecithin to a diet already containing krill phospholipids could be related to the relatively small dietary phospholipids contents of both diets or to the better efficiency of krill phospholipids in comparison to soybean lecithin. Indeed inclusion of krill phospholipids significantly improved growth in terms of total length, body weight, and SGR in comparison with the control diet. Survival was very high during the whole experiment and no significant differences were found among larvae fed the different diets. No differences were found after 24 hours of stress exposure.

Histological study showed that inclusion of krill phospholipids markedly reduced vacuolization of hepatocytes (Fig. 1A), suggesting an enhanced utilization of hepatic lipids. Despite dietary soybean lecithin increases lipid transport from the enterocytes (Salhi et al., 1999) and contributes to lipoprotein production

(Koven et al., 2001), krill phospholipids proved to be more effective in promoting hepatic lipoprotein synthesis. Moreover, dietary inclusion of soybean oil (control and PLO+SBL diets) was found to be associated with a higher incidence in the number of larvae showing enterocyte damage (Fig. 1B) in comparison with larvae fed krill phospholipids. Gut damage or impaired enterocyte membrane observed in the present study could be responsible for the reduction in the activity of the enzyme alkaline phosphatase found in larvae fed soybean lecithin as a single phospholipid source by Wold et al. (2009).

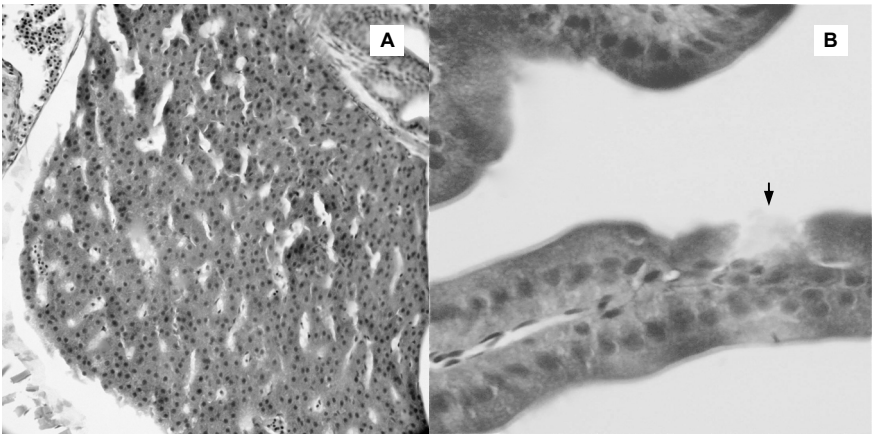

Fig. 1. Hematoxilin and Eosin staining (×400). A- Gilthead seabream larvae with extensive hepatocyte vacuolization; B- Injured enterocytes (arrows) in larvae fed diet PLO+SBL.

Conclusions

The results showed the high quality of the three products assayed, recommending their introduction in substitution of soybean lecithin in diets for gilthead sea bream larvae.

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CIRCADIAN RHYTHMS OF LOCOMOTOR ACTIVITY AND SPAWNING IN ZEBRAFISH (*DANIO RERIO* L.)

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Introduction

Zebrafish (*Danio rerio*) has been extensively used in biomedical research in vertebrates (Fishman, 2001), but little information about its reproduction behaviour is available. The purpose of this paper is to investigate daily spawning and locomotor rhythms in zebrafish exposed to different light conditions, and to observe whether mealtime affects the locomotor and spawning daily patterns.

Material and methods

We used male zebrafish with a size of 31±1.7mm (mean ± standard deviation) and female zebrafish of 38±2mm. The experiments were carried out in the chronobiology laboratory located at the Faculty of Biology, University of Murcia. Seven plastic tanks with a capacity of 5 litres each were used to make the crossings, thus becoming spawning boxes. These spawning boxes had a slope of 40 degrees by which the eggs got to the collector immediately after spawning. The experiments were developed under different light conditions: 14:10 LD cycle with lights on at 8:00 (ZT0, Zeitgeber Time), and continuous light (LL). Fish were fed once a day ad libitum with a commercial food (Prodac). Animals were separated according to sex before crosses were made, so locomotor activity in males and females were monitorized.

In experiment 1 fish were exposed to LD cycle and fed either at ZT4 or at ZT19, to know if feeding time influences spawning. In experiment 2 a pulse of darkness of 1h duration was given either at ZT3 (the peak of spawning previously observed) or at ZT7, to test the light effect on spawning. Finally, fish were exposed to LL to test whether spawning rhythms persisted.

To collect the eggs, we connected five spawning boxes to a programmable fraction collector (Gilson, FC203B, Middleton, USA). Daily spawning rhythms were studied by means of COSINOR's analysis. All the tanks had an infrared (IR) photocell to control the fish locomotor activity. The data analysis was carried

about using Excel® 2003 and a chronobiology software called "ElTemps", designed by Professor Díez Noguera, University of Barcelona, Spain.

Results and discussion

In experiment 1 (14L:10D) there appeared a significant daily spawning rhythm (COSINOR, $p<0.05$) with an acrophase at ZT3 (Fig. 1A). Locomotor activity increased aaround the time of spawning. These findings agree with Spence et al. (2006), who also reported spawn during the first hours of light in zebrafish in their natural environment.

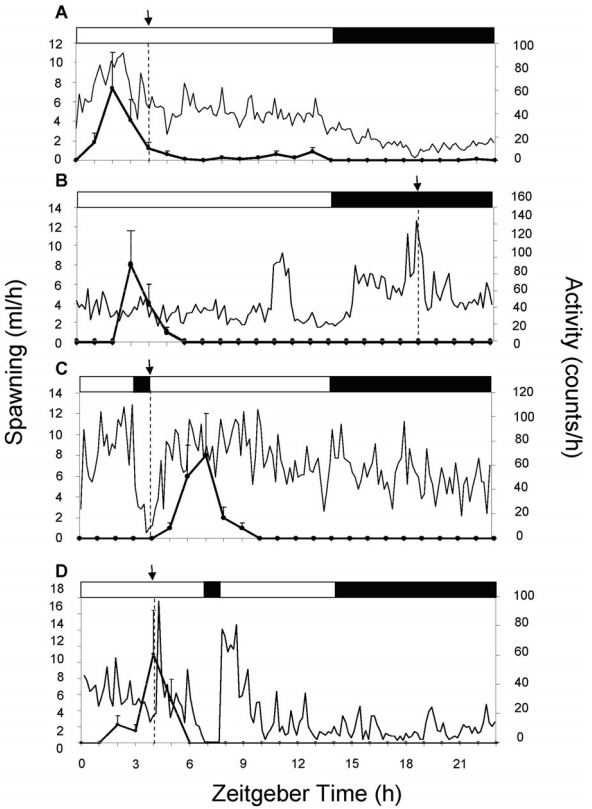

Fig. 1. Average values of daily locomotor activity and spawning rhythms in zebrafish under artificial 14L:10D photoperiod, on the days on which spawning was obtained. Values expressed as volume of eggs/hour. The thick line represents total of eggs. The thin line refers to the activity measured in counts.h^{-1}. Upper black and white bars represent the dark and light phases, respectively. The vertical discontinuous line and the arrow in the top refer to the moment of feeding. In 3C and 3D, the white and black bars at the top indicate the light and dark phase, respectively, of the LD cycle.

When food was provided at night (ZT19), the nocturnal percentage of locomotor activity significantly increased from 25.6±5.8% to 50.4±2.2% ($p<0.05$, t-test), though the acrophase of spawning did not change (Fig. 1B).

In experiment 2, a pulse of darkness at ZT3 induced a shift in the daily spawning rhythm, which started after the pulse of darkness, the peak of highest volume of spawning occurring at ZT7 (Fig. 1C). When the pulse of darkness was provided at ZT7, spawning returned to ZT3.

Under LL locomotor activity rhythms persisted with a periodicity of 22.3h, the spawning rhythm showing a circadian pattern with its acrophase at CT3 (Fig. 2). These results agree with those of Wang and Ge (2004), who reported in the gonad of sexually mature zebrafish daily rhythm of oocyte development and its controlling hormones (activin βA, activin βB and follistatin).

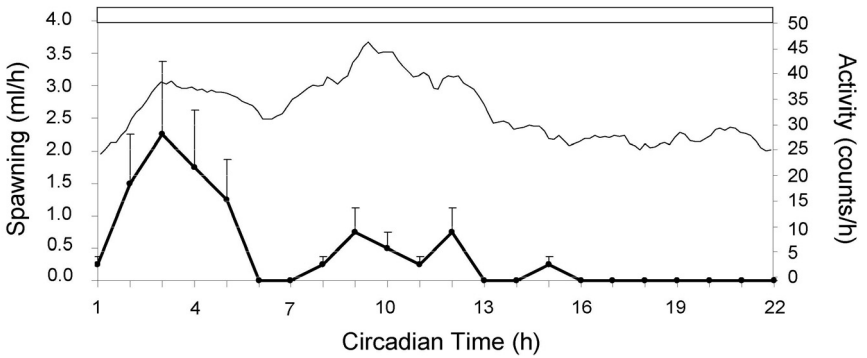

Fig. 2. Average values of daily locomotor activity and spawning rhythms in zebrafish with continuous light photoperiod, on the days on which spawning was obtained. Values expressed as volume of eggs/circadian hour. The thick line represents total of eggs. The thin line refers to the activity measured in counts/h. Upper white bar represents the continuous light photoperiod.

Conclusions

Light, but not feeding, seems to be the key environmental factor in timing the daily spawning in zebrafish, since this study revealed that spawning rhythms can be shifted by dark pulses. Besides, spawning rhythms are circadian, because they persisted under LL.

Acknowledgements

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DIFFERENT TOOLS TO OPTIMIZE THE WELFARE AND THE MORPHOLOGICAL QUALITY OF REARED FINFISH LARVAE

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The mass rearing of fish in aquaculture needs a predictable supply of high quality juvenile fish for high performances in the grow-out phase and to achieve the marketable quality. The relatively low survival rates, mostly observed in new candidate species for aquaculture, and the presence of sublethal, morphological deformities both represent bottlenecks for the aquaculture industry, determining economic losses and inducing negativity in consumers for aquaculture products. It is estimated that between 15 and 50% of gilthead sea bream juveniles with deformities are culled out from the productive cycle at the end of the hatchery phase. Nonetheless, actual sorting methods do not eliminate the presence of deformities in on-grown fishes that need further sorting before harvest. For this reason, new approaches are necessary. Literature indicate that deformities are the consequence of so many influential factors that interdisciplinary studies integrating anatomic, genetic, biomolecular, and physiologic data on larvae welfare conditions are needed. Vertebral malformations may now be the norm in hatcheries so, it's clear that all reared fish should be considered as 'distressed' fish, in which epigenetic and genetic factors try to buffer the environmental effects, but not always efficiently.

In our lab, we are investigating the problem of skeletal deformities using three approaches:

1) Building on knowledge of the locomotor, sensory, and digestive capabilities for the different species, considered an essential tool to properly fit the rearing methodology to the species' and developmental stages' needs. Further, this can give more advanced answers to new generation questions like the identification of the environmental conditions for animal welfare or how to build up protocols in a framework of organic aquaculture. The development of three Sparidae species – gilthead sea bream (*Sparus aurata)*, sharpsnout sea bream (*Diplodus puntazzo)*, and pandora (*Pagellus erythrinus*) – was studied from hatch to juvenile phase, with particular emphasis given to sensory organ differentiation. The data

were compared in order to find differences and similarities in the larval and juvenile trophic ecology of these species. Observations were obtained using scanning electron microscopy to follow the differentiation pattern and timing during development, with special emphasis given to chemo- and mechano-receptors. Eco-functional extrapolations are proposed.

2) The monitoring of morpho-anatomic quality in mass-produced gilthead seabream, from different Mediterranean hatcheries and larval rearing methodologies. This is carried out in the framework of a EU research Project (SSP-2005-44483 "SEACASE - Sustainable extensive and semi-intensive coastal aquaculture in Southern Europe"), and is directed to the production of juveniles with wild-like phenotype. As many as 4217 juveniles of gilthead sea bream, belonging to 60 lots characterized by different origin, were analysed for anomalies in shape (skeletal deformities) and number (meristic counts) of skeletal elements and compared to wild counterparts. The results obtained suggested the possibility of wide ranges of increased morphological condition using semi-intensive larval rearing.

3) The development of a model of the occurrence of skeletal anomalies in reared juveniles of different species, both in terms of type and quantity. Data on skeletal deformities and meristic counts on wild and reared gilthead sea bream and on dusky groupers (*Epinephelus marginatus*), reared under different hatchery conditions, were performed with a particular type of artificial neural network, known as Kohonen's Self-Organising maps (SOMs), assigning a different weight for the different anomalies to obtain a score between 0 and 1 for every group/rearing condition. SOMs were then performed on the matrix of mean values for every different group/species and rearing condition in order to investigate the qualitative and quantitative relationships between rearing approach and occurrence of skeletal anomalies in reared finfish juveniles.

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MICROBIAL MANAGEMENT IN FISH AND SHELLFISH LARVICULTURE: FROM GNOTOBIOTIC EXPERIMENTS TO APPLICATIONS

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Microorganisms play an important role in aquaculture and in larviculture in particular. Large inter-individual variation, both quantitatively and qualitatively has been observed in fish larvae. Very little is known about the temporal changes (using culture independent techniques) in microbial communities associated with larvae, and whether or not small or large changes are beneficial to larvae. It has recently been suggested that functional stabile microbial communities require a certain degree of temporal change in composition. Whether or not this is the case for larvae remains to be established. Despite the poor background in fundamental knowledge, many researchers have tried to improve survival in larviculture through the application of probiotics. Typically, beneficial results have been observed without strong evidence for the mode of action in vivo.

This review will summarise the results that have been obtained in gnotobiotic experimental systems (avoiding largely stochastic and temporal interference) and where possible complement these observations with results obtained under non-gnotobiotic experiment conditions. Gnotobiotic experimental conditions have been developed for *Brachionus*, *Artemia*, and European sea bass. Using these gnotobiotic systems, parameters have been tested that might influence the outcome of host microbial interactions. With respect to the microbial end of the interaction, the effects of quorum sensing and polyhydroxybuyrate (PHB) have been investigated. Using gnotobiotic *Artemia* as experimental model, a *Vibrio* challenge test could be developed. The first results revealed that quorum sensing is indeed important in vivo. Using *Vibrio* strains carrying single or double mutations in its quorum-sensing systems, it could be demonstrated that a mutation in the AI2 system (either in the production or the sensor) considerably reduced the

virulence towards Artemia. Encouraged by these results, we have developed systems to demonstrate that quorum-sensing molecules (mainly acyl homoserine lactone, AHL, also called AI1 molecules) are important in other systems. In turbot and *Macrobrachium* larviculture it could be shown that the daily addition of a mixture of AHL molecules ($1mg.l^{-1}$) can have a strong negative effect on the larval survival. Microbial communities able to degrade AHL molecules in vitro and in vivo have been shown to mitigate the negative effect of added AHL molecules. This suggests that influencing the balance between AHL production and degradation is a possible option to manipulate the standing microbial community, reducing its virulence towards target organisms. However it has yet to be proven that quorum-sensing interference by AHL degradation has a direct effect on the virulence of gut micro-organisms, a problem that can only be solved when an in vivo sensor will be developed.

Further work with the *Vibrio* challenge system in *Artemia* revealed that ingested PHB particles are able to protect Artemia against *Vibrio*. This effect is most probably mediated by PHB degradation in the gut where the resulting short chain fatty acids inhibit the proliferation of *Vibrio* by intracellular acidification. PHB is naturally occurring as a storage compound in many micro-organisms, especially when the latter are grown under nitrogen limiting conditions. Hence it is conceivable that part of the probiotic effect of added micro-organisms is due to the delivery of PHB. In a series of unpublished experiments there is now good evidence that PHB can have beneficial effects on larvae as well as in juveniles. However, the underlying mechanisms operational in these non-gnotobiotic environments, need to be further documented. Rather than trying to manipulate bacterial numbers or their activity, it is also possible to stimulate host-defence mechanisms. Using the gnotobiotic *Artemia* model, it could be demonstrated that offering bacterial feed enriched with HSP70, a heat-shock protein known to stimulate the innate immune response, could protect against *Vibrio*. Finally yeast-bound glucan has been evaluated as a protective agent in the Artemia gnotobiotic model. Using a series of isogenic yeast strains, only differing in the composition of the cell wall due to gene deletion involved in cell-wall synthesis, it could be demonstrated that especially the mnn9 strain has protective characteristics. This yeast strain lacks long mannose chains in mannoproteins and compensates for this defect by an increased production of cell-wall bound beta-glucan. Because immunological data are lacking at the moment, it remains to be proven that such kind of yeast strains can assist in pathogen protection because of a glucan-based stimulation of the host's immune system.

In conclusion, gnotobiotic systems are useful experimental systems that allow conceptual verification of novel treatments and their mode of action. Such knowledge will indefinitely contribute to a knowledge-based application of novel concepts and products in larviculture.

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EFFECTS OF VITAMIN C (L-ASCORBATE 2-TRIPHOSPHATE CALCIUM) ON THE GROWTH, BIOCHEMICAL COMPOSITION, AND TISSUE ULTRASTRUCTURE OF HYBRID CARP

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Introduction

Carps are the major component of freshwater aquaculture throughout India and this sector is increasingly characterized by intensification. To promote the current growth of carp culture the supply of healthy seeds are of paramount significance. However, high mortality, stunted growth rate, skeletal deformities, and frequent disease outbreaks are some of the common problems encountered in carp culture. Ascorbic acid is an essential dietary component that regulates numerous metabolic activities. Several studies have shown that vitamin C is essential to most fishes (Dabrowski et al., 1988) as gulonolactone oxidase enzyme (GLO) is lacking in fishes. Vitamin C plays important role in numerous physiological processes such as growth, bone formation, reproduction, and wound healing (Lee and Dabrowski, 2004). It also prevents immunosuppression, aids in inflammatory response, and enhances phagocyte activity. Supplementing fish feed with high vitamin C doses has prevented diseases and reduced losses during fish production (Affonso et al., 2007). The present investigation aims to study the influence of dietary supplementation of vitamin C (L-ascorbate 2-triphosphate Ca, LATP, HiMedia) on survival, growth, biochemical composition, and tissue ultrastructure of hybrid carp.

Materials and methods

Hybrid larvae (0.28±0.04g) of bighead carp, *Aristichthys nobilis* (♀) × silver carp, *Hypophthalmichthys molitrix* (♂) were cultured (100 fish.m^{-3}) in cemented tanks under four feeding regimes for 56d. Experimental diets (40% protein) were prepared using fish meal, wheat flour, and cod liver oil along with three different doses of vitamin C: 100mg.kg^{-1} (D-1), 200mg.kg^{-1} (D-2), and 400mg.kg^{-1} of diet (D-3). The diet without vitamin C served as control (C). Fish were fed once daily at 9.00 a.m. at the rate of 2% of body weight. Three replicates were used for each feeding scheme. Water temperature and pH ranged from 26.5 to 29.8°C and 7.4 to 7.8, respectively, throughout the study period. The specific growth

rate (SGR) was calculated using the formula: $SGR = 100 \times (I_n W_t - I_n W_i).t^{-1}$, where W_i and W_t were the initial and final body weights and t time in days. For frequency distribution study, weight of individual fish was measured at the end of the experiment; fish were divided into different weight classes in the range of 1.0 to 1.5g, 1.6 to 2.0g, 2.1 to 2.5g, 2.6 to 3.0g, 3.1 to 3.5g, 3.6 to 4.0g, 4.1 to 4.5g, and 4.6 to 5.0g. The number of fish representing each weight group was enumerated and the values were converted to per cent. Vitamin C contents of brain, liver, anterior kidney, and body muscles were assayed (Dabrowski and Hinterleitner, 1989). SGOT and SGPT of blood serum were determined. ALP was determined by using kit (Bayer Diagnostics India Ltd., India). For transmission electron microscopic study, liver and head kidney were surgically removed from anesthetized fish. Tissues were cut into 1-mm pieces and processed following standard protocols. Stained sections were studied and documented photographically with a Philips 201 Transmission electron microscope. All data were analyzed using one-way ANOVA and Duncan's Multiple Range test. The level of significance was accepted at $P<0.05$.

Results

Percent survival of carp was recorded in all treatments. The final average weight was significantly ($P<0.05$) higher in the carp fed with diet D-3 followed by fish fed with diets D-2, D-1, and C. The final average weight was 39.6-69.5% higher in the fish fed with D-3 diet compared to others. A direct relationship was found between the dose of vitamin C in diet and the specific growth rate of carp (Table I). A wide variation in the final average weight of carp was recorded in different treatments. In the control group, all fish were belonging to the weight class of 1.0 to 1.5g. In the D-1 diet fed group, 53.3 and 46.7% fish were belonging to the weight classes of 1.0 to 1.5g and 1.6 to 2.0g, respectively. In the D-2 diet fed group, 68.9% carp belonged to the 2.1 to 2.5g weight class and the remaining 31.1% were in the 2.6 to 3.0g weight class. It is interesting to note that the weight of all fish fed with diet D-3 ranged from 3.1 to 5.0g. A direct relationship was found between the dose of vitamin C in the diet and the concentration of vitamin C in various tissues of fish. Vitamin C level was significantly ($P<0.05$) higher in the carp fed with diet D-3 compared to the others regardless of tissues. SGOT and SGPT levels were significantly ($P<0.05$) higher in fish fed with control diet. Among the treated fish, minimum SGOT and SGPT were found in D-3 diet fed group. Alkaline phosphatase level was significantly ($P<0.05$) higher in the group fed with D-3 diet followed by others (Table I). Ultrastructural study of hepatocytes of hybrid carp cultured under various feeding regimes showed interesting features. The number of mitochondria in control diet fed fish ranged from 3 to 7, while the number in D-1, D-2, and D-3 diets fed fish ranged from 3 to 13, 5 to 15, and 4 to 24, respectively. The numbers of lipid droplets increased in liver as the dose of vitamin C increased in the diet. The number and size of vacuoles in the cytoplasm gradually decreased as the dose of vitamin C in-

creased in the diets. Vacuole was absent in the group D-3. Interesting ultrastructural variations were recorded in the head kidney of treated and control hybrid carp. The number of mitochondria in the micrographs of C, D-1, D-2, and D-3 diets fed fish were 27, 34, 58, and 87, respectively. The number of lysosomes was variable in the head kidney; 7, 2, 3, and 7 lysosomes were found in C, D-1, D-2, and D-3 diets fed carp, respectively. In the control diet fed fish total 93 vacuoles were found, which occupied 18.41% area of the micrograph. The numbers of vacuoles were 46, 68, and 27 in D-1, D-2, and D-3 diets fed fish, respectively; vacuoles occupied 10.67, 13.22, and 7.74% areas of micrographs in D-1, D-2, and D-3 diets fed fish, respectively. Significant differences were also found in the convoluted renal tubules of head kidney among the various treatments. Lymphocyte was found only in the D-3 fed carp. Monocytes occupied 3.56, 7.61, and 11.66% areas of D-1, D-2, and D-3 diets fed fish, respectively. Neutrophilic granulocytes were present in all the micrographs of head kidney.

Table I. Average weight (g), SGR, vitamin C concentrations in various tissues ($\mu g.ml^{-1}$), SGOT, SGPT, and ALP levels (IU l^{-1}) of serum of hybrid carp cultured under four different feeding regimes. Means in the same row followed by different letters are significantly ($P<0.05$) different.

Parameters	Diets			
	C	D-1	D-2	D-3
Average weight	1.26 ± 0.2^{d}	1.59 ± 0.22^{c}	2.49 ± 0.35^{b}	4.12 ± 0.1^{a}
SGR	1.16 ± 0.08^{d}	1.34 ± 0.11^{c}	1.68 ± 0.09^{b}	2.08 ± 0.09^{a}
Vitamin C brain	63.83 ± 8^{d}	107.46 ± 9^{c}	195.17 ± 13^{b}	310.89 ± 18^{a}
Vitamin C liver	117.10 ± 15^{d}	166.24 ± 20^{c}	335.23 ± 25^{b}	487.23 ± 25^{a}
Vitamin C head kidney	29.39 ± 2^{d}	47.30 ± 6^{c}	92.30 ± 12^{b}	163.48 ± 12^{a}
Vitamin C muscles	16.53 ± 6^{d}	33.06 ± 3^{c}	77.15 ± 8^{b}	110.67 ± 14^{a}
SGOT	1170.33 ± 35^{a}	838.47 ± 51^{b}	242.77 ± 61^{c}	49.70 ± 9^{d}
SGPT	123.20 ± 6^{a}	105.53 ± 4^{b}	80.37 ± 4^{c}	37.80 ± 3^{d}
ALP	59.97 ± 7^{d}	134.20 ± 27^{c}	385.57 ± 25^{b}	784.47 ± 60^{a}

Discussion

The average weight and specific growth rate of hybrid carp increased as the dietary inclusion of vitamin C increased in the present study. Dabrowski *et al.* (2004) reported that the growth rates were significantly affected by dietary ascorbic acids levels and growth of fish fed the high-ascorbate diet was greater than low or required-ascorbate level diets. Lee and Dabrowski (2003) showed a typical growth trend in which growth rate was higher in the fish fed vitamin C supplemented diets than in fish fed a diet devoid of vitamin C. The concentration of vitamin C in various tissues of carp increased as the dose of vitamin C increased in the diet. Among these various tissues, significantly ($P<0.05$) higher vitamin C concentration was found in liver compared to other tissues regardless of treatments. Liver ascorbic acid content is usually considered as an indicator of the vitamin C status. Ibiyo et al. (2007) found that the vitamin C concentrations

in various tissues of *Heterobranchus longifilis* were positively correlated with dietary level of the vitamin C. In the present study, significant ultrastrustural modifications are recorded in fish fed with vitamin C incorporated diets compared to the control one. The number of mitochondria increased as the dose of vitamin C increased in the diets. Sagun et al. (2005) reported that the oxidized form of vitamin C, dehydroascorbic acid, enters mitochondria via facilitative glucose transporter and accumulates mitochondrially as ascorbic acid (mtAA) and confers mitochondrial protection against oxidative injury. Therefore, the enhanced number of mitochondria may help in the better performance of liver. The head kidney is a key tissue for immunity in fishes because it is the main site of development and proliferation of B lymphocytes and macrophages and has an organized cluster of cells involved in endocrine function. In conclusion, incorporation of vitamin C in diet of hybrid carp improved the physiological conditions of fish.

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CHARACTERIZATION OF GENE EXPRESSION IN ORANGE-SPOTTED GROUPER LARVA METAMORPHOSIS

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Introduction

Grouper, the most popular table fish for Orientals, Arabics, and Europeans, live in coral reefs threatened by divers, poisons, or explosives. Wild capture of grouper often results in mass damage to the coral reef; therefore, farming of grouper is not only important to supply food for human consumption but is also beneficial to the maintenance of the ocean environment. Hatchery technology of grouper has been established and could supply grouper fingerlings globally. However, the molecular mechanisms essential for growth and development, particularly in their larval stages, are less studied and unclear. Thus, hatching and farming of grouper are still at the stage of "experience art" rather than modern industrial production, often resulting in poor growth and high larval mortality.

Orange-spotted grouper (*Epinephelus coioides*) is the most popular cultured grouper. Some genes such as *Mx* – which is an IFN-induced cytoplasmic protein with antiviral activity against a number of RNA viruses (Leong et al., 1998) – or *Myostatin* – a member of the transforming growth factor-β superfamily with a genetic determinant of skeletal muscle growth (McPherron and Lee, 1997), have been cloned from orange-spotted grouper and analysed. In present study, we used these genes as markers to observe the relationship between gene expression and metamorphosis in grouper larva.

Materials and methods

In this study, orange-spotted grouper (*E. coioides*) were obtained from spawning of broodstock kept in concrete tanks. Spawning occurred within the holding

tanks with reproductive cycles controlled through temperature manipulation as previous (Chen et al., 2006). Larval grouper were collected from 1 to 40 days of post-hatching (dph) in concrete tanks.

Real-time RT-PCR was then used to quantify the expression of mRNA for *IGF*2 (growth factor as growth indicator), *Mx*1 (antivirus factor as innate immunity indicator), CXCR4 (chemokine as lymphocyte indicator), *SPARC* (bone growth factor as bone development indicator), and *HSP90* (protein folding protein as stress indicator) with expression of *actin* as control (Chen et al., 2006; Chen et al., 2007). All primers used in real-time PCR were designed to determine the gene expression of grouper at different stages shown in Table I. Total RNA was separated from larval grouper (1-40dph), following the single-step acid guanidinium thiocyanate-phenol-chlorofrom extraction method (Chomezynski and Sacchi, 1987). First-strand cDNA was synthesized using 2μg total RNA and the SuperScript First Strand cDNA synthesis kit (Invitrogen). The amplification was performed using the qPCR core kit for SYBR Green (Qiagen, Valencia, CA, USA) and Step One Real Time system PCR (Applied Biosystems, Foster City, CA, USA). Typical profile times used were initial step, 95°C for 15min, followed by a second step at 94°C for 15s, 60°C for 30s, and 72°C for 30s for 40 cycles with melting curve analysis. The level of target mRNA was normalized to the level of *actin* and compared to control (healthy grouper) and the values were calculated by $2^{-\Delta\Delta CT}$ method, where ΔCT is the difference in threshold cycles for target and the housekeeping gene, and ΔΔCT is the differences in ΔCT and the threshold cycle for the control.

Table I. Position and sequence oligonucleotide primers used in the study.

Name	Location	Sequence 5' to 3'	Product size	Direction
CXCR4-QRT-S	932~956	CACACTGCTGCCTGAACCCACTGCT	173 bp	Forward
CXCR4-QRT-A	1078~1104	GAGTCCTCCAGTTTACATTCCAGCTAG		Reverse
Hsp90 QF	235~254	TTAGTGCGGGCATCACGGGTAT	216 bp	Forward
Hsp90 QR	431~450	ATCATTGTGTTTAGTGATGA		Reverse
SPARC-QRT-S	1~23	ATGAGGGTGTGGATTGTCTTCCT	173 bp	Forward
SPARC-QRT-A	151~173	TCGATGGCCTCGTCAAACTCTCC		Reverse
Mx1-QRT-S	1338~1358	TTCATACAGCTGGCCCACAGT	226 bp	Forward
Mx1-QRT-A	1540~1563	ATCCTCCTCTCTTTTCCTGTCACT		Reverse
IGF2-QRT-S	15~37	AAGATACGGACACCACTCACTTT	398 bp	Forward
IGF2-QRT-A	382~403	TTAGTGCGGGCATCACGGGTAT		Reverse
β-ACTIN-QRT-S	527~554	TGCCTCTGGTCGTACCACTGGTATTGTC	265 bp	Forward
β-ACTIN-QRT-A	791~768	GGCAGCAGTGCCCATCTCCTGCTC		Reverse

The orientation is indicated as sense (S) and antisense (A).

Results and discussion

As a primary step in the characterization of grouper *IGF*2, *Mx*1, CXCR4, *SPARC*, and *HSP90*, we examined their transcript expression from 1 to 40dph. Real-time quantitative RT-PCR using SYBR Green dye demonstrated high lev-

els of *IGF*2 and *Mx*1 expression in the pro-larva, larval shape, kitting, and dis-kitting stage, but low expression in the morphological shape changed stage between kitting, and dis-kitting stage (Fig. 1). On the other hand, we also found higher expression levels of CXCR4 and *HSP90* in the pro-larva, kitting, and dis-kitting stage. However, lower expression levels of *SPARC* were observed in the pro-larva, kitting, and dis-kitting stage, but interestingly higher and very different from other four gene expression types in the morphological shape changed stage between kitting and dis-kitting. From these results, we found gene expression and metamorphosis in grouper larvae has a very tight relationship. Most genes correlated with innate immunity or growth shut off in the morphological shape changed stage between kitting and dis-kitting (20-26dph). This period is key to larval success and very susceptible to nodavirus. Our results may provide a clue to answer this problem, as innate immunity stopped at 20-26dph, decreasing pathogenic defence. More experiments, however, are needed.

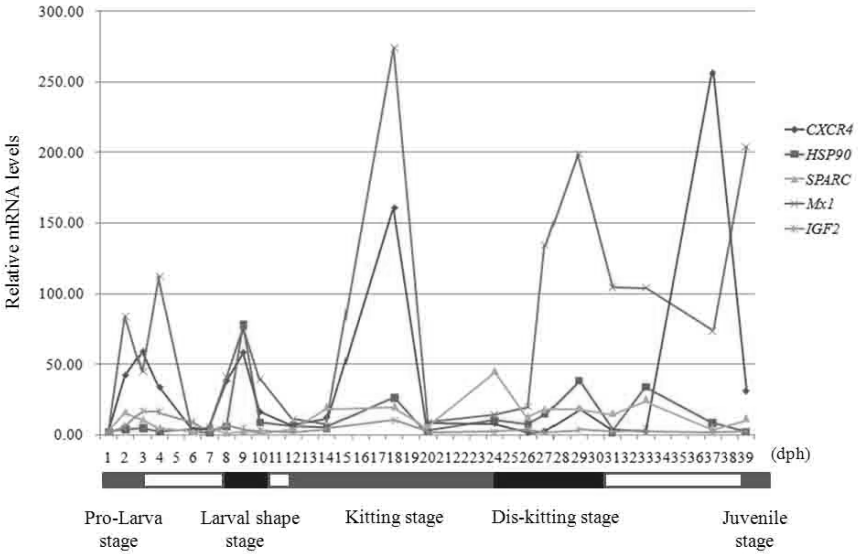

Fig. 1. Expression levels of *IGF*2, *Mx*1, CXCR4, *SPARC*, and *HSP90* transcript in different metamorphosis of grouper larva. Larval grouper were harvested and total RNA extracted at designated time points and reverse transcribed into cDNA. The resulting cDNA was used as a template for quantitative PCR using primers specific for grouper *IGF*2, *Mx*1, CXCR4, *SPARC*, and *HSP90*.

Conclusions

In the present study, we found relationship between gene expression and metamorphosis in grouper larvae. Most genes increase expressions that correlated with morphological stage including pro-larva, larval shape, kitting, and dis-kitting stage, but decrease in morphological shape changed stage between near

stages. In the future, the genome-wide approach may be used to observe and get more information on the relationship between gene expression and metamorphosis in grouper larvae.

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FACTORS INFLUENCING SKELETAL MALFORMATIONS IN AUSTRALIAN CULTURED MARINE FINFISH

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The culture of marine finfish is a growing industry in Australia. The major species currently produced from hatchery-reared stock are Barramundi, Yellowtail Kingfish, and Mulloway. Several other species, ranging from tropical to temperate species are emerging or under investigation as potential candidates for culture in Australia. Reliability of supply of high quality hatchery-produced juveniles is a limiting factor for the development of the finfish aquaculture industry, coinciding with a target to increase finfish aquaculture production in Australia from 27,000 tonnes in 2005 to 100,000 tonnes by 2015. One of the factors impacting upon the quality of hatchery-produced fish in Australia, as it is worldwide, is skeletal malformations. A survey of Australian finfish hatcheries revealed skeletal malformations are a significant impediment to production efficiency in several species and facilities. In some species, such as Yellowtail Kingfish and Striped Trumpeter, jaw malformations are a recurrent issue. For other species, including Barramundi and Flowery Grouper, high incidences of malformation are rare but do occur is some batches in a range of hatcheries.

Striped Trumpeter is a candidate species for aquaculture with jaw malformations a persistent issue in larval culture since the 1980s. Fish were reared in a series of experiments to test nutritional and environmental factors from rotifer feeding through to metamorphosis, largely in a system of 24 × 300-l tanks. Lipid content (DHA and ARA) and vitamins (C and E) in live feeds had little effect on malformations. When cultured at a semi-commercial scale in 3000-l tanks, with commercial enrichment products that provided for good growth and survival, post larvae continued to exhibit a high incidence of malformations from 85-100%. Striped Trumpeter display a high level of active 'walling' behaviour from metamorphosis which appears to directly or indirectly contribute to jaw malformations. Tank colour and culture type (greenwater/clearwater) were used to manipulate the light environment in culture tanks. A novel cost-effective method

was developed to assess tank colour, using adhesive covering that was quick to apply and did not damage tanks. Both tank colour and culture type had a significant effect on the incidence of larval walling behaviour which was positively correlated with the incidence of jaw malformation in metamorphosed larvae. Jaw malformation was high (70% at 29 days post-hatch, dph) in white tanks where walling started from first feeding, and was lower (18-21% at 44dph) in black or marble coloured tanks where walling was minimal. Greenwater reduced walling and jaw malformation in larvae compared with clearwater. Fish fed enriched live prey exhibited a higher incidence of jaw malformation than those fed non-enriched diets, due to the poor quality, reduced activity, and lower incidence of walling in fish fed the non-enriched diets. Walling behaviour is therefore a contributing factor to malformations which can be ameliorated through manipulation of environmental factors. Graded levels of vitamin A (retinol palmitate) in rotifers are being tested to examine the effects on expression of retinoid receptor genes in developing larvae and to compare malformation morphology associated with vitamin A deficiency or excess with those linked to walling behaviour.

In commercial Yellowtail Kingfish culture, malformations persist in all hatcheries (Australia, New Zealand, Japan). Several types of cranial malformations (e.g., longer or short lower jaw; lower jaw bent down on one side; fusion of upper jaw; lower jaw twisted laterally to one side; open mouth; bent and thickened upper jaw; loose maxilla; short operculum) have been described and many are apparent from the rotifer-feeding phase. The early stages of the 'fusion' malformation were apparent in larvae during rotifer feeding as a misalignment of the upper jaw bone (maxilla) inside the lower jaw (dentary). In larger fish, the continued opening and closing of the mouth and the resultant pressure applied to the misaligned upper jaw caused the maxilla and premaxilla bones to twist and curl upward, coinciding with an overgrowth of soft tissue between the upper and lower jaws. Some jaw malformations with similar morphology to those of Striped Trumpeter may be linked to walling behaviour. Vertebral malformations possibly linked with abnormal swimbladder inflation were identified. Biochemical composition of eggs and live feeds, prey density, and system design has been assessed to investigate contributing factors. In Barramundi, jaw malformations were linked to variable nutritional quality of algal pastes used in greenwater and rotifer culture. Jaw and spinal malformations have occurred sporadically in Flowery Grouper with the causative factor(s) as yet unknown. The recent breakthrough by Clean Seas Tuna in the production of Southern Bluefin Tuna juveniles has provided a new opportunity to assess larval quality in this species.

Through evaluation of larval culture protocols, characterisation of malformations in different species, surveys of most research and commercial hatcheries, and increased communication between commercial hatchery operators and scientists, research effort has been focussed to address improving fish quality to further finfish aquaculture development in Australia.

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A DYNAMIC MODEL FOR DIETARY AMINO ACIDS UTILISATION IN FISH LARVAE

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Further improvement of growth performance in fish larviculture is closely linked to a better understanding of the dietary amino acid (AA) requirements, and therefore of the processes involved in AA metabolism. In recent years major advances in the understanding of fish larvae amino acid metabolism have been accomplished, in particular through the use of tracer studies.

Modelling is a holistic approach to integrate knowledge on growth and metabolism and identify gaps. A dynamic mechanistic model that simulates AA metabolism of fish larvae was developed. It aims to improve the understanding of larval digestion and absorption of dietary AA, and the postprandial AA metabolism and growth. The model may also assist in the interpretation of results obtained using tracer studies. The model is driven by amino acid intake, with the absorbed dietary AA being used for energy production or for biosynthetic processes. The model is here used with Senegalese sole (*Solea senegalensis*) fed *Artemia*, and was parameterized using literature data.

The model allows integration of the results obtained after feeding a single meal with tracer AA, and following these tracers AA in the free AA and protein pools of larval gut and larval body at different time points after the meal. Model simulations permit to study the dynamics of the changes in the larval free AA and protein pools. Consequences for larval feeding strategies and amino acid requirements will be discussed.

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GROWTH AND DEVELOPMENT OF DUSKY GROUPER *EPINEPHELUS MARGINATUS* LARVAE IN MESOCOSM OF SEMI-INTENSIVE TECHNOLOGY

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Introduction

Dusky grouper *Epinephelus marginatus* (Lowe, 1834) is a threatened high market value species. Research on its cultivation has been intensified but rates of mortality during larval stages remain high. The present work describe a successful rearing experiments with *E. marginatus* larvae in mesocosm systems of semi-intensive technology and present the minimum feeding requirement per day for larvae from the opening of the mouth until the beginning of metamorphosis.

Materials and methods

The mesocosm experiment was performed outdoors in one shaded 3-m^3 circular tank during July and August 2008 using seawater from the Ria Formosa coastal lagoon (Algarve, Portugal). The water was left stagnant for 6 days under natural light and temperature to allow the microzooplankton to develop before seeding the tanks with newly hatched fish larvae. After the opening of the mouth, enriched rotifers (*Brachionus plicatilis*) were added daily to the tanks (first 3 weeks) and later on newly hatched and enriched *Artemia* sp. (1.5 weeks). Live feed addition lasted for five weeks. *Nannochloropsis oculata* and *Isochrysis galbana* (10 l) were added in equal parts to the tank in a pseudo-green water methodology and whenever the tank became transparent more algae were supplied. Addition of algae had three objectives: i) to feed the plankton; ii) to create a more protected environment for early larvae (they avoid direct sunlight); and iii) to reduce cannibalism in older larvae by shading the water. After larval settlement larger types of natural food (sardine muscle) were delivered until complete weaning into artificial diets. Formulated feed in micropellets (500µm) were distributed manually 3 times daily, in early morning, midday, and late afternoon.

Larval growth in length was determined from the exponential regression of standard length on days after hatching. Specific growth rate, in length (percent per day), was determined as $100(e^{G-1})$. Larval minimum energy requirement for growth was estimated by adapting the methodology followed by Yoshinaga et al (1994) where metabolism and ingestion were assessed through respiration and dry weight. To calculate grouper larvae dry weight (DW) we first converted larval length into ungutted wet weight (WW) using a power regression obtained with older larvae and juveniles (33-123mm) and assumed the DW to be equal to 20% the WW. Respiration was estimated based on the regression equation between respiration rate, R (in µl O_2 ind^{-1}.h), and body dry weight, DW (in mg dry weight $ind.^{-1}$) for red sea bream (*Pagrus major*) post larvae and juveniles at 20°C (Yoshinaga et al., 1994). These respiration rates can be considered as routine metabolism (R_{rout}) since they did not include either active swimming or feeding energy loss as described by Yoshinaga et al. (1994). To account for daily energy losses of larvae routine metabolism was multiplied by 2.0 to get the active feeding metabolic rate (F) and this conversion factor was applied only to daytime (14.5 light hours during the rearing period). The resulting equation to calculate food ingestion was $F = 2.0 \bullet 1.6\ R_{rout} = 3.2\ R_{rout}$. Grouper is a carnivorous fish and the respiration quotient should be close to 0.8 implying that 1µl of oxygen combusts 0.43µg of organic carbon. Similarly to *Pagrus major* we assumed a carbon weight:dry weight conversion factor of 0.4.

Based on the estimated respiration rates and body weights and on the assumptions described above, minimum daily food requirements (in terms of carbon) were calculated for grouper larvae at the time of: mouth opening, oil globule exhaustion, beginning of notochord flexion and beginning of metamorphosis. Individual dry weights of the main zooplankters (groups or species) present in the mesocosms were estimated according to the weight/length relationships in van der Meeren (1991) and in Uye (1982) and the mean length of the pertinent taxa was taken from the literature. Dry weight was converted into carbon content assuming a conversion of 43% and 53% in the case of copepods and bivalvia and polychaeta, respectively (Uye, 1982).

Results

There were two different trendlines in the growth curve of dusky grouper larvae: one for the growth of larval stages from first feeding to beginning of metamorphosis and the second for growth of larval stages during transformation. Metamorphosis occurred at 25dph and 20±1.2mm (SD) in total length and settlement started to occur at 22±1.3mm (30dph). Before metamorphosis, the percentage of daily growth was 9% but declined significantly during transformation to 3% per day. Survival at the beginning of transformation was estimated to be 25-50% in 2008. 60dph, when all benthic juveniles were already well weaned onto dry food, survival was 6%. There was high mortality during transformation.

The relationship between juvenile grouper body wet weight and standard length was highly significant and the power coefficient for SL was 3.156±0.020 SE (t=154.648, df=276, P<0.001) and the intercept (log a) was -2.114±0.039 SE (t=-54.675, df=276, P<0.001). This relation was used to determine individual dry weight of the smaller larvae that was then converted into carbon weight assuming a conversion factor of 40%. Individual dry weight was also used to calculate the respiration rate as described for red sea bream (*Pagrus major)* post larvae and juveniles at 20°C (Yoshinaga et al, 1994). Information on mean larval length, body weight, metabolism, and minimum daily food requirements (in terms of carbon) for larvae at the time of mouth opening (2dph), oil globule exhaustion (5dph), beginning of notochord flexion (15dph), and beginning of metamorphosis (25dph) is shown in Table I. Body carbon weight at each larval stage was, respectively, 15, 32, 412, and 5311µg C.larvae^{-1}. With increasing body size both metabolism and ingestion per unit body weight decline from 12.6 to 7.9 and from 40.3 to 25.2% respectively, although the minimum daily carbon requirement also increases with age.

Table I. Carbon budget for metabolism and ingestion of dusky grouper larvae based on estimated respiration rate at 20°C.

Age (dph)	Standard length (mm)	Body weight (µg C ind.$^{-1}$)	Routine metabolism		Ingestion	
			(µg C ind. day^{-1})	(% body C.day^{-1})	(µg C ind. day^{-1})	(% body C.day^{-1})
2	2.7	14.8	1.9	12.6	6.0	40.3
5	3.5	31.9	3.8	11.8	12.1	37.9
15	7.9	411.9	39.8	9.7	127.2	30.9
25	17.7	5311.7	417.9	7.9	1337.2	25.2

Assuming that at the opening of the mouth (i.e., at 2dph) grouper larvae eat only individuals between 100 and 160µm, each larva will need to eat 96 individuals within this size range to fulfil its minimum daily carbon requirement (Table II). As the larvae grow they eat larger prey and the minimum number of prey required per day decrease. However at the beginning of metamorphosis (~25dph) daily food requirement is high and, even considering only the larger zooplankters in the mesocosms and *Artemia*, the minimum number of prey per larva is very high at 513 individuals.

Table II. Characteristics of prey size and estimated minimum number of daily prey required for different stages of pre-metamorphic dusky grouper larvae.

Age (dph)	Standard length (mm)	Minimum food required (µg C ind.$^{-1}$ day)	Size of prey		Minimum prey required (ind. day^{-1})
			Length range (µm)	Mean weight (µg C ind.$^{-1}$)	
2	2.7	6.0	100-160	0.06	94
5	3.5	12.1	100-275	0.24	51
15	7.9	127.2	275-860	1.9	67
25	17.7	1337.2	610-860	2.6	513

Discussion

The results of this study show that dusky grouper larvae can be successfully reared in semi-intensive mesocosms using the natural bloom method with addition of rotifers and *Artemia* in later developing stages. In the mesocosm, grouper larvae had available a large variety of prey of different sizes from phytoplankton to different larval stages of copepods to meet their basic nutritional needs. Pre-metamorphic specific growth rates, in length, of dusky grouper larvae were high ($9\%.day^{-1}$), and at 10dph the larvae attained an average length of 5.2mm, a size at which *Brachionus* spp. could easily be ingested. According to our data, daily growth was positively related to the amount of endogenous prey and *Artemia* available to the larvae. Considering a density of 1 larva per litre, the density of endogenous prey at seeding should be at least 100 $ind.l^{-1}$ of which 25% are small ciliates, 40% copepod nauplii, and 20% copepodids. These numbers appear to be appropriate to propagate nauplii for first feeding larvae up to a size compatible with rotifer feeding at the exhaustion of the oil globule. If the concentration of endogenous zooplankton does not reach the above densities additional filtered zooplankton (55µm) may be added to fulfil this requirement. At an age of 10dph small amounts of recently hatched *Artemia* (5 $ind.l^{-1}$) should start to be delivered to the tanks in order to check their acceptability by the larvae. If *Artemia* are well accepted, the density should be increased steadily so that the total prey density larger than 275µm should be at least 70 $ind.l^{-1}$ per larva at 15dph. Minimum food requirements from this time until beginning of metamorphosis (~25dph) increase exponentially and the number of larger prey organisms in the mesocosm at this age should be adjusted daily to attain a value higher than 520 per larva at 25dph. Rotifer feeding should not be discontinued until 20dph in order to keep the level of encounter rates high. Specific growth rates of dusky grouper are high and at this age they are well into the post-flexion stage and have a mean total length of 11.8mm, a size compatible with larger prey like 24h-enriched *Artemia*. Simultaneously shredded polychaetes and/or fish muscle pellets and high protein formulated feed in micropellets should be distributed to larvae in controlled quantities to meet the high energy requirement at these ages and to decrease mortality and cannibalism during larval metamorphosis.

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POTENTIAL VALUE OF *NAVICULA INCERTA*, *PROSCHKINIA* SP., *NITZSCHIA* SP., AND *AMPHORA* SP. AS FEED FOR *HALIOTIS TUBERCULATA COCCINEA* POST-LARVAE: EFFECT OF INOCULUMS DENSITY ON ALGAL GROWTH RATES

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Introduction

Benthic diatoms are the principal food source for postlarval abalone (Kawamura, 1996,). Various studies found a relation between benthic diatom density and postlarval abalone settlement, growth, and survival (Searcy-Bernal et al., 2003, Daume et al., 2004, and Roberts et al., 2007). However, only few studies have focused on their nutritional quality and the influence of algal biochemical composition on newly settled abalone (Daume et al., 2003; Gordon et al., 2006; Uriate et al., 2006; Viana et al., 2007). The present study examines the characteristics of four benthic diatoms species and the effect of density on their growth response and nutritional quality for abalone, in terms of their proximate and fatty acid composition.

Material and methods

Diatom cultures
Four species of diatoms *Navicula incerta*, *Proschkinia* sp., *Nitzschia* sp., and *Amphora* sp. were selected and grown in f/2 medium (Guillard, 1975) plus silicate under continuous light of 62±8µmol photon.m^{-2}.s and 28.5±1.4°C. Growth curve and cell attachment capacity were determined at initial inoculums of 0.05×10^6 cells.ml^{-1}, 0.10×10^6 cells.ml^{-1}, and 0.25×10^6 cells.ml^{-1} for each species, and growth was monitored during 7 days. Each species, inoculated at 0.10×10^6 cell.ml^{-1}, were harvested in logarithmic and stationary phases of growth for biochemical analysis.

Cell counts and growth
Average growth rates were estimated using the following formulae:

$\mu = \ln (N_1/N_0)/ t_1-t_0$ (Guillard, 1973).

Ten randomly chosen fields of view were photographed at 400× and attached cells were then counted. The number of cells.mm^{-2} was calculated.

Analytical methods

Triplicate samples of freezed cells from each of the experimental cultures were analyzed for total lipids (Folch et al., 1957), protein (AOAC 1995), carbohydrate, ash, and total fatty acids (Izquierdo et al., 1989).

Data analysis

An analysis of variance (one-way ANOVA) was performed with growth rate, cell number, lipid, protein and fatty acids as the source of variance. Data showing significant differences ($P<0.05$) were analyzed by paired comparisons using Tukey's HSP test. Equality of variance was assessed with Bartlett's test.

Results

Biometric parameters and growth rates

GR were significantly higher ($P<0.05$) for all species when inoculated at 50×10^3 cells.ml^{-1}, the highest ($P<0.05$) being for *Proschkinia* sp. in all treatments (0.47-0.65μ.day^{-1}), while cell production at stationary phase harvest was higher for all species when inoculated at 100×10^3 cells.ml^{-1} (Table I) The GR found in the present study suggest a nutrient limitation and/or a shadowing effect when original inoculums density was increased as well as the effect of cell size on nutrient assimilation (Richmond, 1986). Attachment capacity was the highest at low inoculums' density for all species except *Proschkinia* sp. and *Amphora* sp. had the highest cell attachment capacity.

Table I. Growth rate and cell count at harvest of four species of diatoms grown at three different densities, (mean±SD, n=9)

Species	**Densities (10^3 cells.ml^{-1})**		
	50	**100**	**250**
Growth rate μ (day^{-1})			
Amphora sp.	0.47±0.01^a	0.43±0.00^b	0.29±0.01^c
Nitzschia sp.	0.47±0.00^a	0.44±0.00^b	0.29±0.00^c
Proschkinia sp.	0.65±0.01^a	0.58±0.01^b	0.47±0.01^c
Navicula incerta	0.35±0.01^a	0.30±0.00^b	0.16±0.02^c
	Cell count at stationary phase harvest ×10^6 (ml^{-1})		
Amphora sp.	1.37±0.09^b	2.02±0.05^a	1.92±0.16^a
Nitzschia sp.	1.31±0.05^c	2.22±0.05^a	1.92±0.04^b
Proschkinia sp.	4.61±0.37^b	5.81±0.43^a	6.57±0.42^a
Navicula incerta	0.60±0.04^b	0.83±0.06^a	0.80±0.10^a

Different superscripts across a row indicate difference between growth rates and densities at 95% level (ANOVA Tukey's, test; $P<0.05$)

Production of nutrients

All diatoms except *N. incerta* showed a significantly ($P<0.05$) higher lipid content during the exponential phase (Table II). Protein content followed a similar trend. Carbohydrate content followed a trend inverse to that of lipid content. No significant differences ($P>0.05$) were found in the ash content among all diatoms cultures. Brown et al. (1996, 1997) reported these facts as typical of cultures becoming nutrient limited. *N. incerta* increased its storage products as lipid instead of carbohydrate that could be an indication of silicon deficiency. *Amphora* sp. and *Proschkinia* sp. presented the highest protein, lipid, and carbohydrate content in their logarithmic growth, as well as good energy contents. These values being within the range needed for juvenile abalone they can be considered suited to cover abalone postlarvae nutritional requirements.

Table II. Proximate chemical analysis (% dry weight) and gross energy (GE) at an initial inoculum of 1x 10^5 cells.ml^{-1} and harvested in logarithmic (Exp) and stationary (Sta) phase of growth, (mean±SD, n=9)

Species	Growth Phase	Lipid (%)	Protein (%)	Ash (%)	Carbohydrates (%)	GE (kJ.g^{-1})
Amphora sp.	Exp	9.74±1.70^{a}	19.70±0.95^{a}	57.10±3.40	13.50±5.01^{cd}	10.82
	Sta	7.31±0.68^{cd}	13.07±0.71^{bc}	60.50±0.90	19.15±1.77^{bc}	9.24
Navicula incerta	Exp	6.11±0.17^{de}	13.00±0.60^{bc}	53.70±3.80	27.23±3.77^{a}	10.16
	Sta	8.88±0.63^{ab}	13.00±1.07^{bc}	58.00±0.43	20.20±0.70^{abc}	10.00
Nitzschia sp.	Exp	4.90±0.55^{ef}	14.50±0.40^{b}	62.00±1.50	18.70±1.70^{bc}	8.56
	Sta	3.11±0.24^{g}	14.20±1.22^{b}	61.00±0.00	21.80±1.21^{ab}	8.33
Proschkinia sp.	Exp	7.82±0.72^{bc}	20.72±1.40^{a}	61.40±8.02	10.05±6.70^{d}	9.68
	Sta	4.00±0.56^{fg}	11.60±1.93^{c}	60.80±2.80	23.73±5.03^{ab}	8.39

Different superscripts down a column indicate means which differ significantly at 95% level (ANOVA tukey's test; $P<0.05$)

Fatty acid composition

Polyunsaturated fatty acids (PUFA) constituted the largest fraction of the total fatty acids (TFA). The proportion of the various PUFAs varied among the diatom species and between growth phases. All of the analyzed diatoms had significant quantities of 20:5n-3 (eicosapentaenoic acid - EPA) *Proschkinia* sp. had the highest quantity of 20:4n-6 (arachidonic acid - ARA). *Proschkinia* sp., *Nitzschia* sp. and *Navicula incerta* showed a decrease in EPA and ARA between the logarithmic and the stationary phase. Levels of 22:6n-3 (docosahexaenoic acid - DHA) (0.19%-1.90% TFA) increased between logarithmic and stationary phase and were generally low among the diatoms tested and are also reported to be low in abalone tissues. The fatty acid profiles of the diatoms tested were characteristic of most diatoms. Based on previous abalone nutritional studies (Mai et al., 1995, Daume et al., 2003, Gordon et al., 2006, Viana et al., 2007), the levels of PUFA, n-3 PUFA and more specifically EPA and DHA found in this study suggest the diatoms tested could be suited for *H. tuberculata coccinea* postlarvae and fulfill their nutritional requirements.

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DIETARY VITAMINS C AND D AFFECT THE SKELETAL DEVELOPMENT OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) LARVAE

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The induction of deformities due to inadequate amounts and proportions of vitamins has been demonstrated in several fish larvae species. This is mostly caused by the disruption of some molecular mechanisms underlying the skeletogenesis process. Experiments conducted in European sea bass showed that lower proportions of vitamins stimulated PPARγ expression in conjunction with low BMP4 expression, suggesting the differentiation of adipocytes from stem cells in detriment of osteoblast formation during the first two weeks of life. This regulation of osteoblasts differentiation pathway by dietary vitamins could likely cause skeletal deformities. Although the level of vitamin mix adapted by the NRC (1993) for the fish larval period gave the best larval growth, survival, and morphogenesis, the percentage of malformations remained still too elevated. The investigation therefore of the effects of each vitamin separately on European sea bass larval ontogenesis is needed to determine more accurately the dietary vitamin levels inducing adequate larval morphogenesis.

Vitamin C (ascorbic acid, AA), a water soluble vitamin, acts as a co-substrate for hydroxylase and oxygenase enzymes involved in the biosynthesis of procollagen, carnitine, and neurotransmitters, among other numerous physiological functions such as antioxidant or pro-oxidant. In general, fish are unable to synthesize AA due to the lack of the last enzyme of the biosynthetic pathway (L-gulonolactone oxidase). AA must therefore be supplied through the feed. For European sea bass, among different AA (L-ascorbic acid phosphate) levels evaluated, 50mg $AA.kg^{-1}$ diet gave the best larval growth and skeletal performance and lower and upper levels ranging from 0 to 400mg.kg^{-1} diet, promoted cartilage damage. Due to the influence of AA on cartilage formation, most of deformities were found in skeletal elements that underwent chondral ossification, especially jaws and some structures of the caudal fin complex (epurals and specialized neural arch). Absence of AA caused 100% mortality at the end of the

larval period, while insufficient amounts, revealed by an over stimulation of the vitamin C receptor (Svct1) expression, led scorbutic larvae characterized by cartilage reduction and high frequency of specimens with one vertebra lost. Additionally, high levels of AA produced specimens with supernumerary vertebrae. Both too low and too high levels of AA negatively affected the normal BMP4 and VDR expression patterns for suitable skeletogenesis. Besides, the expression of PPARγ was stimulated in concomitance with low levels of osteocalcin expression leading to a poor mineralization. In fact, there was an inverse correlation between skeletal development and incidence of malformations, and specimens fed with 50mg $AA.kg^{-1}$ diet presented the most ossified skeleton and the fewer incidences of skeletal deformities at the end of the larval period.

Vitamin D is crucial for maintaining calcium and phosphate homeostasis and protecting skeletal integrity. This hormone functions through the vitamin D receptor (VDR) inducing the expression of various calcium binding and transport proteins in the intestine to stimulate active calcium uptake, thus preserving normocalcemia and, indirectly, maintaining bone mineralization. Besides, vitamin D also acts directly on osteoblasts to inhibit proliferation, modulate differentiation, and regulate mineralization of the extracellular matrix. As it happens with AA, fish obtain the required vitamin D by the ingested food. Dietary vitamin D_3 (1,25-dihydroxy vitamin D, VD_3) had a great impact on the European sea bass digestive system ontogenesis that was reflected at bone level. Dietary VD_3 levels lower and upper than $27.6IU.g^{-1}$ diet induced a delay of the intestinal maturation affecting TRPV6 expression, the major transcellular mediator of Ca^{2+} uptake from the intestinal lumen, and therefore, the following pathways of the ossification process. Besides, the level of VD_3 during the early stages of development was critical since it influenced the differentiation of osteoblastic cells via the BMP4 pathway, this also affecting VDR and osteocalcin expression levels, necessary for bone mineralization. Low VD_3 produced poorly mineralized larvae, while higher VD_3 levels promoted a retarded mineralization causing several malformations. Vertebral and branchiostegal deformities were maximised at the lower ($11.2IU.g^{-1}$ diet) and higher VD_3 levels (42 and $120IU.g^{-1}$ diet), whereas pugheadness and light caudal-fin deformities were maximised at only the lower VD_3, suggesting that skeletal elements developed at the early developmental stages (jaw and caudal elements) are more resistant to the high VD_3 levels than those developed in the later stages (vertebrae and branchiostegal rays).

Although other vitamins such as vitamin A, E and K are also essential for an adequate skeletal development, these findings indicate that vitamin C and D requirements vary during the larval development and skeletogenesis and, therefore, dietary vitamin levels should be formulated accordingly.

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ONTOGENY OF CLOCK MECHANISMS IN RAINBOW TROUT (*ONCHORHYNKISS MYKISS*) AND SEA BASS (*DICENTRARCHUS LABRAX*) DURING EARLY DEVELOPMENTAL STAGES

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Introduction

The study of the molecular clock mechanism in fish species other than Zebrafish (*Danio rerio*) is still in its infancy (Davie et al., 2009). However, processes as diverse as spawning, embryonic development, and larval metamorphosis are all known to have strict temporal order in many species. Clearly descriptions of the molecular time keeping mechanisms that may drive these processes are of great importance. The aim of this work was to identify the presence of clock systems in the embryos/larvae of Rainbow trout and European sea bass and describe their expression through development windows as well as investigate the impact of continuous photoperiod regimes on their expression.

Materials and methods

Rainbow trout embryos were maintained at constant temperature, from fertilisation, under either a 12h:12h Light:Dark photocycle (LD) or continuous illumination (LL) until just post hatch (Hatching occurred at 350°C.days). At fertilisation and 60, 150, 300, and 420°C.days embryos were collected and stored at -70°C every 4 hours over a consecutive 24-hour period. qRT-PCR assays were established to measure *Clock*, *Period 1*, and *aanat-2* expression in all conditions with results being normalised to *β-actin*. European sea bass embryos were maintained from hatch under either continuous illumination (LL), continuous darkness (DD) or a 12h:12h Light:Dark photocycle with the light being either white (LDw), Blue (LDb) or red (LDr). The presence of a diel cycle in mRNA expression of *Period 1* was measured by qRT-PCR from 0-5 days post hatch (DPH) as well as at 16 DPH.

Results and discussion

In rainbow trout both the clock genes (*Clock* and *Period 1*) were actively expressed in rainbow trout embryos. *Period 1* mRNA expression cycled under both

12:12 and LL conditions with expression peaking at ZT 02:00 under the 12:12 photoperiod while the same cycling expression under LL appeared to be dampened and phase advanced by approximately 4 hours. However, *Clock* mRNA expression was arrhythmic under both photic conditions. The rate limiting enzyme in pineal melatonin synthesis is *Aanat-2* and its functional expression is used to monitor the embryonic development of the pineal system (Gothilf et al., 1999). *Aanat-2* mRNA abundance showed a diel cycle elevating in the night under the 12:12 photoperiod as early as 150°C.days, while no such similar cycling was apparent under the LL treatment. This confirms the independence of melatonin synthesis cascade from clock mechanisms in salmonids.

In European sea bass, *Period 1* mRNA expression was influenced by both the photoperiod and light spectral content. Where as in rainbow trout, *Period 1* cycling was evident in embryos, no clear rhythmic expression was apparent until 5DPH in sea bass larvae reared under LDb or 16DPH under LDw. In all other treatments while *Period 1* is actively expressed there was no clear diel cycling to the expression.

Conclusions

Overall these results represent valuable insights into the potential significance of clock mechanisms in teleost larval development. Clearly due to the diversity of life history strategies in teleosts there is potential for variability in the way different species use this system.

This is the first functional demonstration of clock rhythms in rainbow trout embryos and as such it highlights a number of interesting possibilities. *Period 1* cycling appears before the development of classical photoreceptors (Osthlom et al. 1987) suggesting this cycling is initiated either by as yet unidentified photoreceptors or is passed to the oocyte via maternal mRNA (Delaunay et al. 2000). However the mismatch in *Period 1* expression between photic conditions adds further weight to the suggestion that unidentified photoreceptor mechanisms are in operation.

The lack of clear cycling of *Period 1* in Sea bass does not dismiss the role of clock mechanisms in the early stages of development of this species. Rather, it highlights that other candidates from the canonical clock mechanism must be investigated.

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THE BACTERIAL STORAGE COMPOUND POLY-β-HYDROXYBUTYRATE (PHB) INCREASES GROWTH PERFORMANCE AND INTESTINAL MICROBIAL DIVERSITY IN JUVENILE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX)*

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Introduction

The bacterial storage compound poly-β-hydroxybutyrate (PHB) has potential use as alternative anti-infective strategy for aquaculture rearing (Halet et al., 2007). However, its positive impact has only been reported for the aquaculture model organism *Artemia fransiscana.* In this research, the effects of (partially) replacing the feed for juvenile sea bass with PHB were investigated regarding the fish growth performance and changes in the fish intestinal microflora.

Materials and methods

During a 6-week trial period, juvenile sea bass with an initial average weight of 1.8g were fed 6 different PHB treatments: a non-fed treatment and a 0%, 2%, 5%, 10%, and 100% substitution of the normal feed (w/w) by PHB. All the diets were dosed at 3% fish wet body weight per day. At weekly intervals, the fish wet weight and intestinal pH was determined. Samples of the intestinal matter were taken for analyzing the shifts in the microbial community composition and the bacterial diversity. The latter is expressed as the range-weighted richness (Rr), a new parameter calculated for a microbial community based on its DGGE band pattern (Marzorati et al., 2008).

Results

About 90% of the fish fed with the 0%, 2%, 5%, and 10% PHB diet survived. For the non-fed sea bass, the survival decreased to 40% while feeding with

100% PHB resulted in a survival of 75% (survival data not shown). The diets with 2% and 5% PHB induced in a significant increase of the average fish weight gain to 243% and 271%, respectively, relative to 216% in the 0% PHB treatment (Fig. 1). The PHB level of 10% in the diet resulted in the lower weight gain of 209%.

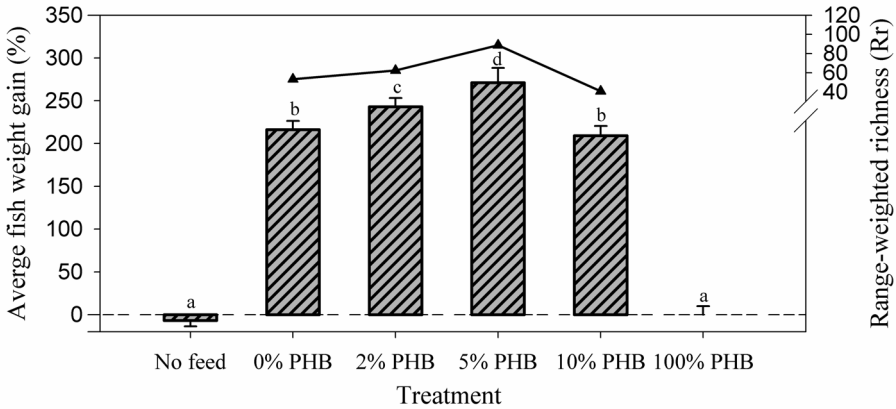

Fig. 1. Average fish weight gain of European sea bass juveniles (hatched) and range weighted richness of the gut microbial community (line) after a 6-week feeding trial with diets containing different levels in PHB (n = 8, different letters indicate significant differences for the average weight gain)

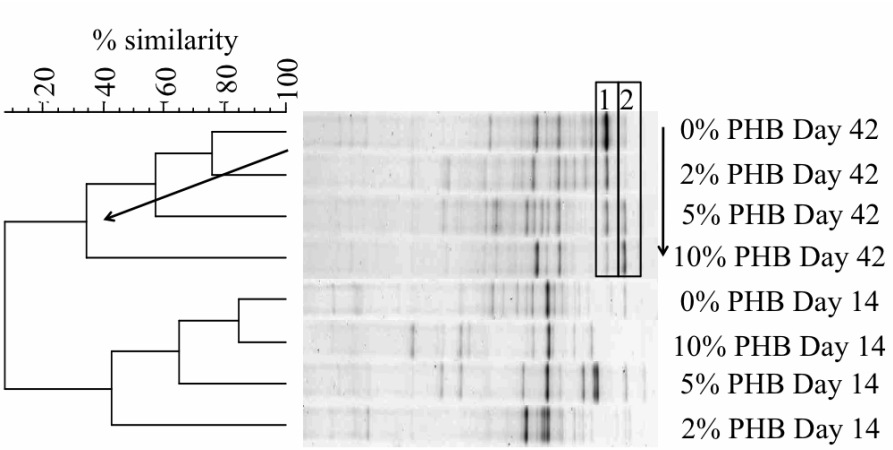

Fig. 2. DGGE band patterns based on the amplified bacterial DNA extracted from the gut of European sea bass juveniles. After 42 days, higher PHB levels induced larger changes in the microbial community (see arrows).

The diversity of the gut microbiota, calculated as the range-weighted richness (Rr) from the DGGE band patterns on day 42 in Figure 2, revealed a highly similar trend to that of the average fish weight gain (Fig. 1). For the microbial community composition, a trend of larger changes (= lower % similarity) at higher dietary PHB levels could be observed after 42 days of feeding (Fig. 2). Also, the presence of PHB in the gut affected individual microbial species (Fig. 2, frames 1&2).

Finally, lower gut pH values were observed at higher PHB levels (Fig. 3).

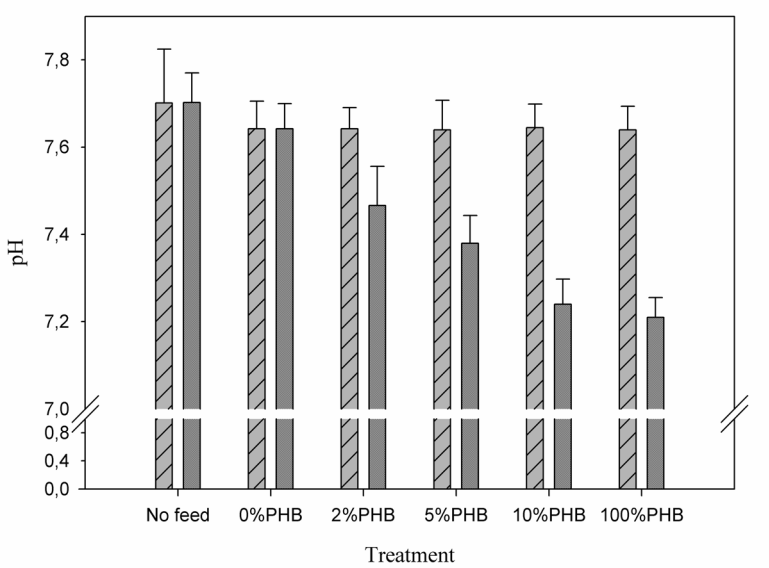

Fig. 3. pH values in the gut of European sea bass juveniles before (hatched) and after a 6 weeks (solid) feeding trial with diets containing different levels in PHB (n = 8, different letters indicate significant differences)

Discussion and conclusions

PHB showed the ability to act as an energy source for survival in the absence of feed, confirming that PHB was degraded and taken up by the fish during gastro-intestinal passage (Defoirdt et al., 2007). The increasing shifts in the microbial community at higher PHB levels and the corresponding decreases in pH make it likely that microbial activity was closely related to the intestinal transformation of PHB. The presence of PHB at the 2% and 5% level seemed enough to increase the growth performance of the fish, whereas the lower growth at 10% PHB indicated a limited availability of the basic feed. Based on the similar trends between the average fish weight gain and range-weighted richness, it is hypothesized that higher bacterial diversity was causal to the increased growth

performance. More research is currently being performed to evaluate host-microbial interactions by these biodiversity-functionality analysis parameters.

Acknowledgements

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THE CULTURE OF ROTIFERS IN A HIGH-DENSITY FLOW-THROUGH SYSTEM USING A COMPLETE DRY CULTURE DIET

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Introduction

Rotifer production remains a labour intensive and uncertain activity for marine fish hatcheries. One of the main problems in rotifer production is the unpredictability of the batch culture system because of the unexpected occurrence of crashes. Therefore the system requires several duplicate and back-up cultures. Semi-continuous culture systems primarily increase productivity since a daily harvest is allowed compared to batch cultures with harvests after 2 or 3days of culture. Several alternative systems to batch culture are already in use but still some bottlenecks exist, mainly dealing with tank management and the culture diet. Rotifer culture food should have a long shelf life and result in a high rotifer performance at reasonable production costs. In this work, some results are presented culturing rotifers (*Brachionus* biotype *Cayman*) in a flow-through system for several weeks on a new diet S.tream® (Inve Aquaculture NV, Dendermonde, Belgium).

Material and methods

Rotifer cultures were performed in 1200-l cylindroconical tanks, equipped with a central aeration and 1 central oxygen diffuser. The water-inlet was supplied through a header tank, containing pre-heated (25°C) and clean diluted seawater of 25g.l^{-1}. Daily water renewal was done at 100-200%. The outlet filter in the tank was made of stainless steel material (Trislot®) with slit openings of 50micron. Besides this, 2 flock filters based on the air-water lift principle and Scotch Brite® material were suspended in the tank to keep the culture water clean. The feeding was done manually, 4 times per day. The daily amount of S.tream® supplied was ranging between 0.35 and 0.42g.million^{-1} rotifers.

Rotifers were inoculated at a density between 2000 and 3000.ml^{-1}. The working density was maintained at 3000.ml^{-1}, meaning that daily the amount exceeding 3000.ml^{-1} was harvested. Several runs were performed, each over a culture pe-

riod of 7 days, doing a daily flush and a daily cleaning of the water-outlet filter and flock filters. After 1 week, all the rotifers were filtered off and rinsed and the culture tanks were thoroughly cleaned. The same rotifers, after rinsing, were put back in the tank to start a new cycle of one week. After having optimized the culture performance in the 1-week cycles, culture periods were progressively prolonged to runs of several weeks each. A comparison was made between the performance in 3-day batch cultures starting at different densities versus flow-through cultures of 21-day cycles.

Results and discussion

The performance of an average 1-week run is presented in Table I.

Table I. Performance of rotifers fed on S.tream® during a 1 week cycle in a 1200-l flow-through system.

Start Density (R.ml^{-1})	2 827
Avg density before harvest (R.ml^{-1})	3 615
Total harvest d1-d7 (million)	6 979
Avg daily harvest (million)	997
Avg daily harvest (mill.m^{-3})	831
Total feed consumed d1-d7 (g)	9680
FCR d0-d8 (g.mill^{-1})	1.39
SGR d0-d7(%.day^{-1})	16
Avg egg ratio d0-d7(%)	25

During this 1-week cycle, the average egg ratio was 25% and the specific growth rate (SGR) was 16% per day, resulting in an average daily harvest of almost 1billion rotifers. Longer culture periods were performed and the most suitable duration was established at 3weeks. This time span was a good compromise to keep the growth performance of the rotifers optimal, maintaining the culture tank sufficiently clean and the competition with ciliates under control. The comparison between the semi-continuous culture of 21days using S.tream® and the traditional batch cultures are presented in Table II.

Table II. Comparison of rotifer performance in batch cultures and semi-continuous culture in flow-through (FT) system. LD: low density batch culture, MD: medium density, HD: high density.

	FT	**LD**	**MD**	**HD**
Working density (R.ml-1)	3000	-	-	-
Start density (R.ml-1)	-	500	750	1000
End density (R.ml-1)	-	1400	1973	2700
Avg daily harvest (mill.m-3)	800	300	400	567
Tank life span (days)	21	3	3	3
FCR (g.mill rotifers-1)	1.5	1.2	1.2	1.13
SGR (%.day-1)	15	32	32	33
Avg egg ratio (%)	25	25	26	22

The average daily harvest is much higher working in the semi-continuous system compared to batch culture systems, even when the latter are performed at high densities. The higher productivity in the system and the increased life span of 21 days compared to 3 days of a batch culture results in a considerable labour reduction in the rotifer section of the hatchery. The specific growth rate (percent population increase per day) is lower in the semi-continuous system because rotifers are harvested on a daily basis (without waiting until the stationery phase is reached). The feed conversion rate (FCR) expressed as the total amount of feed used per million rotifers produced, is slightly higher in the semi-continuous system compared to the batch culture system.

Conclusions

Culturing rotifers on a complete dry diet in a semi-continuous system (flow-through) for a duration of several weeks has proven to be feasible. This method of rotifer production is reducing the workload considerably. Thanks to the intensification and stable method of production, the output per volume culture water has been increased as compared to results obtained in batch culture. Further optimization could be done to improve the FCR by using a continuous feeding system instead of 4 manual feedings, reducing the feed quantity lost by water-exchange.

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MICROBIAL MANAGEMENT IN ASIA-PACIFIC FISH REARING: DATA FROM LAB AND COMMERCIAL OPERATIONS

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Introduction

The success of intensive aquaculture comes from improved genetics, nutrition, and management (including biosecurity and disease control). The high density of larvae and the heavy load of organic matter lead to a fast deterioration of the hygienic conditions in the larval rearing tanks. This creates a favourable environment for bacterial growth, often harmful to the sensitive larval fish that only have partially the innate immune system operational and hence a low resistance against diseases. As a consequence, strict hygienic measures have been recommended (Moretti et al. 1999). Hygiene measures should aim at reducing the chance of pathogen contamination, or the adverse activities of pathogens, rather than sterilizing the rearing environment. A strategy based on the non-selective reduction of bacteria, followed by the selective control of bacterial composition in water and the colonization of the larvae, and the enhancement of the ability of larvae to sustain bacteria in the environment has been advocated by Skjermo and Vadstein (1999).

There has been a trend to move away from prophylactic use of chemicals due to the pressure from the public and regulatory agencies, but also the decreasing efficacy of these treatments.

The benefit of favourable bacteria (i.e., probiotics) has been discussed in numerous scientific publications, but their effect under commercial conditions has been seldom demonstrated, either due to difficulties of working in the field, or due to the high variability in collected data partially due to interactions with other parameters. Furthermore, their use does not remove the necessity of having suitable biosecurity measures or management. Here we report the benefit of using mixtures of *Bacillus* spp. The Sanolife strains of *Bacillus* were checked for safety, and specifically selected for their ability to directly inhibit bacteria pathogenic to fish, to produce exoenzymes for digestion improvement and waste product degradation, and to grow under a wide range of conditions (i.e., pH,

temperature, salinity). Similar mixtures of *Bacillus* are successfully applied in shrimp larviculture (Decamp et al. 2008b)

Materials and methods

Commercially available mixtures of *Bacillus* strains were evaluated in the lab and under commercial conditions:

Inhibition of S. iniae *strains by the 5 Sanolife* Bacillus *strains (Bac 1-5).*
Performance of selected strains against fish pathogens by the cross-streaking method was done at the University of Queensland (Australia) and at INVE Thailand (Thailand), using strains from UQ culture collections and INVE isolates. The inhibition tests were performed on freshly prepared and thoroughly dried TSA plates. *Bacillus spp.* were streaked vertically down the center of the plate using a 10-µl disposable loop, dipped in the prepared standard solution (optical density of 1.2 at 600nm in phosphate buffered saline). *Streptococcus iniae* of 4 concentrations (10^6, 10^7, 10^8, 10^9cfu.ml^{-1}) were streaked away from the central *Bacillus* streak using a 1-µl disposable loop. To investigate the impact of growth phase, *Bacillus* streaks were prepared 24 hours in advance of, or simultaneously with, the *S. iniae* cross streak. Plates were incubated at 27°C for 24 hours, then photographed for digital analysis.

Survival of red tilapia larvae in sex reversal pond
Pond water treatment in a red tilapia *(O. niloticus x O. mossambicus* hybrids*)* sex reversal farm, Thailand. After the typical pond preparation, the pond water was treated using Sanocare PUR disinfectant (INVE), prior to the application of the mixture of *B. subtilis* and *B. licheniformis* strains, giving a final concentration in the pond of 1×10^3cfu.ml^{-1}. After stocking with fry (2000 fry.m^{-2} of average weight 0.12g), the same product was applied at the same dosage once per week, for the 3-week long trial. Another pond was treated with unspecified commercial probiotic (labelled probiotic X)

Performance of red tilapia fingerlings in nursery
Combined water and feed application during red tilapia (*O. niloticus* × *O. mossambicus* hybrids) nursery, Thailand. After the typical pond preparation, Pond 2 received weekly applications of the mixture of *B. subtilis* and *B. licheniformis* strains, giving a final concentration in the pond of 1×10^3cfu.ml^{-1}. Pond 1 was the negative control and did not receive any water probiotic. In each pond, 6 happas were stocked with 300 fish (mean weight 0.54g – total biomass 0.162kg). For each pond, 3 happas were fed with probiotic treated feed and the other 3 happas received non-treated feed. Feed probiotic treatment was the addition of selected strains of *B. subtilis*, *B. licheniformis* and *B. pumilus* at a final concentration of 2×10^7cfu.g^{-1} feed.

Results and discussion

Inhibition of S. iniae *strains by the 5 Sanolife* Bacillus *strains (Bac 1-5)*

Table I. Performance of selected *Bacillus* strains against *S. iniaie* strains. Levels of inhibition: 0= no inhibition; 1=1-5mm inhibition zone; 2=6-10mm inhibition zone; 3= 11-20mm inhibition zone; 4= >21mm inhibition zone; 5= >21mm after 24hr incubation, and 11-20mm without pre-incubation

Code	Source	Bac 1	Bac 2	Bac 3	Bac 4	Bac 5
QMA0083	*L. calcarifer,* Australia	2	4	2	4	4
QMA0177	*L. calcarifer*, Australia	3	4	1	5	2
QMA0165	*L. calcarifer*, Australia	4	1	3	4	2
QMA0186	*O. mykiss*, Israel	3	3	0	5	2
QMA0187	*Channa striata*, Thailand	3	2	2	5	3
QMA0188	*O. mykiss*, Israel, Israel	3	1	3	4	4
QMA0189	*O. mykiss*, Reunion Island	1	0	3	4	4
QMA0191	*L. calcarifer*, Australia	2	3	0	4	2
QMA0231	*L. calcarifer*, Australia	3	2	2	5	3

This in-vitro test confirmed that all the *Streptococcus* isolates were strongly inhibited by at least 1 of the *Bacillus* strains used in the Sanolife formulations.

Survival of red tilapia larvae in sex reversal pond

Table II. Survival of tilapia in sex reversal pond, under commercial conditions. Individual data were not collected by the commercial operator.

Treatments	Number of happas	Average survival
Negative control	21	82.08%
Sanolife probiotics	14	92.35%
Probiotic X	25	89.31%

This test documented the benefit of pond water treatment with effective microbial products. Within a short period of time, this led to an improvement in survival (up to 12%).

Performance of red tilapia fingerlings in nursery

Table III. Survival, biomass and FCR of fingerlings stocked in happas in 2 ponds. Pond 1 did not receive water probiotic whereas pond 2 received weekly application of probiotics. Within each pond, 3 happas received probiotic-supplemented feed and 3 happas received control feed.

Pond	Treatments	Data after 7 weeks		
		Biomass	Survival	FCR
Pond 1	Negative control	2.56±0.99kg	38.56±18.02%	5.57±1.74
Pond 2	*Bacillus* mixture in pond water only	1.66±0.60kg	39.56±13.73%	2.89±0.87
Pond 1	*Bacillus* mixture in feed only	3.28±0.59kg	57.22±18.92%	3.49±0.24
Pond 2	*Bacillus* mixture in pond water and feed	2.10±0.24kg	59.89±23.56%	2.37±0.30

The data showed:

- large variability between ponds, not necessarily explained by the treatment.
- for the non-treated pond water (pond 1), the benefit of applying the probiotic in the feed with improved survival and FCR
- for the treated pond water (pond 2), the benefit of applying the probiotic in the feed was also documented
- feed with improved survival and FCR

Conclusions

These tests document the ability of selected *Bacillus* strains to directly inhibit pathogenic *S. iniae* strains. The application of *Bacillus* strains in the water and/or in the feed led to improved performance during tilapia nursery and early growout.

Similar applications led to clear benefits in commercial Australian barramundi hatcheries, i.e. cleaner rearing tanks and faster growth (Decamp et al. 2008a), but also in experimental seabream larviculture, i.e. lower expression of HSP70 and glucocorticoid receptor, a higher expression of IGFI and lower levels of myostatin in groups fed on Sanolife probiotics, resulting in a significant increase in fingerling growth (Carnevali and Makridis, pers comm.).

Acknowledgements

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THE NATURAL FURANONE (*5Z*)-4-BROMO-5-(BROMOMETHYLENE)-3-BUTYL-2(*5H*)-FURANONE DISRUPTS QUORUM SENSING IN *VIBRIO HARVEYI* BY DECREASING THE DNA-BINDING ACTIVITY OF THE MASTER REGULATOR LUXR

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Introduction

Vibrio harveyi is becoming increasingly recognised as an important pathogen in aquaculture. Because of antibiotic resistance in these bacteria, alternative biocontrol strategies are needed. One of them is the disruption of bacterial cell-to-cell communication, called quorum sensing (QS). *V. harveyi* uses a three-channel QS system. The three channels of this system are mediated by the Harveyi Autoinducer 1 (HAI-1), the Autoinducer 2 (AI-2), and the Cholerae Autoinducer 1 (CAI-1). The three autoinducers are detected at the cell surface by membrane-bound, two-component receptor proteins that feed a common phosphorylation/dephosphorylation signal transduction cascade (Henke and Bassler, 2004). Recent investigations have pointed out that QS disruption in these bacteria by using halogenated furanones could be a promising alternative biocontrol strategy (Defoirdt et al., 2006). Here, we aimed at defining the molecular mechanism by which these compounds disrupt QS-regulated gene expression in vibrios.

Materials and methods

Vibrio harveyi wild type (BB120) (Bassler et al., 1993) and mutants were grown in LB20 at 28°C under constant agitation. Mutants used were BNL258 (*hfq::*Tn5*lacZ*) (Lenz et al., 2004), JAF375 (*luxN::*CmR *luxQ::*KanR) (Freeman and Bassler, 199a), JAF483 (*luxO* D47A linked to KanR) (Freeman and Bassler, 199a), JAF553 (*luxU* H58A linked to KanR) (Freeman and Bassler, 199b), JMH597 (*luxN::*Tn5 *cqsS::*CmR) (Henke and Bassler, 2004), and JMH612

(*luxPQ::*Tn5 *cqsS::*CmR) (Henke and Bassler, 2004). Spectrophotometry at OD_{600} was used to measure growth. Luminescence was measured with a Lumac Biocounter M2500 luminometer (Lumac b.v., Landgraaf, The Netherlands). Reverse transcriptase realtime PCR with specific primers (designed based on the *luxR* sequences in GenBank) was performed according to standard procedures. LuxR was purified as described previously (Miyamoto et al., 1996). SDS-PAGE and mobility shift assays with radioactively labelled *luxR* promoter DNA were performed as described previously (Miyamoto et al., 1996).

Results and discussion

Bioluminescence is one of the QS-regulated phenotypes in *V. harveyi.* The furanone blocked bioluminescence in signal molecule receptor double mutants that only respond to one of the three signals (Fig. 1). Importantly, the compound has no effect on the biochemistry of bioluminescence and thus, these results indicate that all three channels of the QS system are blocked by the furanone.

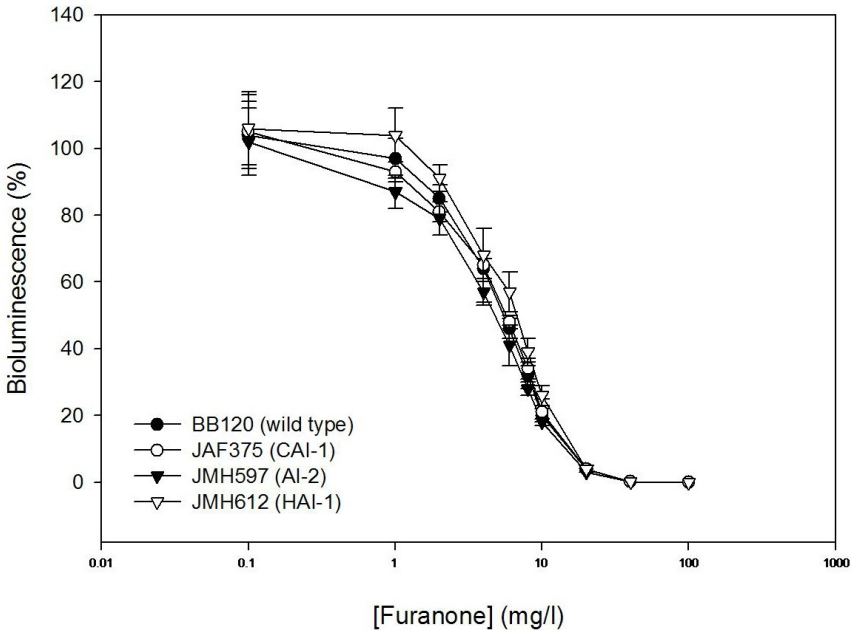

Fig. 1. Bioluminescence of *Vibrio harveyi* wild type and signal receptor double mutants as a function of the furanone concentration. Signals that are still detected by the mutants are between brackets.

Further bioluminescence tests with mutants that are locked in the high cell density configuration at different stages in the signal transduction cascade revealed that the luminescence of constitutively luminescent *luxU , luxO,* and *hfq* mutants

was blocked. This indicated that the furanone target is located downstream of Hfq in the signal transduction cascade. Reverse transcriptase realtime PCR with specific primers showed that the furanone does not affect *luxR* mRNA levels (data not shown). SDS-PAGE of furanone-treated purified *LuxR* protein showed that the furanone does not affect *LuxR* stability, whereas mobility shifts of the same samples showed significantly (± 20-fold) less shifts with furanone-treated *LuxR* (Fig. 2). This indicated that the furanone decreases the DNA-binding activity of *LuxR*.

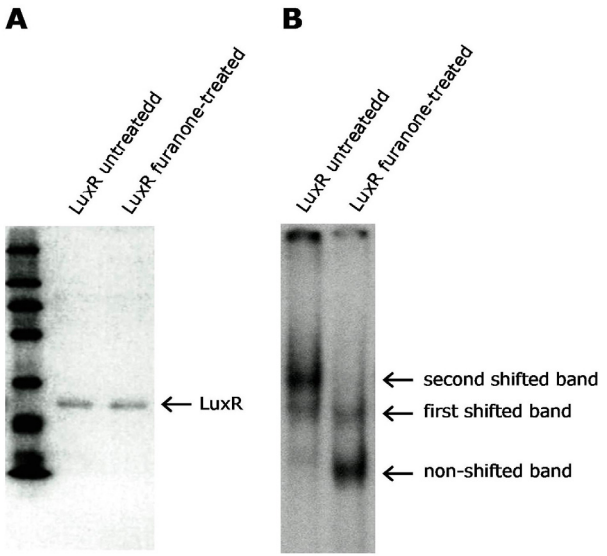

Fig. 2. (A) SDS-PAGE of purified LuxR, before and after treatment with 50 mg l^{-1} furanone and (B) LuxR DNA binding of the same samples as in panel A, as determined by mobility shifts with the *luxR* promoter DNA.

The fact that the furanone affects the master regulator rather than selectively blocking one of the channels of the *V. harveyi* QS system is quite important with respect to practical applications since there seems to be a difference in the relative importance of the three channels for a succesfull infection of different hosts. Indeed, disrupting only the AI-2-mediated channel has been shown to significantly increase survival of the brine shrimp *Artemia franciscana*, whereas the HAI-1-mediated channel had no effect on infection of the shrimp (Defoirdt et al., 2005). In contrast, both the HAI-1- and AI-2-mediated channel needed to be disrupted in order to decrease mortality of gnotobiotic rotifers (*Brachionus plicatilis*) caused by *V. harveyi* (Tinh et al., 2007). Since the furanone blocks all three channels of the system at once by acting at the end of the QS signal transduction cascade, it will not be necessary to develop different furanone compounds to protect different hosts. Consistent with this, the natural furanone was

shown to protect both brine shrimp and rotifers from luminescent vibrios (Defoirdt et al., 2006; Tinh et al., 2007).

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SPAWNING PERFORMANCE AND OFFSPRING QUALITY OF DOMESTICATED BLACK TIGER SHRIMP *PENAEUS MONODON* FED A SEMI-MOIST MATURATION PELLET

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Introduction

Research into shrimp broodstock nutrition is a key element in the development of domesticated stocks for aquaculture. This is certainly the case for *Penaeus monodon* broodstock, because (a) experimental domestication projects with black tiger spawners report low fecundity and poor egg hatching results (Coman et al., 2005; 2007), and (b) *P. monodon* broodstock do not ingest dry maturation pellets as readily as *Litopenaeus vannamei*. Nevertheless, the advantages of formulated broodstock feeds compared to fresh food are generally accepted: biosecurity, availability, consistent nutritional value and easy of use. In addition, essential nutrients, active substances (e.g., hormones) and therapeutics can be added easily.

The present study investigates the potential of a newly-developed formulated broodstock pellet to replace a typical broodstock diet based on fresh-food components. The formulated feed is a semi-moist pellet (BREED-S® FRESH, INVE Aquaculture), intended to be formulated to the specific needs of domesticated broodstock in terms of nutrients, attractants and palatability. The semi-moist feed has a soft texture to promote pellet ingestion by the animals.

Materials and methods

In two independent experiments, spawning and reproductive performance of 11 and 13-month old F2-domesticated black tiger shrimp fed different feeding regimes were evaluated. Females were tagged for individual monitoring, and a stocking density of 4 females and 4 males per 5-m^3 recirculation tank was applied in experiment 1 with 4 tank replicates per treatment. In experiment 2, the tanks were stocked with 6 males and 4 females, with 6 replicate tanks per treatment.

In experiment 1, the shrimp were fed three feeding regimes: 100% mixed fresh food (100FF), 40% soft pellet plus 60% mixed fresh food (40SP), and 60% soft pellet plus 40% mixed fresh food (60SP). The soft pellet BREED-S® FRESH (SP) with 60% protein, 15% lipid (on dry matter) and 28% moisture was imported from INVE Thailand Ltd. In experiment 1, the fresh-food mixture was composed of squid, oyster, marine worm and pork liver and was formulated to resemble ARA/EPA, DHA/EPA, and n-3:n-6 fatty acid ratios of mature ovaries of wild black tiger shrimp (Marsden et al., 1992). In experiment 2, treatments 100FF and 60SP were repeated but marine worms were omitted from the fresh food mixture to reduce the risk of disease transmission (Vijayan et al., 2005).

The shrimp were fed 3-4% (on DW basis) of their body weight a day. The experimental feeding started approximately 2 months before ablation, and was continued 5 weeks post-ablation during which the evaluation parameters were monitored.

Results and discussion

The result of both trials (Tables I and II) demonstrated that up to 60% of the fresh food mixture could be replaced with the commercial soft pellet without loss in spawning performance of the domesticated *P. monodon* shrimp: the spawning frequency and fecundity results were very similar (P>0.05). This confirms that the pellets were readily ingested and assimilated, and that the formulated lipid level of the diet supported normal spawning performance.

Table I. Spawning performance and offspring quality of domesticated shrimp breeders fed different maturation diets in experiment 1

	100FF	**40SP**	**60SP**
Female weight (g)	114.5 ± 6.6^{a}	100.9 ± 7.4^{b}	106.8 ± 5.0^{ab}
Spawns per female	3.6 ± 0.5^{a}	3.3 ± 0.5^{a}	3.6 ± 1.1^{a}
Fecundity (eggs/spawn)	$291\,346 \pm 31\,972^{a}$	$277\,647 \pm 71\,736^{a}$	$306\,636 \pm 18\,865^{a}$
Egg hatching rate (%)	62.5 ± 5.74^{a}	66.7 ± 6.0^{ab}	75.0 ± 1.9^{b}
Metamorphosis into zoea (%)	93.1 ± 1.7^{a}	93.6 ± 2.06^{a}	96.3 ± 0.8^{b}

Table II. Spawning performance and offspring quality of domesticated shrimp breeders fed different maturation diets in experiment 2

	100FF	**60SP**
Female weight (g)	119.6 ± 13.8^{a}	120.8 ± 11.6^{a}
Spawns/female	3.1 ± 1.16^{a}	3.3 ± 1.2^{a}
Fecundity (eggs/spawn)	$265{,}949 \pm 56{,}819^{a}$	$273{,}888 \pm 37{,}008^{a}$
Egg hatching rate (%)	66.1 ± 7.6^{a}	77.9 ± 4.8^{b}
Metamorphosis into zoea (%)	92.3 ± 1.5^{a}	93.6 ± 1.6^{a}

Interestingly, feeding the pellet to the broodstock resulted in improved egg and larval quality. The treatment with the highest level of fresh-food replacement (i.e., 60SP) yielded significantly improved ($P<0.05$) egg hatching rates (experiment 1 and 2) and larval metamorphosis rates (experiment 1 only), as compared to the treatment 100FF. This positive effect persisted during the hatchery cycle, as can be seen from the PL15 survival rates in Figure 1. We speculate that this is due to essential nutrients such as carotenoids, vitamins and highly-unsaturated fatty acids provided through the formulated feed as was also shown in previous studies (reviewed by Wouters et al., 2001).

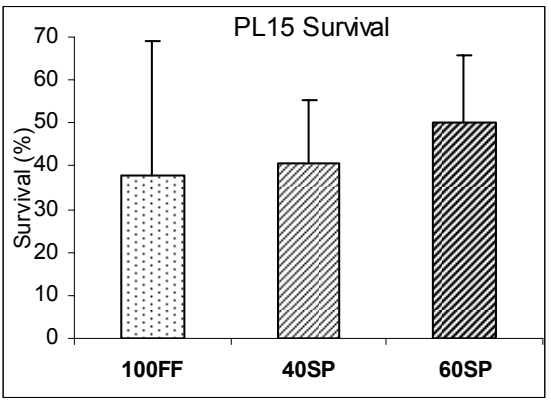

Fig. 1. Survival at postlarval stage PL15 of *Penaeus monodon* offspring originating from treatments 100FF, 40SP and 60SP of maturation experiment 1.

Conclusions

To our knowledge, this is the first study reporting successful results with a soft – yet storable and commercially available – maturation pellet for *P. monodon* broodstock at a fresh-food replacement level above 50%. Furthermore, the study demonstrates that a combination of diversified fresh-food components with a balanced maturation pellet can significantly improve nauplii production, therefore improving the success rate of domestication programs as well as the profitability of shrimp hatcheries.

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SENEGALESE SOLE IS ABLE TO ADAPT PROTEIN METABOLISM WHEN CO-FED WITH *ARTEMIA* REPLACEMENT

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Introduction

Digestibility and protein retention are key issues in defining larval growth performance as well as survival rate. In fact, high digestibility has been shown to correlate with better growth and survival rate in Western Atlantic sea bream larvae (*Archosargus rhomboidalis*) (Houde and Schekter, 1983). The aims of this study were to evaluate the effects of *Artemia* replacement by an inert diet on Senegalese sole growth performance, and understand how protein digestibility and protein retention efficiency may explain these effects.

Material and methods

Larval rearing
Senegalese sole eggs were obtained by natural spawning of captive broodstock kept at the Ramalhete facilities (University of Algarve, Faro, Portugal). Newly hatched larvae were reared in 100-l cylindroconical fibreglass tanks in a closed recirculation system with an initial density of 100 larvae.l^{-1}. Two experiments were performed in order to test several feeding regimes. First, three feeding regimes were randomly assigned to 9 tanks: ST - standard live feed feeding regime (ST treatment); ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening feeding regime (ArtRL treatment); Rot9 – rotifer for a longer period that the ST until larvae reached 9 days after hatching (DAH) feeding regime (for further details see Engrola et al., 2009a). Secondly, three feeding regimes were randomly assigned to 12 tanks: ST - standard live feed feeding regime (ST treatment); ArtRL - live feed and 20% *Artemia* replacement with inert diet (dry matter basis) from mouth opening feeding regime (ArtRL treatment); ArtRH - live feed and 58% *Artemia* replacement with inert diet (dry matter basis) from mouth opening feeding regime (ArtRH treatment) (for further details see Engrola et al., 2009b). Larvae rearing in the first experiment lasted from hatching up to 68DAH, while the second experiment lasted from hatching up to 20DAH.

Postlarvae rearing

The postlarvae rearing done in Experiment 1 was performed to determine if the feeding regimes during the pelagic and benthic phase of sole may influence post-larvae quality and consequently weaning performance. The experimental units consisted of 9 21-l white flat-bottomed fibreglass tanks (width 30cm × length 70cm × height 10cm). Each tank was stocked with 630 postlarvae, corresponding to a density of 3000 individuals.m^{-2}, with three replicates per treatment in a total of 9 tanks. Postlarvae were fed according to the feeding regime tested: frozen *Artemia* metanauplii (50%) and inert diet (60%) until 40DAH (ArtRL), and frozen *Artemia* metanauplii until weaning (ST and Rot9). Postlarvae from ST and Rot9 were sudden weaned at 40DAH, meaning fish were fed inert diet after fasting one day, when their weights were within 5-10mg dry weight (DW) as recommended by Engrola et al. (2007). At 40DAH postlarvae from ArtRL were exclusively fed with inert diet.

Protein utilization trial

Artemia [U-^{14}C] labelling

Artemia was radiolabelled with a [U-^{14}C] uniformly labeled protein hydrolysate (1.85 MBq mL Amersham Pharmacia Biotech Ltd., UK) according to the method developed by Morais et al. (2004a). *Artemia* nauplii were enriched at a density of 200 *Artemia*.ml^{-1} in a sealed incubation system at 28ºC, with a dose of 3.3µl of the above [U-^{14}C] protein hydrolysate per mL of seawater.

Sole metabolic trials

Sole larvae and postlarvae were allowed to eat the radiolabelled *Artemia* during 30 minutes; this period allows the sole to eat during a period of time shorter than gut transit time. After this period fed sole were carefully transferred, one by one with a Pasteur pipette, through two tanks with clean seawater, and subsequently transferred to an incubation vial.

Results and discussion

The *Artemia* replacement with 20% of inert diet from mouth opening (ArtRL) promoted better sole growth and quality at weaning (68 DAH) (see also Engrola et al., 2009a). Previous work already indicated that sole larvae could be co-fed with inert diet from mouth opening (Cañavate and Fernández-Díaz, 1999). Nevertheless the sole weight observed by those authors at the end of the experiment was two-fold smaller than in the present study.

Still, growth of 20-DAH sole fed the *Artemia* replacement (ArtRL) was depressed compared to fish feed exclusively on live feed (both Experiments 1 and 2). Moreover, a high *Artemia* replacement (ArtRH) had a negative impact on sole growth (Experiment 2). Sole weight at 20DAH was 42% lower in relation to

fish fed live feed alone. Sole dry weight at 20DAH decreased with increasing levels of *Artemia* replacement (see also Engrola et al., 2009b).

Sole larvae that were co-fed with inert diet from mouth opening had a higher *Artemia* intake than postlarvae fed *Artemia* alone (Experiment 1). This might indicate that an *Artemia* replacement regime promotes appetite or that the larvae were at sub-optimal feeding status. In fact, the quantity of *Artemia* supplied daily was progressively reduced for co-fed fish, and although larvae were observed ingesting the inert diet, growth was depressed in younger stages (Experiment 1 and 2). Most likely the abundant presence of *Artemia* nauplii in the metabolic trial tanks promoted feed intake, and these results may be taken as an indication of improved appetite. On the other hand, the higher feed intake observed in 21DAH ArtRH sole might be an attempt to restore energy reserves depleted during metamorphosis (Experiment 2). Growth during sole metamorphosis climax is sustained by using energy reserves accumulated during the earlier stages (Parra and Yúfera, 2001).

Sole *Artemia* protein digestibility ranged between 56.97% (16DAH – Experiment 1) and 83.08% (6DAH – Experiment 2). The higher values are comparable to those reported by Morais et al. (2004b) for the same species. During metamorphosis climax (14-18DAH) the *Artemia* protein digestibility of both the live feed and co-fed sole were lower than at younger or older ages (Engrola et al., 2009b). Therefore, ontogeny of the sole digestive capacity was affected by feeding regime. A significant decrease, 0.06-fold and 0.12-fold, of sole protein digestibility was observed between 6 and 15DAH, concomitant with a 0.16-fold and 0.10-fold increase of protein retention efficiency in ST and ArtRL sole, respectively (Experiment 2).

Conclusions

In conclusion, a high co-feeding strategy impairs sole larvae protein utilization and thereby leads to lower larval growth. This depression in sole protein digestive capacity and protein retention efficiency occurs mostly during metamorphosis climax. In addition, the present study confirms previous findings that sole is able to adapt the protein metabolism to a low level of *Artemia* replacement (Engrola et al., 2009c), and such a precocious exposure to dry feed may promote better adaptation to inert diets when weaning is completed.

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WILD ZOOPLANKTON FOR *OCTOPUS VULGARIS* LARVAL REARING

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Introduction

Octopus vulgaris is considered an important species for aquaculture in Spain. Ongrowing is carried out in cages specially designed for the species with 750g subadult octopus separated according to sex and cultivated during a 4-month period until they reach 2.5-3.0kg. However, life cycle has been rarely completed due to the high larval mortality mostly due to the absence of an appropriate life prey. Ongrown *Artemia* (1 to 4mm size) alone or complemented with spider crab zoeae have been used as prey, being the survival of the paralarvae only 1 among 10000 (Carrasco et al, 2006; Iglesias et al, 2004). Several attempts to rear the larvae by means of micro- or encapsulated diets have resulted in no survivors (Villanueva et al, 2002)

We present here the results obtained in octopus paralarval rearing using zooplankton (copepods and *Palaemon* sp. zoeae) captured from a lagoon in which water coming from fish ongrowing area of IRTA facilities have been used.

Materials and methods

Octopus paralarvae were obtained from a matured female generously donated by the Institute of Oceanography in Vigo. The larvae were reared in 500-l, black-coloured tanks connected to a recirculation unit at 20 larvae.l^{-1} using UV-filtered seawater, 18ºC temperature, a 16hL:8hD photoperiod and 500 lux intensity. *Artemia* metanauplii (1-3 nauplii.ml^{-1}) and zooplankton (0.05-0.1 *Palaemon* zoeae.ml^{-1} and 0.02 copepods.ml^{-1}) were used as food. During the first week of larval rearing "green water" technique was used, whereas later water was renewed daily (0.7-1 l.min^{-1}). Prey was administered 4 times per day, 3 during the day and 1 at night.

Larvae were measured every 10 days, whereas samples for biochemical analysis were taken at the beginning and at days 15 and 20. Total and mantle length were

measured using an image analysis whereas wet and dry weight (oven dried at 60ºC for 24h) were measured in a balance.

Zooplankton was captured from the lagoon by means of a light trap in which a 150-µm mesh basket with an air-lift was placed. Plankton was collected at night during July and August 2008 and in the morning counted and separated according to the size of the prey. Only the less than 400µm fraction was used to feed the larvae.

Results

Temperature, oxygen, and salinity in the lagoon where the zooplankton was produced was kept at 26.4±1.2ºC, 6.0±1.2mg.l^{-1}, and 32.8±1.2ppt, respectively. Phytoplankton production was highest from mid to end July (maximum values of 9.54µg.l^{-1}). Quantities of zooplankton collected in the light trap varied between 3750-100 000 zoeae of *Palaemon* and 1400-27 500 copepods per day, as shown in Figure 1.

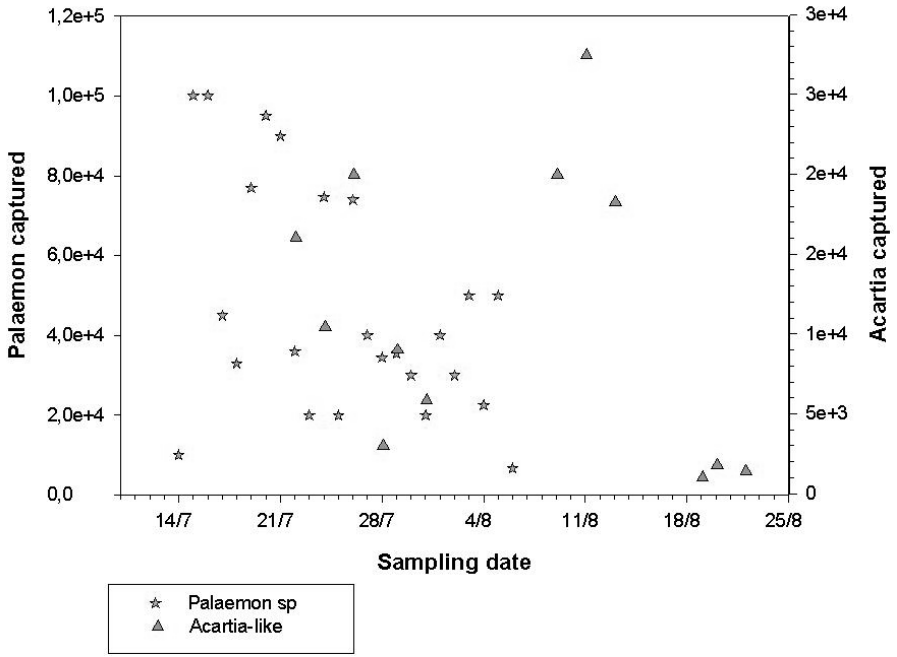

Fig. 1. Zooplankton captured (units per day) and used for larval feeding.

The larvae were fed everyday with *Artemia* metanuplii and zooplankton captured with occasional addition of spider crab zoeae. Although larvae showed ac-

tive feeding on the prey administered, all the larvae died by day 48. Growth in weight of Octopus paralarvae from day 0 to 48 is shown in Figure 2.

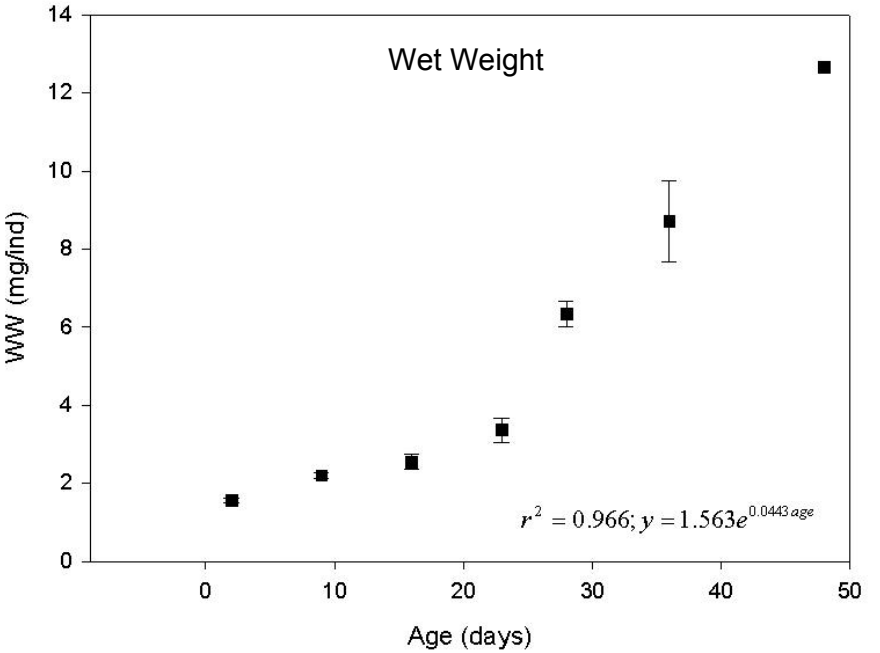

Fig. 2. Wet weight of Octopus paralarvae from day 2 to 48.

Although growth was considered acceptable, data from literature of larvae fed either *Artemia* metanauplii or exclusively spider crab zoea, showed higher results in wet weight than those obtained using wild zooplankton. Considering the results obtained, future experiments will be carried out using lower larval density and adjusting the quantities of zooplankton provided everyday and reducing handling and disturbance to the paralarvae.

Table I. Growth curves (wet and dry weight) obtained in published literature and the present work

	DW vs. age	**WW vs. age**
Carrasco et al. (2006)	$y= 0.3215\ e^{0.0725x}$	
Moxica et al. (2002)	$y= 0.4408\ e^{0.0568x}$	
Iglesias et al. (2004)	$y= 0.3563\ e^{0.0745x}$	
Villanueva (1995)		$y= 1.2468\ e^{0.0808x}$
This work	$y= 0.286\ e^{0.00511x}$	$y= 1.563e^{0.0443x}$

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EGG QUALITY AND BIOCHEMICAL COMPOSITION FROM MEAGRE BROODSTOCK (*ARGYROSOMUS REGIUS*)

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Introduction

The meagre (*Argyrosomus regius*), in common with a number of species of Sciaendae, has good aquaculture potential. The culture of meagre has steadily increased and Spain, France, Italy and Portugal produced 884 tons in 2006 with a value of 4.1 million Euros. Under Mediterranean cage culture conditions meagre grow rapidly, one kilo per year and produce a good quality white fillet with low fat content that has a good price in the market. Duncan et al. (2008) described the acclimatisation of 12 wild broodstock to obtain advanced stages of maturity (vitellogenesis and spermiation) and spawning with the GnRHa induced spawning therapies. The present paper presents for meagre eggs percentage fertilisation, percentage hatch, survival of starved larvae, egg diameter, egg dry weight, larval length, bio-chemical composition (proteins, carbohydrates, and lipids), and fatty acid composition.

Materials and methods

In the present study six wild broodstock (four females and two males average weight of 20kg) were induced to spawn with GnRHa implants or injections (Duncan et al., 2008). The following egg quality parameters were determined: egg diameter, percentage fertilisation, percentage hatch, survival of starved larvae, egg dry weight, larval length, bio-chemical composition (proteins, carbohydrates, and lipids), and fatty acid composition. A sample of the eggs was plated onto a 96-well EIA plate (EIA plates, Nunc) as in Giménez et al. (2006) and incubated in darkness at 17ºC in a refrigerated incubator. EIA plate samples, eggs and larvae were inspected and counted on a daily basis to determine and hatching rate, percentage survival after hatching, mortality rate at 3 and 5 days post hatching (dph), percentage of deformities and time to 100% mortality. Considering that first feeding and point of no return occur in this species at 3 and 7dph, respectively, it was considered that high quality batches (good) of eggs were those with a mortality rate less than 10% between 3 and 5dph and low quality batches (bad) of eggs were those with mortality rates greater than 35% at 3dph.

Samples of floating eggs (n=50) and newly hatched larvae (n=20) were taken for biometry and individual dry weight determination. Dry weight (DW) determination was after oven-drying at 60ºC for 24h. For biochemical analysis samples of about 1 g of eggs and newly hatched larvae were taken in cryovials and frozen at -80ºC. Protein and carbohydrates were analysed, total lipids were extracted from samples, and fatty acid methyl esters (FAME) prepared by acid-catalysed transmethylation. FAME were then extracted and purified (Tocher and Harvie, 1988). FAME were separated and quantified by gas-liquid chromatography (Thermo Trace GC, Thermo Finningan, Milan, Italy) using a 30m×0.25mm ID capillary column (BPX 70, SGE Europe Ltd., UK) with on-column injection and flame ionization detection with Helium as carrier gas (1.2ml.min^{-1} constant flow rate). Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid) and a characterised fish oil, and quantified by the response factor to the internal standard, 21:0.

Results and discussion

A total of 19 spawns were collected of which 14 spawns (10 spawns after the 26th March and 4 spawns after the 4th May) were from the fish treated with implants and 5 spawns (3 spawns after the 26th March and 2 spawns after the 4th May) were from fish treated with a single injection. With the exception of one spawn (0% fertilisation on 7th May from the injected fish, data excluded) fertilisation rates ranged from 82-100% with an average of 94.5±5.4%. Hatching rate was variable, being from 2.4-95% in the eggs of implanted fish and from 12-67% in eggs from injected fish. Larval survival was also variable and 3dph survival of larvae from implanted fish ranged from 16.7-94.5% and from injected fish ranged from 54.4-92.2% and 5dph survival from implanted fish ranged from 0-88.3% and from injected fish ranged from 39.1-78.8%. Good quality spawns (<10% mortality between 3 and 5dph) were obtained from implanted fish on 30th March, 1st, 2nd, 9th and 11th April and from injected fish on the 1st April. Poor-quality spawns (mortality rates >35% at 3dph) were obtained from implanted fish on the 29th March and 4th April and from injected fish on the 29th March. Generally the first spawn on the 29th March from each group was of poor quality followed by one or two good quality spawns and finally in the fish treated with implants some spawns of variable and medium quality. Eggs from implanted fish had an average diameter of 0.99±0.02mm and were significantly ($P<0.05$) larger than eggs from injected fish that had an average diameter of 0.93±0.01mm. No significant differences were observed in dry weight and the average was 54±3.8µg. The average length of newly hatched larval was 2.95±0.2mm. No significant differences were observed in biochemical composition and respective average content of lipid, carbohydrate, and protein were 17.9±2.5% (min 14.8, max 22.8), 5±1.8% (min 2.5, max 8.7), and 31.9±6.7% (min 24.2, max 47.3), respectively. Differences were observed in fatty acid proportions (Table I).

Table I. Average total lipid content (mg g^{-1} DW), total fatty acid content (mg g^{-1} lipids) and fatty acid composition (percentage total fatty acids, %TFA) of the spawnings obtained in April and May from meagre (*Argyrosomus regius*) broodstocks by the use of hormone implant or injection. Different superscript letters indicate significant differences (t-test, P<0.05) a, b between the type of hormonal induction, A, B between months using the same type of hormonal induction.

Fatty acids	**Implant**		**Injection**	
(% TFA)	**April (N=10)**	**May (N=3)**	**April (N=3)**	**May (N=2)**
16:0	21.4±2.9$^{a, A}$	16.6±2.2^{B}	16.2±2.8^{b}	15.7±2.0
18:0	3.6±0.6	4.4±0.9	3.7±0.4	4.0±0.8
Total saturated[1]	**27.5±3.4^{B}**	**23.2±2.2^{A}**	**22.3±5.1**	**20.7±2.2**
16:1n-7	10.5±0.8^{a}	6.9±2.8	6.6±1.7^{b}	4.8±0.6
18:1n-9	13.6±2.0	14.0±2.4	13.9±1.6	13.8±2.9
Total monounsat[2]	**28.9±2.3^{B}**	**25.0±1.2^{A}**	**26.5±4.0**	**23.1±4.9**
18:2n-6	2.6±0.6^{A}	3.7±1.0^{B}	3.4±0.9	4.0±1.3
20:4n-6	0.9±0.2	0.9±0.1	0.9±0.4	1.0±0.1
Total n-6[3]	**3.9±0.8^{A}**	**5.1±0.6^{B}**	**4.3±0.7**	**5.2±1.7**
18:3n-3	0.8±0.1^{A}	1.4±0.2^{B}	1.2±0.6	1.1±0.5
20:5n-3	7.7±1.9	9.8±1.2	10.4±1.9	9.2±2.1
22:5n-3	2.1±0.4^{b}	2.1±0.3	2.8±0.5^{a}	2.3±0.3
22:6n-3	28.6±3.8	31.9±0.6	31.4±5.8	35.4±2.1
Total n-3[4]	**39.8±5.7^{A}**	**46.7±1.3^{B}**	**46.9±8.3**	**51.1±5.4**
Total PUFA	43.6±5.6^{A}	51.7±1.2^{B}	51.2±9.0	56.3±7.0
Total lipids/DW (mg g^{-1})	192.7±27.3^{A}	161.1±64.2^{B,b}	161.1±11.4	187.5±12.2^{a}
FAMES/DW (mg g^{-1})	700.8±120.9	560.0±113.9	659.9±122.6	576.5±18.2

[1]Includes 14:0 and 22:0; [2]Includes 18:1n-7, 20:1n-9, 22:1n-11; [3]Includes 22:5n-6; [4]Includes 18:4n-3

The eggs from implanted fish had significantly (P<0.05) more total lipid (expressed as DW mg.g^{-1}) in April compared to May. The balance of the lipids was also significantly different in eggs from implanted fish obtained in April and May. Significantly higher content (% total fatty acids) of total saturated and monosaturated fatty acids and significantly lower content of PUFA were found in eggs spawned in April compared to eggs spawned in May. A similar trend was observed when fatty acid composition was compared between "good" and "bad" quality eggs (Table II). A higher content (% total fatty acids) of total saturated and monosaturated fatty acids and a lower content of PUFA were observed in "good" eggs compared to "bad" eggs. However, n was very low (n=5 for good eggs and n=2 for bad eggs) and statistical analysis could not be applied to determine if a difference existed. These are the first results to be published on the quality of eggs from meagre and give an indication of the biochemical composition and particularly fatty acid composition for future studies.

Table II Average fatty acid composition of "good" and "bad" quality spawnings obtained after the use of implants to spawn meagre (*Argyrosomus regius*), considering the larval survival at 3 and 5dph as the main quality factor.

Fatty acids (% TFA)	Implant Good (N=5)	Implant Bad (N=2)
16:0	23.2±2.5	19.2±5.3
18:0	3.9±0.6	3.0±1.0
Total saturated[1]	**29.3±3.0**	**24.4±6.3**
16:1n-7	10.8±0.8	9.6±0.8
18:1n-9	14.0±1.9	11.6±1.0
Total monounsaturated[2]	**30.0±1.8**	**26.3±3.5**
18:2n-6	2.8±0.5	2.1±0.1
20:4n-6	0.9±0.3	1.0±0.3
Total n-6[3]	**3.7±0.6**	**3.7±0.9**
18:3n-3	0.7±0.1	0.7±0.1
20:5n-3	7.1±1.5	8.7±3.3
22:5n-3	1.9±0.3	2.3±0.6
22:6n-3	26.7±3.3	33.5±5.3
Total n-3[4]	**36.9±4.9**	**45.6±8.9**
Total PUFA	40.7±4.7	49.3±9.8
Total lipids/DW (mg g^{-1})	196.5±27.4	197.6±12.2
FAMES/DW (mg g^{-1})	676.8±105.8	799.2±216.9

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FIRST FEEDING OF LOBSTER LARVAE (*HOMARUS GAMMARUS*)

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European lobster (*Homarus gammarus*) is a new species in marine aquaculture. Today there are only a few hatcheries in whole Europe and the reason is probably that lobster is a complicated species to culture in closed land based systems. Lobsters, and even lobster larvae, are very aggressive and considered as cannibalistic. To eliminate this problem, the lobsters must be kept isolated in single units to prevent them from killing and eating each other. Lobster farming therefore requires technology and facilities that are designed for this type of production and completely different from those used for fish farming. This also complicates feeding, rinsing and it requires water systems with relatively high temperature (18°C). The first feeding concepts for lobster larvae tested is either based on an open system, where the larvae are grown for 20 days before they transferred to single units, or a closed system if the larvae are transferred to single units immediately after hatching.

During the first feeding period, lasting from hatching until day 16-20, larvae pass through five stages (I – V) and change from a pelagic to a benthic state. In the initial feeding experiments the larvae were either given *Artemia*, different commercial, or experimental diets. The results showed that *Artemia* was preferred compared to all formulated diets and the survival ranged from 91-94% compared to <31% in the groups fed formulated diets. The larvae fed formulated diets also showed very low growth and reached only stage II after 20 days.

Following the first feeding period the lobster juveniles were gradually fed an increasing share of formulated diets through weaning. This was often associated with high mortality (>50%), but resent findings, performed in commercial scale units, have shown that if the weaning period increase and the formulated diets are introduced in the middle, and not at the end of the live food period, both the survival and growth might increase. Experimental waning diets containing raw material from the copepod *Calanus finmarchicus* have led to reduced mortality related to moulting and improved pigmentation of the lobster.

Like marine fish larvae there seems to be a close relationship between the content of highly unsaturated fatty acids in the diet (DHA, EPA and ARA) and the survival of the lobster larvae. The use of n-3 HUFA enriched *Artemia* increased survival twice through the live food period (20 days) compared to that obtained with unenriched *Artemia*. Another important variable in a closed system is the larvae and prey density in the first feeding units where it is important to slightly overfeed in order to reduced cannibalism.

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EFFECT OF THE BROODSTOCK DIETS UPON THE FECUNDITY AND THE QUALITY OF EGGS OF *ENTEROCTOPUS MEGALOCYATHUS*

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Introduction

The reproductive cycle of the southern octopus, *Enteroctopus megalocyathus*, begins in winter, peaks in spring, extends during the summer, and finishes with a rest period in autumn. The reproductive peaks are observed in December and January (Chong et al., 2001), with a fecundity of 20 000 to 100 000 eggs per female. Oyarzún (2001), applying a gonadosomatic index (GSI), determined the length of first sexual maturity of *E. megalocyathus* at 77cm for females with an approximate weight of 1250g; whereas Chong et al. (2001) found first maturity at 71.7cm length for females and 69.9cm length for males.

Viable eggs of this species have been obtained under controlled culture indicating that embryonic development lasts 150 days until hatching (Uriarte et al. 2008), whereas in the nature the advanced embryos and first paralarvae until 5 days after hatching of this species has been described (Ortiz et al., 2007).

Currently there are few studies on *E. megalocyathus* embryonic development, so it is not known if there are differences in the success of progeny when the eggs have been laid by females fed different diets. In this work, we studied the effect of the broodstock diets on the fecundity and quality of eggs of *E. megalocyathus.*

Materials and methods

12 wild immature females of *E. megalocyathus*, 1.4±0.2kg of total fresh weight, collected off Hueihue (41°52´S; 73°51´W), Region X, Chile, were maintained at 12ºC under three experimental feeding regimes: 1) fresh fish at 7% body weight, 2) mixture (3:1) of fresh fish and fresh crab at 10% body weight, and 3) fresh fish at 10% body weight, until gonad maturation and laying of egg clutches.

Two samples of 12 eggs were sampled from every clutch from every female to analyze the soluble perivitelline protein content and fatty acid composition of egg yolk. Growth in length and wet weight of eggs throughout the embryonic development were evaluated in every clutch.

Results and discussion

Spawning periods varied between 545 and 1100 degree-days, during which the females increased 1.5 times their body weight. Eggs were only obtained in the females of fed a mixture of fish and crab at 10% body weight and only fresh fish at 10% body weight, with an average of 2100 eggs (±480) per octopus female. This means that clutches could be obtained in the treatments with presence or absence of crustaceans. So the conditioning in this experiment was affected by the value of daily ration. Rations of 7% of the corporal weight per day reduced the percentage of females that laid eggs and the number of laid eggs per clutch (Table I). When the ration was 10% body weight per day, both only fresh fish or mixture (3:1) of fresh fish + fresh crab had similar values as diets measured as females laying eggs and eggs per clutch (Table I). However, there was highest variability in the number of eggs per clutch when the diet was only fish.

The number of eggs obtained per female represented only 10% of the expected fecundity reported by Chong et al. (2001) for natural populations. The eggs of different ages and from different females showed an exponential relationship ($WWt = 2.07*TL^{1,4887}$) between wet weight (WWt) and total length (TL). Egg weight increased throughout embryonic development only in eggs coming from females fed with 10% fresh fish and those that arrived until 134 days after laying with a specific growth rate of $0.25\%.day^{-1}$, this value only a third of the $0.96\%.day^{-1}$ observed in other cold water octopodids (Uriarte et al., 2009). The eggs from clutches coming from the other diets lost weight throughout development, and the few eggs produced in 7% fresh fish diet only reached the tenth week of development, after which they showed visible signals of deterioration.

Table I. Female survival during reproductive conditioning and success of conditioning measured as % of females with clutches and number of eggs laying per female. Each value is the mean and standard error.

Broodstock diet /daily ration	Female survival during conditioning experiment (%)	Females with egg clutches (%)	Fecundity (eggs.female^{-1})
Fresh fish at 7% body weight	43	33	<100
Mixture (3:1) of fresh fish and fresh crab at 10% body weight	43	67	1388 ± 188
Fresh fish at 10% body weight	40	67	1600 ± 1000

A reduction of the protein content in the perivitelline fluid was not observed during the egg incubation throughout 130 days after laying. The total content of the

lipid was not reduced either during the egg incubation. Furthermore, samples or signals of embryonic development were not observed at 130 days when they should have displayed very advanced embryos (Uriarte et al. 2008).

The fatty acids of eggs were only compared during the first 6 days after laying, and there were no significant differences in the contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) between eggs of the different broodstock diets and rations (Table II). The EPA contents varied between 0.3-2.5% of the dry weight of yolk, and between 0.4 and 5.9% of the dry weight of yolk for DHA.

Table II. Fatty acids of eggs (percentage of yolk dry weight) immediately after they were laid in the tanks by females from three different diets. Each value is the mean and standard error.

Broodstock diet / daily ration	DHA (%)	EPA (%)	18:0 (%)	16:0 (%)
Fresh fish at 7% body weight	1.34±0.44	0.56±0.17	0.36±0.08[a]	1.56±0.71
Mixture (3:1) of fresh fish and fresh crab at 10% body weight	1.63±0.37	0.91±0.22	0.48±0.10[a]	1.95±0.46
Fresh fish at 10% body weight	2.93±0.50	1.55±0.29	1.13±0.30[b]	3.47±0.63

Both fresh fish and fresh crab showed a total lipid of 4.9% of muscle, the fresh fish richer in FAME and DHA than fresh crab. The DHA:EPA ratio was 3.7 for fresh fish and 0.4 for fresh crab. The DHA/EPA ratio in the eggs ranged between 1.8 for both 10% body wt diets and 2.4 for 7% body wt diet.

It can be concluded that diets for broodstock conditioning allowed egg production without significant differences in the lipid and fatty acid profile of *E. megalocyathus* eggs, but wild eggs are needed to compare the effect of experimental conditions upon eggs with respect to their natural performance.

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NEW INSIGHTS OF THE EFFECTS OF DIETARY VITAMIN A ON FLATFISH SKELETOGENESIS: THE CASE OF SOLEA SENEGALENSIS

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Morphogenesis and skeletogenesis are complex processes that take place from embryogenesis and continue until the completion of the juvenile phenotype. These processes depend on the differentiation of multiple cell types to generate elements with distinct forms and functions throughout the body in a short period of time, ranging from a few weeks to several months depending on the fish species and water rearing temperatures. In flatfish, skeletogenesis is especially complex due to the dramatic changes in morphology during their metamorphosis and transition from the pelagic to the benthic environment. Thus, the larval body has to change from a symmetrical to an asymmetrical form, including the relocation of one eye and the rotation of the vertebral column.

Several factors have been extensively reviewed in order to evaluate their effects on skeletogenesis and bone remodelling. Among them, Vitamin A (VA) is a morphogenetic nutrient that has a key role in many biological processes; e.g., cell proliferation and differentiation, growth, body patterning, etc. As fishes are not able to synthesize it, they have to obtain it from the diet, not only in the optimal amounts but also in the proper form. Special attention has been paid to VA in relation to skeletal abnormalities affecting fry production in hatcheries. Thus, a dietary imbalance (deficiency or excess) of VA has a negative effect on skeletogenesis, leading to an increase in the incidence of skeletal deformities.

The Senegalese sole is a promising flatfish species for diversifying European marine aquaculture. However, high levels of skeletal deformities in hatchery-reared individuals may hamper the production in proper quantity and quality. Thus, the information gathered from other flatfish species in relation to the effects of different nutrients, and in particular VA, on flatfish skeletogenesis is very important in order to achieve a better understanding of the dietary effects of VA on Senegalese sole morphogenesis and provide a clue for reducing the incidence of skeletal disorders affecting the quality of juveniles.

Different experimental approaches have been used to evaluate the effects of VA on flatfish skeletogenesis. The use of dietary dose/response experiments using live preys was adapted to study by nutritional approach the VA effects on skeletogenesis. The present work aims to review the literature related to the effects of dietary VA imbalance in the morphogenesis of flatfish larvae and early juveniles, and compare those results with recent results in Senegalese sole.

Different studies have shown that the form of inclusion of VA (retinyl esters or carotenoids) into the diet is also important, since this affects their transport, accumulation, mobilization, and transformation into retinoic acid. Consequently, this information is also considered when interpreting the results from different dose/response experiments. In Japanese flounder, the safe level of VA in *Artemia* nauplii for preventing the development of skeletal abnormalities was less than 45,200IU.kg^{-1}. In summer flounder and Atlantic halibut juveniles, a diet containing less than 52,873 and 8,333IU.kg^{-1} has been described as the best for assuring proper juvenile development. In Senegalese sole, larvae fed *Artemia* metanauplii containing 4,333IU.kg^{-1} showed a high incidence of skeletal deformities, showing that this species is quite sensitive to low levels of VA. However, levels of VA lower than that obtained in live prey were not tested due to the impossibility of reducing VA content in the commercial emulsion. In addition, a deficiency effect was quite impossible to describe with experiments using live prey. Thus, the recent development of a balanced compound microdiet for Senegalese sole opens a new frame for studying the effects of low VA levels.

Although different flatfish species share the final juvenile phenotype, they are extremely variable in metamorphosis and skeletogenesis patterns. For example, jaw elements ossify early in metamorphosis in Atlantic halibut and Senegalese sole, but at the end of metamorphosis in winter flounder. Proper skeletogenesis requires proper timing of cell differentiation and ossification of bony structures. As VA regulates chondrocyte and osteoblast maturation, proliferation and function, the effects of dietary VA imbalance in the development of deformities needs to be considered in this context as well. Recent results showed that in Senegalese sole, intramembranous bony structures are less sensitive to an excess of VA than chondral ones. Besides, a dietary VA imbalance exerts different effects on morphogenesis depending on the larval stage of development, being the premetamorphic stages more sensitive than the older ones. The molecular pathways involved in flatfish morphogenesis and metamorphosis affecting skeletal deformities is also discussed in this review.

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INFLUENCE OF SWITCHING ROTIFER WITH *ARTEMIA* ON DIGESTIVE ENZYME ACTIVITY OF *PAGRUS AURIGA* LARVAE

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Introduction

The gastric glands appear 16 days after hatching (DAH) in *P. auriga* larvae and around 30DAH gastric function becomes complete (Moyano et al., 2005; Sanchez-Amaya et al., 2007). This rapid digestive development coincides with high growth rates and fast organogenesis and suggests that high energy and nutrient intake may be needed. Thus, an early switch from rotifers to more energetic prey such as *Artemia* seems adequate. However, high larval mortality usually coincides with being fed *Artemia* and it is hypothesized that an inadequate digestive response to *Artemia* may occur at certain ages. Trypsin (TR) and chymotrypsin (CH) activities are good indicators of larval nutritional status. Alkaline phosphatase (AP) has been associated with the absorption of nutrients and its activity decrease usually accompanies the feeding of an inadequate diet. In carnivorous marine teleosts, bile salt-activated lipase (BAL) plays a main role in lipid digestion. Present study aimed at revealing digestive enzymes changes associated to the live prey offered to *P. auriga* larvae during a period when high mortality is frequently found under intensive larval rearing conditions.

Materials and methods

Four replicate 300-l larval tanks were performed at 22ºC, a salinity of 36-38g.l^{-1}, and a permanent surface illumination of 800lux. The initial larval density was 40 individuals.ml^{-1}. From the onset of exogenous feeding (3DAH) until 18DAH, all tanks were daily fed 20 rotifers.ml^{-1}. At 19DAH, the following feeding regimes were applied until 36DAH: 1) larvae fed *B. plicatilis* from 19DAH to 29DAH; 2) larvae fed *Artemia* from 19DAH to 26DAH; 3) larvae fed *Artemia* from DAH24 to 31DAH; and 4) larvae fed *Artemia* from 29DAH to 36DAH. Rotifers were enriched for 12h with *Isochrysis galbana* (T-ISO strain). *Artemia* metanauplii were also enriched for 12h with *I. galbana. Nannochloropsis gaditana* and *I. galbana* were daily added to larval tanks. A daily ration of 20 rotifers.ml^{-1} and 3 *Artemia*.ml^{-1} was used through the experimental period, ensuring similar biomass fed to larvae regardless the type of prey employed. Larval body dry mass

was determined by drying samples of 15-40 larvae at 85ºC to constant weight. Enzyme extracts were prepared by homogenization of pooled whole larvae (20mg.ml-1) in cold distilled water followed by centrifugation (16 000g, 30min, 4ºC). The soluble protein concentration in extracts was determined by the Bradford method adapted to microplate wells using bovine serum albumin (1mg.ml-1) as a standard. Larval extracts were assayed for TR, CH, BAL, and AP determination. TR and AP activities were measured at pH7.8 following Gawlicka et al. (2000) using BAPNA and p-nitrophenyl phosphate as the substrate, respectively. CH activity was determined according to Moyano et al (2005) using a fluorimetric method with succinyl-Ala-Ala-Pro-Phe as substrate. CH activity was expressed in units (UF), as percentage increase of emission.min-1. BAL was measured according to Perez-Casanova et al. (2004) with p-nitrophenyl myristate as substrate.

Results and discussion

Similar growth rates were found for *P. auriga* larvae under all experimental feeding regimes (Fig. 1). Comparing the regression growth equations did not reveal any difference due to the use of either *Artemia* or rotifers as food within the same period (P>0.05). Growth was similar to other results reported for *P. auriga* (Prieto et al., 2003). Survival was over 90% from first feeding until 19DAH. It was unaffected (P>0.05) by any of the experimental feeding regimes used, averaging a mean value of 2.1±1.1% at day 36. Low survival for the experimental period indicate that larval rearing conditions of *P. auriga* still need significant optimization, particularly beyond 19DHA.

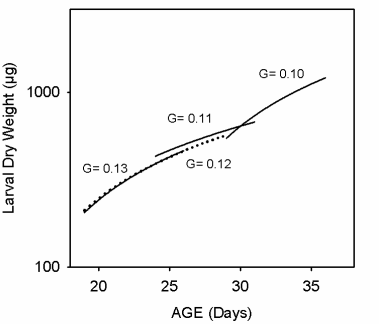

Fig. 1. Growth of *P. auriga* larvae fed on rotifers (dotted line) and after shifting to *Artemia* at 19, 24 and 29 DAH, respectively (solid lines).

The specific activities of TR, CH, and AP decreased from 19DAH to 24DAH and were unrelated to the feeding regime used (Fig. 2). BAL activity, however, increased within this period. A peak of TR was measured at 27DAH, followed by a decrease to very low levels after the change from rotifers to *Artemia* at 24DAH (Regime 3). The lowest TR activity was found when feeding *Artemia* in relation to feeding with rotifers, with an only exception at 27DAH (Fig. 2a). CH

activity decreased during the experimental period but was not related to any change in the feeding pattern (P>0.05). Slight changes were observed three days after shifting to *Artemia*. However, CH activity approached to its natural age tendency beyond this period (Fig. 2c). Larvae fed *Artemia* (24DAH onwards) had a 10-40% higher AP activity (P<0.05) than larvae fed rotifers (Figure 2b). BAL activity significantly decreased when larvae were fed *Artemia* from 29DAH (Regime 4) onwards (Fig. 2d).

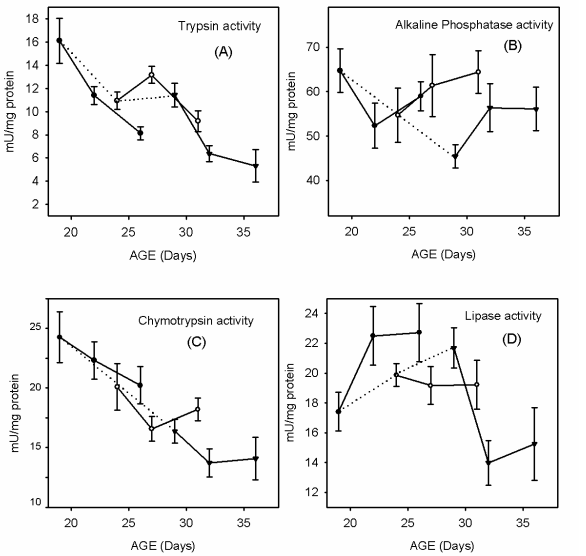

Fig. 2. Digestive enzymes activity in *P. auriga* larvae fed on rotifers (dotted line) and after shifting to *Artemia* at different ages (solid lines).

Present results were indicative that digestive enzyme activity is also triggered by internal mechanisms. The apparition of the gastric gland around 22DAH in *P. auriga* (Sanchez-Amaya et al., 2007) may represent the major change from TR to a combined trypsin-pepsin digestion occurring at this point. BAL secretion seems to be induced by food (Hoehne-Reitan et al., 2001). In present study, BAL levels of *P. auriga* larvae increased when initiating *Artemia* from 19DAH, but a reduced activity was found when the shift to *Artemia* was performed in older larvae. Similar results were reported for turbot larvae which lipase activity decreased with age and showed a main drop coinciding with the shift from rotifers to *Artemia* (Hoehne-Reitan et al., 2001). It has been suggested that a lower lipase activity could be related to a reduced dietary content in sterol esters and wax esters (Perez-Casanova et al. 2004). At this regard, Van der Meeren et al. (2007) demonstrated rotifers to content 11.6% sterols and wax esters, whereas undetected levels were reported for *Artemia*.

AP was the enzyme with the higher extent of variation in response to diet changes. A high enzyme activity after three days of shifting rotifers to *Artemia* and a posterior stabilization was evidenced in *P. auriga* larvae. These results

might suggest better food assimilation for *Artemia* at this time. AP is considered to be a general marker for nutrient absorption and its increased presence in the larval intestine is associated to more functionally developed enterocytes (Gawlicka et al., 2000). Shifting to feeding *Artemia* caused an opposite response for BAL respecting to that of AP activity, suggesting a preferential protein digestion when replacing rotifers by *Artemia* at certain ages.

Conclusions

The digestive pattern here described supports the theory about the dependence of fish larval digestive enzymes on food characteristics. No specific digestive disfunction could be attributed to larval mortality associated to feeding *Artemia*.

Acknowledgements

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EFFECT OF CO-FEEDING REGIMES ON BIOLOGICAL PERFORMANCE AND BIOCHEMICAL COMPOSITION OF MEAGRE (*ARGYROSOMUS REGIUS* ASSO, 1801) LARVAE

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Introduction

Although the use of live food in the early stages of larval culture is considered essential for its success (Sorgeloos et al., 2001), it is one of the most expensive financial costs of production. For this reason, early weaning should be done as soon as possible, and is of great importance in the co-feeding period, since fish have a preference for live feed and this period helps the larvae better adapt to the inert feed once live feed is completely withdrawn (Baskerville-Bridges and Kling, 2000).

Materials and methods

The experiment was conducted in triplicate, using 15 150-l fibreglass tanks. Each tank was equipped with continuous aeration and a continuous flow rate of filtered and UV-sterilized seawater. Water entered the tanks at the bottom and exited at the surface. The experiment was conducted with a daily seawater renewal of 100% until day 15, when it was increased to 600%. The photoperiod was 12L:12D with artificial light. Daily measurements of temperature and oxygen were done, using an OxyGuard Handy Polaris probe (Birkerød, Denmark). Oxygen and temperature values during the experimental period averaged 5.85±0.12ppm and 20.7±0.8°C, respectively, and were consistent between tanks.

Initial stocking was 50 larvae.l^{-1} with larvae aged 9 days after hatching (SL=4.24±0.21mm, WW=0.13±0.008mg) was performed according to the volumetric method described by Borrero (2008). The experiment was conducted to evaluate the effect of different times of co-feeding (0, 5, 10, 15, and 20 days) on growth, survival, and biochemical composition of the larvae. Diets used were microalgae (*Nannochloropsis* sp.), rotifers (*Brachionus* sp.) enriched with DHA Protein Selco, *Artemia franciscana* nauplii (Type AF, INVE, Dendermode, Belgium), *A. salina* metanauplii (Type EG, INVE) enriched with Easy DHA Selco

of INVE, and micro-diets Gemma Micro 150 and 300 (Skretting, France). Table I shows the sequence and quantity of feed used.

Table I. Feed sequence and quantities used.

Treatment	0	5	10	15	20	Feed added
			Days after hatching			
Phytoplankton	9-15	9-15	9-15	9-15	9-15	300 000 cells.ml^{-1}
Rotifer	9-15	9-15	9-15	9-15	9-15	6-7.ml^{-1}
Nauplii		12	12	12	12	0.5.ml^{-1}
Metanauplii		13	13	13	13	0.5.ml^{-1} + 0.5.ml^{-1}
Metanauplii		14	14	14	14	1.ml^{-1}
Metanauplii		15-17	15-22	15-27	15-32	1.5.ml^{-1}
Micro-diet GM 150-300 (50%)	12-30	12-30	12-30	12-30	12-30	15% biomass.d^{-1}
Micro-diet GM 300 (100%)	30-40	30-40	30-40	30-40	30-40	15% biomass.d^{-1}

At the end of the experiment, 30 larvae from each tank were sampled and measured for standard length (SL) and dry weight (DW) as per Fernández-Palacios et al. (2007). The final survival was determined by counting all individual larvae from each tank. These larvae were then kept for posterior analysis according to Fernández-Palacios et al. (2005). At day 40 after hatching, 30 larvae from each tank were sampled for air resistance (Borrero, 2008).

The results obtained were expressed as average ± standard deviation of the mean. The data were compared statistically using the analysis of variance (ANOVA). When statistically significant differences were detected, the differences between means were done using Tukey's multiple comparison test for averages ($P<0.05$) (Sokal and Rohlf, 1981).

Results and discussion

The results shown in Table II show that there were no statistically significant differences in dry weight or in final larvae survival of the different co-feeding regimes tested. Differences were found in standard length, showing that the larvae with a co-feeding time of 10, 15, and 20 days were significantly bigger than those of 0 and 5 days co-feeding. Hernandez-Cruz et al. (2007) found no significant differences in dry weight in two groups of meagre larvae with a co-feeding time of 6 and 11 days. Alves et al. (2006) found no significant differences in survival in 30-day-old fat snook (*Centropomus parallelus*) larvae with a co-feeding period of 5, 10, and 15 days, though they did find significant differences in the standard length, specifically that a co-feeding time of 0 days produced significantly lower standard length than the rest, similar to our experiment.

Table II. Ranges of the calculated parameters in the experiment.

Treatment	ST (mm)	DW (mg)	% S	% S Activity Test
0	14.87±1.90[a]	11.17±1.84	13.11±1.21	53.30±11.50
5	15.95±1.63[a]	13.05±1.74	15.70±4.51	61.02±7.07
10	17.69±2.42[b]	18.03±7.72	16.59±3.64	68.14±12.88
15	18.53±1.36[b]	22.20±5.61	19.90±1.10	75.00±13.80
20	18.03±1.92[b]	17.92±3.77	22.33±7.54	73.52±11.22

*Values in the same column with no superscripts are not significantly different.

Table III shows the major biochemical components of the larvae at the end of the experiment. There were no statistically significant differences in any of them, probably due to the feed used that was the same for all treatments, varying only in duration. In an experiment with larvae of the same species, 35 days after hatching larvae, and a co-feeding period of 5 and 10 days, Borrero (2008) found no significant differences in the biochemical content of the larvae at the end of the experiment.

Table IIIBiochemical composition of the experimental larvae.

Treat.	Protein	Lipìds	ARA	EPA	DHA	n-3 HUFA
0	10.16±0,25	2.06±0.05	1.63±0.05	8.75±0.11	16.31±0.76	27.40±0.51
5	10.31±0.14	2.08±0.04	1.63±0.06	8.35±0.26	16.54±0.36	27.42±0.76
10	10.19±0.17	2.16±0.06	1.76±0.06	8.72±0.41	16.30±0.91	27.50±0.48
15	10.43±0.19	2.10±0.05	1,75±0.05	8.53±0.25	16.09±0.53	26.72±0.39
20	10.14±0.33	2.08±0.06	1.69±0.07	8.65±0.17	16.34±0.22	26.89±0.63

*Values in the same column with no superscripts are not significantly different.

With respect to the activity test of air resistance (Table II), no significant differences between treatments were found, although larger-sized larvae showed better survival. Similar results were observed by Liu et al. (2002) working with sea bream (*Sparus aurata*) larvae, which associated higher survival to the activity test of air resistance with bigger size larvae.

Conclusions

In meagre it is possible to go from rotifer to inert feed at 15 days old, with a survival of 13.11%. But a co-feeding period with *Artemia* implies greater survival, higher growth of larvae, and better resistance to the activity test. If the co-feed period is at least of 10 days the growth in size significantly improves.

Acknowledgments

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CHANGES IN ANTIOXIDANT DEFENCES OF RAINBOW TROUT FRY FED OXIDISED LIPID DURING EARLY DEVELOPMENT

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Introduction

Alteration of the dietary prooxidant/antioxidant balance leading to lipid peroxidation is considered a major cause of diseases including muscular dystrophy in several fish (Lall and Lewis-McCrea, 2007). Given the fatty acid status of fish, it is important to understand the control of in vivo lipid peroxidation that can cause alteration in the development. The objective of the study was to characterise the changes in antioxidant defence system of rainbow trout (*Oncorhynchus mykiss*) fry fed oxidised lipid during early development.

Materials and methods

Rainbow trout (*O. mykiss*) fry were reared at the INRA experimental fish farm in Donzacq (Landes, France) at 17±1°C. From the swim-up stage, which corresponds to the beginning of exogenous feeding (trial 1) or 8 weeks after swim-up (trial 2), fish with a mean initial body weight of 0.1 or 1.0g were hand-fed four or six times per day to apparent satiation. Six semi-purified diets were tested in triplicate for 4 weeks. Diets were isoproteic (56%) and isolipidic (18%) with 12% fresh fish oil (diets R1, R3, and R5) or oxidised fish oil (diets R2, R4, and R6) and 6% soybean lecithin (diets R1 and R2), egg lecithin (diets R3 and R4) or soybean oil (diets R5 and R6). Samples were taken on day 0 and from each tank at the end of the 4-week feeding trial after 24h of fasting. They were anaesthetised in diluted 2-phenoxyethanol for wet weight determination and frozen in liquid nitrogen. Tocopherols were analysed in whole fry according to Akhtar et al. (1999). Antioxidant enzyme activities were assayed in whole fry whereas lipid-soluble fluorescent products (LSFP), anisidine value (AV), and conjugated dienes (E_{232}) and trienes (E_{268}) were determined in total lipid of fry as described previously (Fontagné et al., 2008).

Results and discussion

In both feeding trials, after a 4-week feeding period, rainbow trout fry fed diets with oxidised lipid (R2, R4, and R6) exhibited lower growth rates than those fed

fresh lipid (Fig. 1). This result underlines the necessity to control lipid peroxidation in diets for fish larvae.

Moreover, swim-up fry fed a diet without phospholipid (R5) displayed a reduction of growth compared to fry fed diets with phospholipids (R1 and R3) suggesting that early stages of rainbow trout require higher levels of dietary phospholipids compared to juveniles in agreement with previous observations of Coutteau et al. (1997) for different fish species.

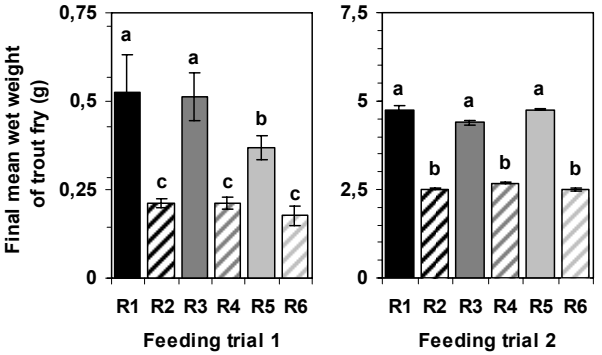

Fig. 1. Growth of rainbow trout fry fed the six diets with different lipid supplements for 4 weeks. Values are means±SD of 3 rearing tanks. Different superscripts indicate significant differences ($p<0.05$).

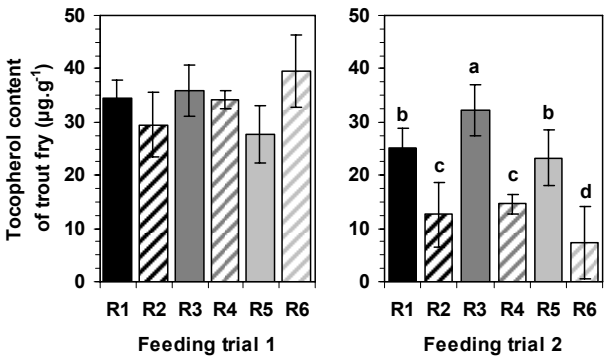

Fig. 2. Levels of vitamin E (α and γ-tocopherol) in whole rainbow trout fry fed the six diets with different lipid supplements for 4 weeks. Values are means±SD of 3 rearing tanks. Different superscripts indicate significant differences ($p<0.05$).

No significant differences in levels of the antioxidant vitamin E were observed between dietary treatments in trial 1 with swim-up fry whereas decreased tocopherol contents were noticed in fry fed oxidised lipid compared to fry fed fresh lipid in trial 2 with older fry (Fig. 2).

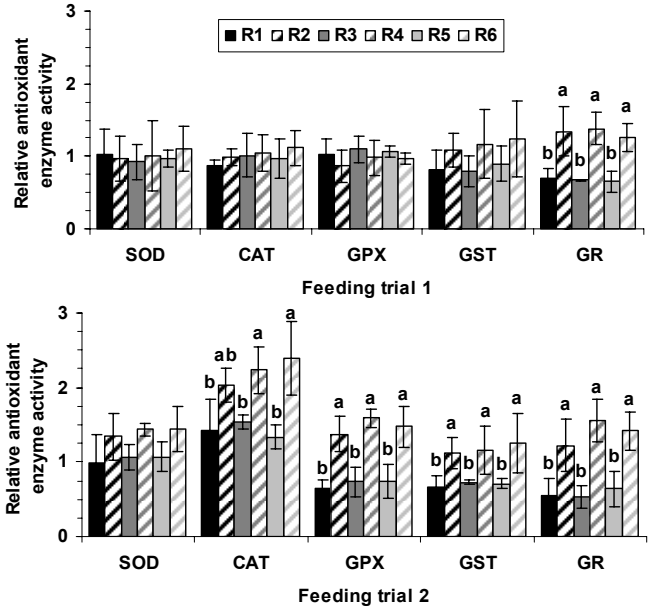

Fig. 3. Relative specific activity of antioxidant enzymes in whole rainbow trout fry fed the six diets with different lipid supplements for 4 weeks. Values are means±SD of 3 rearing tanks and expressed relative to the mean activity for each enzyme in trial 1. Different superscripts indicate significant differences ($p<0.05$).

Likewise, dietary control of the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione-S-transferase (GST) was low in trial 1 with only a higher activity of glutathione reductase (GR) in fry fed oxidised lipid. On the other hand, increased activities of all antioxidant enzymes (even if not significant for SOD) were noted in fry fed oxidised lipid compared to fry fed fresh lipid in trial 2 (Fig. 3).

The level of lipid peroxidation products was significantly higher in fry fed oxidised lipid compared to fry fed fresh lipid in trial 1, especially in fry fed the diet R6 without phospholipid (Fig. 4). This level and differences between dietary treatments were decreased in trial 2 probably due to enhanced antioxidant defence system in later stages of trout fry.

Conclusions

The present study demonstrates that compared to late developmental stages, early stages are more susceptible to dietary oxidative stress, possibly due to lower response of endogenous antioxidant defence system.

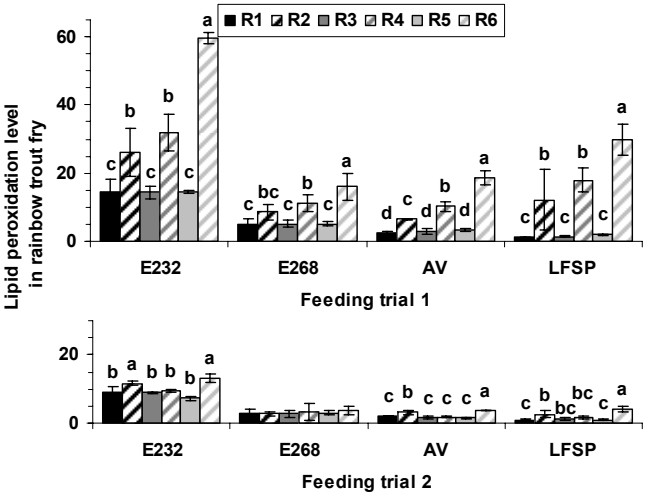

Fig. 4. Levels of lipid peroxidation products in total lipid of whole rainbow trout fry fed the six diets with different lipid supplements for 4 weeks. Values are means±SD of 3 rearing tanks. Different superscripts indicate significant differences ($p<0.05$).

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STRATEGIES TO UNRAVEL GENE EXPRESSION RESPONSES OF HOST-MICROBE INTERACTIONS IN COD (*GADUS MORHUA*) LARVAE

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Introduction

Studies on host-microbe interactions have shown that commensal bacteria in the intestine play essential roles in the development and functionality of the host. The use of gnotobiotic vertebrates has revealed that microbial colonization directly affects a wide range of biological processes, including nutrient processing and adsorption, development of the mucosal immune system, and epithelial proliferation (Rawls et al., 2004)

A gnotobiotic model system to investigate gene responses to microflora has been established for zebrafish (*Danio rerio*) (Rawls et al., 2004; Smith et al., 2006)). However, zebrafish hatch at a fairly developed state and are quite phylogenetically distant compared to most marine fish

We have established a protocol for bacteria-free rearing of cod larvae and chosen two different approaches to investigate the effect presence of bacteria have on gene expression; using Suppression Subtractive Hybridization (SSH) and qPCR targeting genes previously reported to be differentially expressed in gnotobiotic zebrafish by Rawls et al. (2004).

Materials and methods

All cod eggs used were disinfected twice with glutaraldehyde and hatched in sterile seawater containing 10ppm each of rifampicin and ampicillin. After hatching, the cod larvae were transferred to either bacteria-free or bacteria-containing (matured) seawater. Axenic *Isochrysis* sp. was also added, in accordance with the green-water technique (Skjermo and Vadstein, 1993). The larvae were fed gnotobiotic rotifers from day 3 post-hatch, and grown until day 17. Samples from rearing water, rotifers, and algae cultures were taken daily, to

check for contamination by bacteria. Bacterial densities were determined by plate dilutions and by flow cytometry.

SSH-PCR was performed on polyA cDNA from pooled larvae samples (n=8). Both forward and reverse subtractions were performed, yielding two clone libraries with putatively differentially expressed genes. Sequences were analysed using blastn and blastx (www.ncbi.nlm.nih/blast/Blast.cgi). Twenty-two of the identified genes were selected for qPCR, and used to investigate differential expression in a second, replicated cod experiment.

Based on the findings of Rawls et al. (2004) and highly similar sequences available from cod in GenBank, qPCR primers were designed to amplify C3, angiopoietin-4, farnesyl diphosphate synthetase, integrin, Cyp1a1, glutathione peroxidase, and tryptophanyl t-RNA synthetase. Serum ameloid A1 was one of the genes regulated by bacteria in Zebrafish, but as there was no similar sequence available for cod, primers were instead designed for serum lectin.

All qPCR reactions were run on pooled (n=5) cDNA from bacteria-free cod larvae and larvae raised in matured, bacteria-containing water. Plasmids containing the amplicon of each primer were used to generate standard curves, and the results were normalized to β-actin copy numbers (serving as a housekeeping gene).

Results and discussion

Sequencing of the cloned library revealed 90 putatively upregulated genes in the bacteria exposed larvae, and 67 in the bacteria-free larvae. A large percentage of these, 58% and 64% respectively, had no significant similarity to sequences in GenBank. qPCR on the 22 selected genes revealed only slight differences in gene expression between the bacteria free versus exposed larvae. There was however some difference in expression between the two bacteria-containg replicates. This variation between replicates can mean that the sequences from the SSH library may represent genes that were randomly differentially expressed between the pooled larvae sent in for analysis, rather than genes regulated by the presence of bacteria, or that the bacteria present in the matured water in the two experiments were too different to produce comparable data between SSH and the resulting gene expression. In addition, the SSH technique favors the enrichment of high abundance transcripts and is therefore susceptible to a high false-positive rate. Despite the fact that the hybridization was performed in both directions to maximize the detection and identification of differentially-expressed genes, it may still have omitted rare targets. In order to identify differentially regulated genes from the SSH library it may also be necessary to sequence more clones. In a study by Ghorbel et al. (2006), it was shown that of 1152 sequenced clones, only 459 were found to be differentially regulated using microarray analysis.

Gene expression of the eight genes chosen based on Rawls et al. (2004) showed the same trend as SSH, where expression levels varied more between the two bacteria-exposed replicates, compared to the bacteria-free treatment. In the study by Rawls et al. (2004) these genes were shown to be regulated by a subset of bacterial components, as they were expressed differently depending on the type of bacteria present in the zebrafish.

The bacterial composition has not been determined for the matured water replicates, but it is likely that they are not identical, as random events can significantly change the composition. Different bacterial content can explain the different gene expression seen in the bacteria-exposed replicates. In order to investigate the effect on gene expression of bacteria versus no bacteria, it may be necessary to run purely gnotobiotic studies, with known bacterial compositions.

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EFFECT OF VITAMIN A ON THE SHAPE OF EUROPEAN SEA BASS

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Introduction

Environmental conditions during fish ontogeny significantly affect the phenotype of larvae, juveniles and adults. Nowadays it is well known that not only morpho-anatomical abnormalities, but also normal phenotypic variations of body shape, meristic characters, muscle cellularity, and sex (e.g., Sfakianakis et al., 2006; Cahu et al., 2003; Koumoundouros et al., 2009) are frequently the result of factors acting during the embryonic and larval stages of fish. The present study examined the effects of dietary vitamin A during the larval phase on the skeletal shape of normal European sea bass (*Dicentrarchus labrax* (Linnaeus 1758)), during and after the metamorphosis stage.

Materials and methods

Twenty one populations of sea bass larvae were fed on microparticulate diets with different levels of retinol acetate (3, 9, 21, 34, 69, 89, and 155 10^3 IU kg^{-1} dry feed, corresponding to RT0, RT5, RT10, RT15, RT25, RT35 and RT70 groups, respectively), in three replicates. Different dietary regimes were applied during the 9-45 days post-hatching (dph, 20°C water temperature), whereas in the next phase (45-100dph) all population were fed on a common commercial diet (Marin Start-miet AL.0, Le Gouessant, France).

A random sample of ca 50 individuals was taken from each population at the end of the application of the different nutritional regimes (45dph) and at the juvenile stage (100dph). Larval samples were anaesthetised, fixed in 5% phosphate buffered formalin, and stained for bone and cartilage (Park and Kim, 1984). Juvenile samples were anaesthetised, straight positioned along the longitudinal axis, frozen at -20°C, and radiographed.

Geometric morphometrics were used to study the effect of dietary vitamin A on the shape of whole skeleton (juveniles, 100dph) and pelvic fins (larvae, 45dph). Shape analysis was performed on 21 landmarks collected on the photographs of the x-rayed juveniles, as well as on 11 landmarks collected on the ventral photographs of the pelvic fins (larval samples) (Fig. 1). The method of geometric morphometrics and the used software are presented in details elsewhere (Georgakopoulou et al., 2007). Only individuals without skeletal deformities were included in the study.

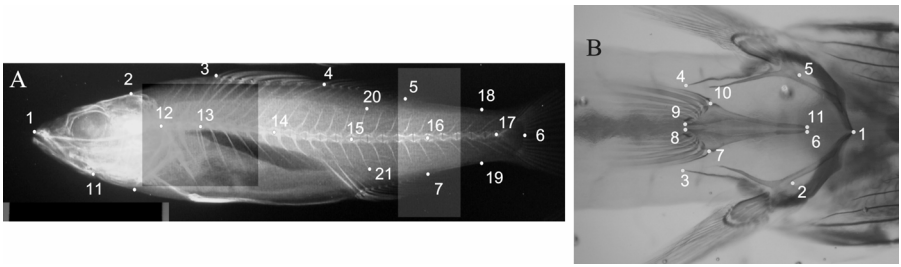

Fig. 1. Landmarks collected for the shape analysis in the juvenile (A) and metamorphosis phase (B).

Results and discussion

Results demonstrated a significant effect of larval nutrition on the shape of juvenile skeleton (Wilk's λ=0.0579, $p<0.001$), 55 days after the application of the different regimes. Shape differences were mainly expressed along the first canonical variable, discriminating RT0 group from RT10, RT25, RT35 and RT70 (Fig. 2). Spline diagrams demonstrated that compared with the rest groups, RT0 group was characterized by an anterior transposition of the first dorsal and anal pterygiophore, an anterior transposition of the caudal peduncle, a posterior transposition of the 5th, 10th, 15th, and 20th vertebrae, and by a posterior-dorsal transposition of the last anal pterygiophore (Fig. 2).

At the end of the application of the different regimes (45dph), the shape of the pelvic fins was significantly differentiated between the different groups (Wilk's λ=0.3087, $p<0.001$). Shape differences were mainly expressed along the first canonical variable, discriminating RT0 from the rest groups (Fig. 3). Spline diagrams demonstrated that compared with the rest groups, RT0 group presented an anterior transposition of the cleithrum, a lateral displacement of scapula and lower metacleithrum, as well as a size reduction of the pelvic basipterygia (Fig. 3).

Environmentally-driven phenotypic plasticity is well studied in fish, including European sea bass (e.g. Georgakopoulou et al., 2007; Koumoundouros et al., 2009). Similarly the role of vitamin A in the development of skeletal deformities

in fish has been demonstrated in a variety of species (e.g., Cahu et al., 2003). However, this is the first evidence of the crucial role of larval nutrition for the normal skeletal structure of sea bass juveniles.

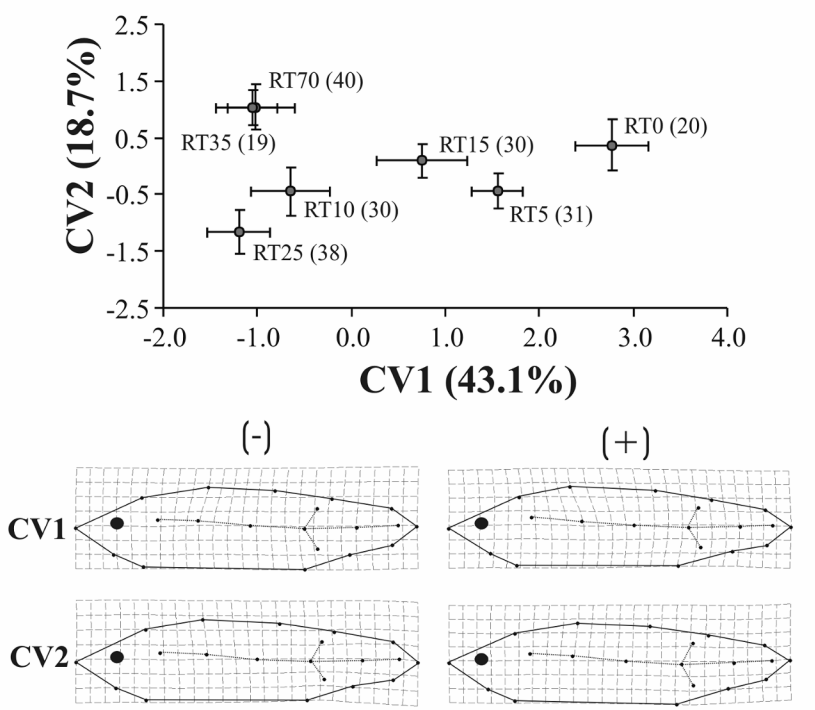

Fig. 2. Top. Distribution of the different nutritional groups along the first (CV1) and second (CV2) canonical axis. Error bars equal to ±2SE. Sample size of each group is given into brackets. Bottom. Spline diagrams demonstrating the components of shape change relative to the extreme values (-, +) of CV1 and CV2.

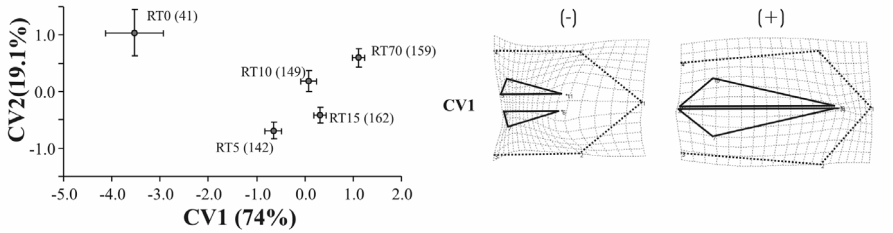

Fig. 3. Left. Distribution of the different nutritional groups along the first (CV1) and second (CV2) canonical axis. Error bars equal to ±2SE. Sample size of each group is given into brackets. Right. Spline diagrams demonstrating the components of shape change relative to the extreme values (-, +) of CV1.

Conclusions

European sea bass has been proven a highly plastic fish to the action of dietary retinol during the larval phase. Future relative research should target on the underlying mechanisms controlling this phenotypic variability, as well as on the relationship of this variability with teratogenesis.

Acknowledgements

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THERMALLY INDUCED PLASTICITY OF BODY SHAPE IN ADULT ZEBRAFISH *DANIO RERIO* (HAMILTON, 1822)

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Introduction

Developmental temperature is one of the most important environmental factors responsible for the appearance of plasticity in fish, either in the form of temporary modification of the relative scaling of development (e.g., Koumoundouros et al., 2001), or of alterations of gender (e.g., Pavlidis et al., 2000), meristic characters, and body shape (e.g., Georgakopoulou et al. 2007), as well as swimming capacity and number of red muscle fibres (e.g., Koumoundouros et al., 2009). With the exception of studies in gender and meristic characters, most studies on fish phenotypic plasticity focus on single developmental stages. However, there is increasing evidence that environmental conditions experienced in early ontogenetic period could affect fish phenotype well after the metamorphosis stage (Georgakopoulou et al., 2007).

In the current study we examined whether water temperature during early ontogenetic stages (embryonic to early larval) affect adult body shape in zebrafish, *Danio rerio* (Hamilton, 1822).

Materials and methods

The effect of temperature on body shape was examined during two different early developmental periods of 280°d, each (DP1, 28-308°d post-fertilization, and DP2, 280-560°d post-fertilization). During these periods, fish were exposed to three different thermal conditions (22, 28, and 32°C), whereas common thermal conditions (28.0±1.0°C) were applied prior to and after the period of 280°d (Fig. 1). All trials were performed in duplicate. The same batch of eggs was used for the constitution of the experimental groups of each replicate. Maintenance and rearing of fish populations were made as described in Westerfield (1995).

The effect of developmental temperature on adult body-shape was studied by means of geometric morphometrics, on a random sample of 30 individuals per sex and experimental population. Samples were taken 5 months after the end of

the application of the different thermal regimes. Shape analysis was performed on 15 landmarks collected on the individual photographs of the adult fish (Fig. 2). The method of geometric morphometrics and the used software are presented in details elsewhere (Georgakopoulou et al., 2007).

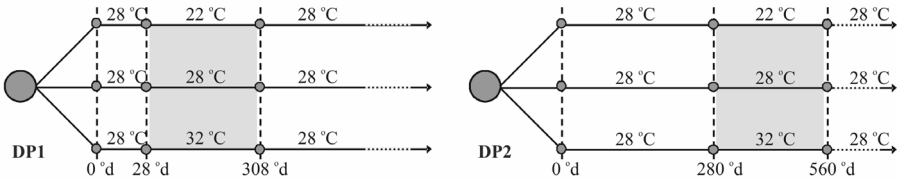

Fig. 1. Experimental design followed in this study.

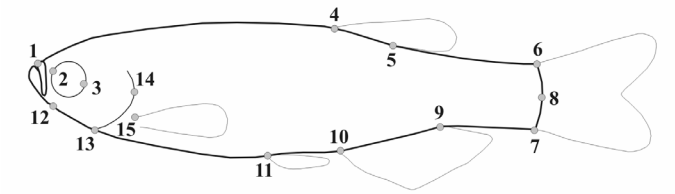

Fig. 2. Landmarks collected on in vivo photographed zebrafish.

Results and discussion

In both developmental periods and replicates examined, developmental temperature and sex had a significant effect on the body shape of adult zebrafish (Wilk's $\lambda = 0.10035\text{-}0.55572$, $p<0.001$, MANOVA). According to the Canonical Variate Analysis, sex-related shape variation was evident in all cases along the first canonical axis (52.53–69.23% explained variance) (Fig. 3). Temperature-related shape variation was mainly expressed along the second canonical axis (16.16-39.82% explained variance), discriminating the 22°C groups from those that were initially developed at 28 and 32°C. In DP1a trial females of 28°C were grouped with females of 22°C and not with females of 32°C (Fig. 3).

In both thermal treatments (DP1 and DP2), shape differences along CV2 (effects of developmental temperature) were mainly attributed to non-uniform shape components, which accounted for the 81.7-98.3% of the between group shape variation. In DP1, vector diagrams showed that 22°C groups where relatively characterised by the anterior transposition of landmarks 4, 5, 9, and 10 (base of the dorsal and anal fins) and by a posterior transposition of landmarks 6 and 8 (base of the caudal fin) (Fig. 4). In DP2, compared with 28°C and 32°C groups, the 22°C groups were mainly characterised by the anterior shift of landmark 12 (angular), ventral shift of landmark 13 (ventral tip of the gill cover), dorsal shift of landmark 11 (base of the pelvic fins), and by the posterior shift of landmarks 14 (posterior tip of the gill cover) and 15 (base of the pectoral fin) (Fig. 4).

Shape differences between the two sexes were mainly attributed to non-uniform shape components, which accounted for the 85.6-91.0% of the between sex shape variation. Compared with the female individuals, male zebrafish were characterised by a proximal transposition of landmarks 4, 10, 11, 14, and 15 (smaller abdominal area), a ventral transposition of landmarks 1 and 12 (upper and lower jaw elements) and by a posterior transposition of landmarks 6, 7 and 8 (longer caudal peduncle) (Fig. 4).

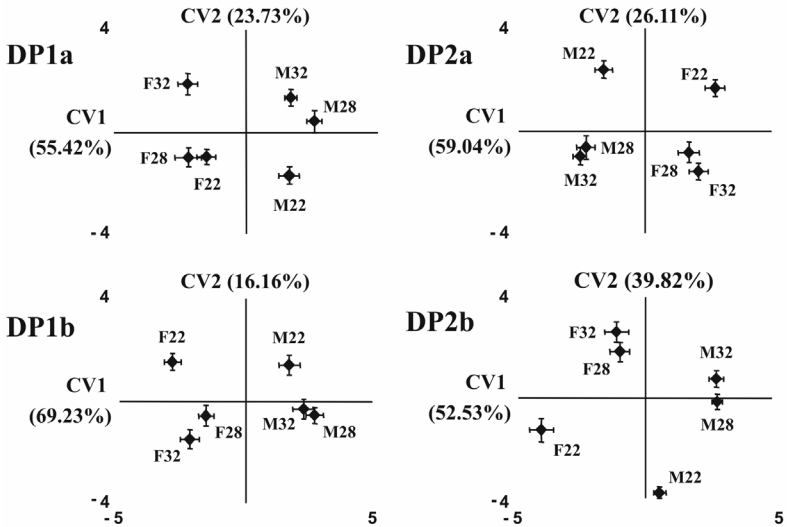

Fig. 3. Effect of developmental temperature (22, 28 or 32°C) and gender (F, M) on the scores (mean±2S.E.) of canonical variate analysis (CV1, CV2). a, b, indicate the replicate of each developmental period examined (DP1, DP2).

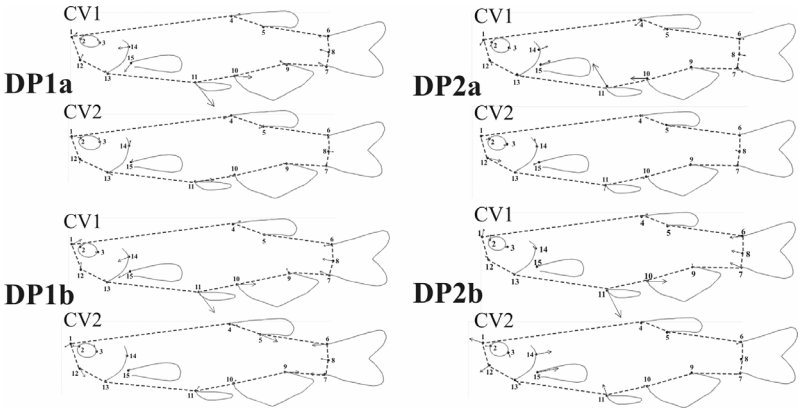

Fig. 4. Spline diagrams demonstrating the components of shape change relative to the negative values of the canonical axes (CV1-CV2). a,b indicate the replicate of each developmental period examined (DP1, DP2).

Environmental effects on fish body shape have been widely studied (e.g., Robinson and Parsons, 2002; Georgakopoulou et al., 2007), but this is the first study, to our knowledge, that demonstrates a significant effect of temperature conditions applied during early life on adult body shape. In a similar study, Georgakopoulou et al. (2007) showed that water temperature during embryonic and larval stages can influence the body shape of the juvenile European sea bass. However, shape differences between the experimental groups diminished during the period following the end of the different thermal treatments, well before the adult stage.

Conclusions

Zebrafish exhibit a significant temperature-driven phenotypic plasticity. Given the wide background on the biology of the species, zebrafish could be a valuable model organism for studying the underlying mechanisms which are involved in this phenomenon.

Acknowledgements

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THE EFFECT OF GRADED DIETARY LEVELS OF VITAMIN A, GIVEN TO EARLY SEA BREAM (*SPARUS AURATA*) LARVAE ON SKELETAL DEFORMITIES AND GENOMIC EXPRESSION

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Morpho-anatomical abnormalities have been considered as the most significant problem in Mediterranean finfish aquaculture. In general, skeletal deformities develop as a result of insufficient knowledge of the optimum environmental preferences (biotic and a-biotic factors) of fish at the different stages of their life. This can be defined as the capacity to continue a normal development until the juvenile and adult stages. There is a close relationship between larval nutrition at first feeding and skeletal abnormalities. This work was the first to systematically study the effect of different vitamin A dietary levels on *Sparus aurata* larval development as to determine whether the dietary effect of vitamin A was associated with a particular developmental larval stage. In addition, this study developed a vitamin A enrichment technology based on the use of liposomes.

The aims of this research were to (1) evaluate the effect of different vitamin A dietary levels on fish development, in terms of optimal growth, and incidence of skeletal malformations; (2) provide an insight into dietary larval requirements, and (3) study the relative mRNA gene expression abundance of RBP, RALDH2, LRAT, STRA6 (four genes that play an important function in vitamin A metabolism and are associated with ossification in fish) during Sea bream larval morphogenesis.

We conducted the experiments by feeding five groups of sea bream larvae, *S. aurata*, with *Artemia* and rotifers enriched with increasing vitamin A levels, at different time periods during the larval metamorphosis: first feeding period (day

4-19), second feeding period (day 20-34), and during the entire larval stage (day 4-34). Based on these results we observed that vertebral deformities in *S. aurata* occurred in a dose-dependent manner. No relationship was found between the dietary Vitamin A in the present study and the relative presence of opercular deformities or swim bladder presence. High vitamin A level resulted in higher incidence of bone deformities, such as vertebral curvature, cranial deformation and compression of vertebra. This study demonstrated for the first time that during the first stages of the metamorphosis (4-19 days post hatching (dph)) higher dietary level of vitamin A caused a high number of cranial deformations, while during the late stages of the metamorphosis (19-34dph) extreme level of vitamin A caused a high number of abnormal vertebrae. These results indicate that the effect of vitamin A is development stage specific in *S. aurata* larvae.

The relative mRNA gene expression of RALDH2, LRAT, STRA6 (three genes which are associated with ossification in fish) was higher during the first three weeks post hatching of Sea bream larval morphogenesis. Furthermore, STRA6 (a multitransmembrane domain protein) is reported for the first time in fish. In the present study with sea bream this gene was widely expressed during the embryonic development and in the adult ovary, which emphasizes the importance of vitamin A in the maternal yolk sac and during embryonic development.

The level of vitamin A which is associated with best performance in terms of growth promotion and reduced rate of deformities is within the range of 0.5-3.9μg retinoid/gr ww in the live food diet and 0.4μg retinoid/gr ww in terms of accumulation in the larval.

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DIGESTIVE PHYSIOLOGY OF BAY SNOOK *PETENIA SPLENDIDA* LARVAE: A SYNTHESIS BETWEEN LIGHT MICROSCOPY AND DIGESTIVE ENZYME ACTIVITY STUDIES

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Introduction

During these last twenty years, most of studies related to the digestive physiology in larvae have been focused in salmonids and marine finfish species due to their important commercial value for the aquaculture industry, but the literature available for freshwater fish species is quite reduced in comparison. In the particular case of cichlids, most of the efforts devoted to study the early development of this group have been focused on tilapia species, since these species are among the most important fish farmed worldwide. However, little is known about the organogenesis in other cichlid species, and in particular of the Central and South American species.

The ontogenetic development and functionality of the digestive system in bay snook *P. splendida* larvae was studied by combining light microscopy and the quantification of several digestive enzymes activities.

Materials and methods

Newly hatched bay snook larvae (n = 2100) were reared until the juvenile stage (45 days post hatch, dph) at 10 larvae.l^{-1} in a closed water system at the Aquaculture Laboratory of DACBIOL-UJAT facilities (Tabasco, Mexico). Larvae were fed to apparent satiation four times per day with *Artemia* nauplii and metanauplii (10 ind.l^{-1}) from mouth opening (3dph) until 15dph. Afterwards and until the end of the study, fish were fed three times per day with a dry trout diet (Silver Cup). The development of the digestive tract was assessed with standard histological procedures (Gisbert et al., 2008a) whereas its functionally was measured by biochemical quantification of digestive (pancreatic, intestinal, and gastric) enzymes as described in Gisbert et al. (2008b).

Results and discussion

Results showed the complimentary roles of both methodological approaches in describing the development and functionality of the digestive system in fish larvae. In particular, data showed that the development of the digestive system in this species was a very intense and asynchronous process, proceeding from the distal to the anterior part of the digestive tract, the intestine being the first region to differentiate at 9dph (6.5mm) and the oesophagus the last (21dph, 8.4mm).

Fast development of the digestive system prior to complete exogenous feeding is also seen in other cichlid species, as well as in other freshwater fish species with large and medium size eggs (e.g., acipenserids, salmonids or siluriformes). However, the fast development of the digestive system is not a generalized feature among other freshwater fish species, such as coregonids, percids, cyprinids, or characids, which are characterized by small size eggs. However, although the ontogenetic pattern of differentiation of the digestive structures is quite similar among cichlids, there are species-specific differences in the timing of differentiation of different digestive structures depending on their reproductive strategy. Thus, ontogeny of the digestive system in bay snook larvae was similar to other substrate spawner cichlids, but faster than in mouth-brooding cichlids. In this sense, the reproductive strategy (parental care) of different cichlid species might have an important effect on the ontogenetic development of the larvae, and consequently on the order of development and functionality of its digestive tract.

Histological observations showed that bay snook larvae retained endogenous yolk reserves until 24dph (8.9mm), likely an advantage for weaning onto inert diet. Stomach and gastric gland differentiation (pepsin activity detection) coincided with the onset of exogenous feeding, indicating that prey were digested by gastric and pancreatic enzyme secretions. These results indicated that at the onset of exogenous feeding, larvae had a well-differentiated and functional digestive system from yolk absorption in which pancreatic (trypsin, amylase, lipase, and chymotrypsin), intestinal (aminopeptidase-N and alkaline phosphatase), and gastric (pepsin) enzymes are present and involved in food digestion processes.

The important lipid accumulation observed in the intestinal mucosa, liver, and pancreas in fish fed a compound trout diet indicated that while fish were able to digest and absorb lipids, diet formulation did not suit the nutritional requirements of early juveniles of this species.

Acknowledgements

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AN ECOPHYSIOLOGICAL AND BIOCHEMICAL APPROACH TO SELECTING THE IDEAL DIET FOR *OSTREA EDULIS* (L.) BROODSTOCK CONDITIONING

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Introduction

Fecundity and initial larval quality have been shown to be related to broodstock conditioning, particularly food supply (Millican and Helm, 1994), while other factors influencing larval growth and survival have been examined by Berntsson et al. (1997). However, the effects of different diets on flat oyster reproduction have mainly been studied from a biochemical point of view. Thus, lowered fecundity related to *Dunaliella tertiolecta* used as diet for breeders was explained by a lack of essential lipids in the food (Millican and Helm, 1994). To be nutritionally efficient, a microalga must be well ingested, assimilated, and allocated to the reproductive compartment. The present work will focus on ecophysiological aspects of feeding in *Ostrea edulis* conditioned with different microalgae, with a study of the biochemical allocation of these diets. The overall nutritional effects on fecundity and subsequent larval development will be used to determine an ideal conditioning diet based on physiological and biochemical data.

Material and methods

Four different microalgae were tested as mono-specific diets: *Isochrysis affinis galbana* (volumetric size ≈ 45µm^3, dry weight 20pg.cell^{-1}, T-ISO strain CCAP 927/14), *Chaetoceros gracilis* (80µm^3, 70pg.cell^{-1}, strain UTEX LB2658), *Skeletonema marinoï* (85µm^3, 50pg.cell^{-1}, strain CCAP 1077/3) and *Tetraselmis suecica* (280µm^3, 225pg.cell^{-1}, strain CCAP 66/4). 18-month-old *O. edulis* (≈5cm length and 0.5g meat dry weight) were distributed homogeneously in translucent 50 l tanks, in triplicate for each diet. They were maintained at 19 °C

in a flow-through system at a flow rate of 12 $l.h^{-1}$, and continuously fed at $1600\mu m^3.\mu l^{-1}$ (equivalent T-ISO). Seawater was previously filtered on 1µm polypropylene filter media following UV treatment. Ingestion and absorption rates were studied according to Beiras et al. (1994) to determine the most efficiently assimilated microalga. Protein and glycogen composition of different tissues (gonad, digestive gland, muscle, and gills) were then measured following standard methods, to determine which microalga led to the most efficient allocation of resources to reproduction.

When a significant spawning was detected, larval rearing was set up in a dedicated 5-l translucent flow-through system. A standard bispecific diet (T-ISO + *C. gracilis*) was fed to larvae from all of the different broodstock batches. Offspring of each parental treatment were reared in triplicate and larval development assessed through survival, growth and competence to metamorphose.

Results and discussion

C. gracilis and *S. marinoï* led to high absorption values (2.1 ± 0.4 and 2.0 ± $0.5mg.dwg^{-1}.h^{-1}$) and high absorption efficiency (AE %: 38.8 and 48.5%: Fig. 1a). In contrast, lower values ($p < 0.05$) were recorded for *T. suecica* (1.3 ± $0.6mg.\ dwg^{-1}.h^{-1}$, 35.9 %) and *I. aff. galbana* ($0.01 \pm 0.03mg.dwg^{-1}.h^{-1}$, 1.5%).

Mean gonad glycogen ranged from 79 to $141mg.dwg^{-1}$. Gonads accumulated glycogen when *O. edulis* were fed any of the microalgae except T. ISO ($p < 0.05$) and the extent depended on the algal species supplied: *T. suecica* < *C. gracilis* < *S. marinoï*. In contrast, gonad protein content of oysters fed T. ISO was the highest, at $528mg.dwg^{-1}$ ($p < 0.05$).

Nevertheless, 1.4 million larvae were harvested during the conditioning period (7 weeks) when flat oysters were fed T-ISO. When they were fed *C. gracilis* or *S. marinoï*, 1 million larvae were released, but only 0.3 million when the breeders were supplied *T. suecica.*

Larval survival measured at the end of the larval rearing period (day 11) was high and similar (91 to 98%), regardless of the broodstock diet. In contrast, initial larval size was related to adult nutrition, with a mean of 173µm ± 7, 179µm ± 7, 178µm ± 6, and 165µm ± 9 for larvae released from oysters fed T-ISO, *C. gracilis*, *S. marinoï* and *T. suecica*, respectively. Larval growth (daily growth rate) was higher for larvae of breeders fed *T. suecica* and T-ISO with 7.5 and $7.2\mu m.d^{-1}$, respectively. In contrast, larvae from broodstocks supplied with diatoms (*C. gracilis* and *S. marinoï*) exhibited the lowest growth: 6.4 and $5.4\mu m\ d^{-1}$, respectively. When these latter were fed the balanced bispecific diet (T-ISO + *C. gracilis*), similar percentages of eyed larvae were recorded on day 11 (46 and

51%). Those batches from breeders fed T-ISO and *T. suecica,* however, produced a lower final number of competent larvae (25 and 26%: Fig. 1b).

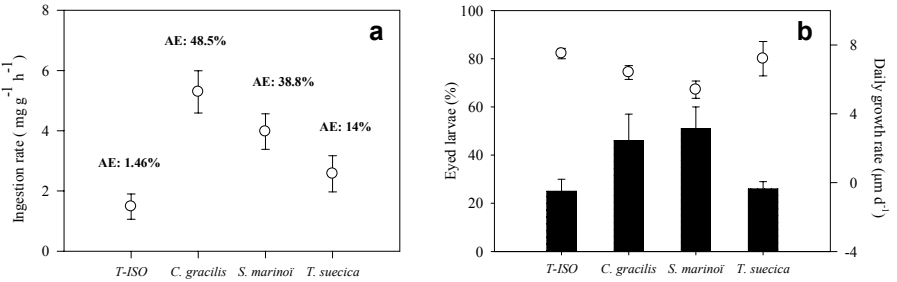

Fig. 1. (a) Ingestion rate and Assimilation Efficiency (AE) of *O. edulis* broodstock fed different diets; (b) Final larval size and percentage of eyed larvae on day 11 according to parental conditioning diet. Larvae were fed T-ISO + *C. gracilis* from release.

Conclusions

When used as single diets, *C. gracilis* and *S. marinoi* are efficient for *O. edulis* conditioning. They both lead to high larval performances, as expressed in the number of larvae competent to metamorphose. In contrast, *T. suecica* is not recommended for *O. edulis* conditioning due to its low AE, low fecundity and low number of metamorphosis-competent larvae. Due to its low assimilation efficiency and the weak larval performances induced, *I. aff. galbana* (T. ISO) appears to be unsuitable for *O. edulis* conditioning. However, a high number of larvae were released with this diet. These conflicting data could be due to an intense use of parental reserves to sustain reproduction. This hypothesis is supported by the observation of a 20% decrease in muscle and gills during conditioning of this broodstock batch.

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STEREOLOGICAL STUDY ON EFFECTS OF *AEROMONAS HYDROPHILA* ON DIGESTIVE TRACT DEVELOPMENT OF EARLY *ARTEMIA* NAUPLII

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Introduction

The brine shrimp, *Artemia*, is one of the most important live feeds for commercial production of fish and shellfish larvae (Sorgeloos et al., 1986). Many scientific studies are performed using gnotobiotic *Artemia* to investigate host-microbe interactions. In the present study, GART (gnotobiotic *Artemia* rearing system) was expanded by incorporating stereological tools to investigate the effects of the bacterium *Aeromonas hydrophila* (LVS3) on the development of the digestive tract of early *Artemia* nauplii.

Materials and methods

Artemia cysts were decapsulated and cultured axenically for four days and fed daily with Baker's yeast together with dead LVS3. This group was considered as control axenic group. The test group (monognotobiotic), was fed daily with the same feed that was supplemented with live LVS3 (5% of the total dead bacterial biomass) only on day 1 after decapsulation. Live nauplii were sampled on day 2 and day 4. They were fixed in AFA (80ml of 100% ethyl alcohol, 15ml of 40% formaldehyde and 5ml of acetic acid) for 5min, dehydrated in a graded series of alcohols and embedded in paraffin wax. Five-µm thick sections of 6 nauplii per group were stained with Haematoxylin/Eosin for stereological examination.

The volumes of the mid- (MGV) and hindguts (HGV) of 6 nauplii per treatment were estimated based on histological sections by applying the Cavalieri method as performed by Casteleyn et al. (2007). Ten sections of each mid- and hindgut of serially sectioned specimen, separated by a fixed interval *(T)*, were selected

randomly by randomizing the position of the first section within the interval T. A point grid with a known fixed area associated with each point (*a/p*) was uniform randomly placed on the histological section of interest using the software Cell*F (Olympus Belgium) (Fig. 1). The gut epithelium together with its brush border, when present and the underlying muscle layer were taken into account. The grid points (*p*) which hit these tissues were counted and the total area of the tissues per section (*Ai*: area of the *i*th section) were calculated by multiplying the area per point (*a/p*) by the total number of points counted per section (*Pi*: number of points hitting the tissues of the digestive tract on the *i*th section).

$$A_i = \frac{a}{p} \cdot P_i$$

Then, the total volume (*V*) of the digestive tract of the examined nauplius was calculated by multiplying the sum of all areas by the section interval (*T*).

$$V = T \cdot \sum_{i=1}^{m} A_i$$

The precision of the volume estimation of each mid- and hindgut was obtained by calculating the coefficient of error (CE) for the Cavalieri method developed by Gundersen and Jensen (1987). The lower the value, the more precise the volume estimation is. To estimate CE, three sums (a, b and c) were first calculated:

$$a = \sum_{i=1}^{m} A_i \cdot A_i \qquad b = \sum_{i=1}^{m-1} A_i \cdot A_{i+1} \qquad c = \sum_{i=1}^{m-2} A_i \cdot A_{i+2}$$

Using a, b and c, it is possible to calculate the CE, as shown in the following equation.

$$CE(V) = \frac{1}{\sum_{i=1}^{m} A_i} \cdot \sqrt{\left[\frac{1}{12}(3a + c - 4b)\right]}$$

Results and discussion

Remarkably, the volumes of the midguts of the nauplii of both the tested days were larger than that of hindguts. The volume differences between the axenic and monognotobiotic treatments were, however, not significant (Fig. 2). Further studies are needed to confirm the results and to find explanations for these observations.

Conclusions

It is likely that differences of the volumes of the mid- and hindgut between axenic and monognotobiotic *Artemia* will appear in the later stages of their life cycle for which it would be necessary to prolong the experiment. Further, the results of this experiment indicate that gnotobiotic *Artemia* with stereological tools is a useful model system for studying the development of *Artemia*.

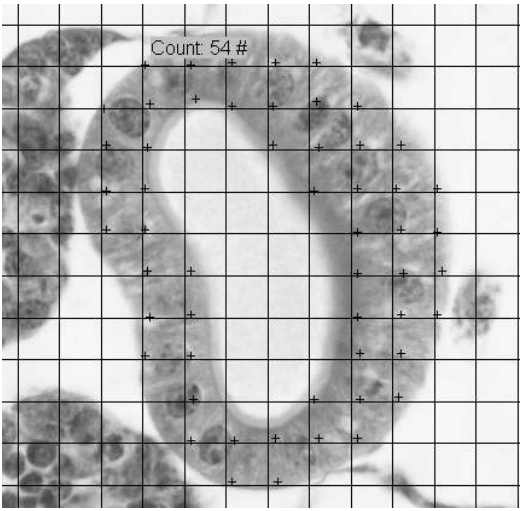

Fig. 1. A cross section of a 2-day-old *Artemia franciscana* nauplius through the midgut showing the point counts using the grid (Cavalieri method).

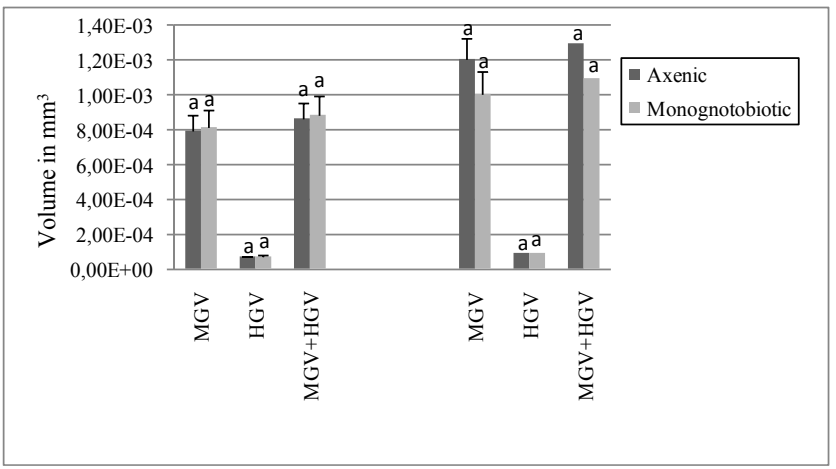

Fig. 2. Average volumes (mm^3) with standard error of the mean of the midgut (MGV), the hindgut (HGV), and both the mid- and hindgut (MGV+HGV) of axenic and monognotobiotic *Artemia* at day 2 and 4.

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DIETARY POLYAMINE PRODUCING YEAST INDUCES DIGESTIVE ACTIVITY AND GROWTH PERFORMANCE IN *PARALABRAX MACULATOFASCIATUS* LARVAE

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Introduction

The use of probiotics in aquaculture has increased in recent years in efforts to improve larval culture yields. *Debaryomyces hansenii* strain CBS 8339 secretes putrescine (put), spermidine (spd), and spermine (sp). These polyamines are valuable molecules for growth and cellular differentiation and ornithine decarboxylase (ODC) is the first enzyme in their biosynthesis. The main objective of this work was to evaluate the effect of dietary inclusion of wild (ODC+) *D. hansenii* and yeast inhibited by ornithine-decarboxylase activity (ODC-) on the growth and digestive enzyme activity of the spotted sand bass *Paralabrax maculatofasciatus* larvae.

Materials and methods

D. hansenii polyamines biosynthesis was monitored during the cell cycle of the yeast in batch culture in the presence or absence of 2% D,L-α-difluoromethylornithine (DFMO), an ODC irreversible inhibitor. Yeast growth was measured as the increase in optical density (OD) at 550nm and polyamines were extracted and quantified by HPLC according to Mallé et al. (1996). A microparticulated diet was designed, and supplemented either with 1.1% (equivalent to 10^6UFCg^{-1}) of ODC+ or ODC- *D. hansenii*. A microparticulated diet without yeast was used as control. Larvae growth, survival, pancreatic (trypsin,

lipase, amylase), and intestinal (aminopeptidase N) enzyme activities were measured from 20 to 35 days post hatching (dph). The experiments were carried out in three replicates and results analyzed by a one-way ANOVA followed by Neuman Keul's multiple range test when significant differences were found at the 0.05 level using SPSS software (ver. 15.0, LEAD Technologies, Inc, USA).

Results and discussion

Our results show that the inhibitor DFMO (2%) did not affect the growth or the production of polyamines in *D. hansenii* ($P>0.05$), likely because an alternative metabolic pathway of polyamines biosynthesis is used (arginine-decarboxylase) by yeast to cover polyamine deficiency (Kallio and McCann, 1981). In the case of fish culture, the larval survival was significantly different in those larvae fed ODC (+) diet (13.1±0.5%) compared to those fed ODC (-) diet (11.6±0.9%) and the control diet (10.3±0.5%). However, the growth in length (Fig. 1) was lower in larvae fed the yeast diets, because the higher larval density permitted a size segregation and cannibalism (Hecht and Peinar, 1993). The activity of trypsin, lipase, and aminopeptidase N were highest in the groups fed with yeast ODC (+) diet. In the case of amylase activity, the control group had the highest levels of this enzyme at 20 and 30dph, which indicates a delay in the maturation process with this diet.

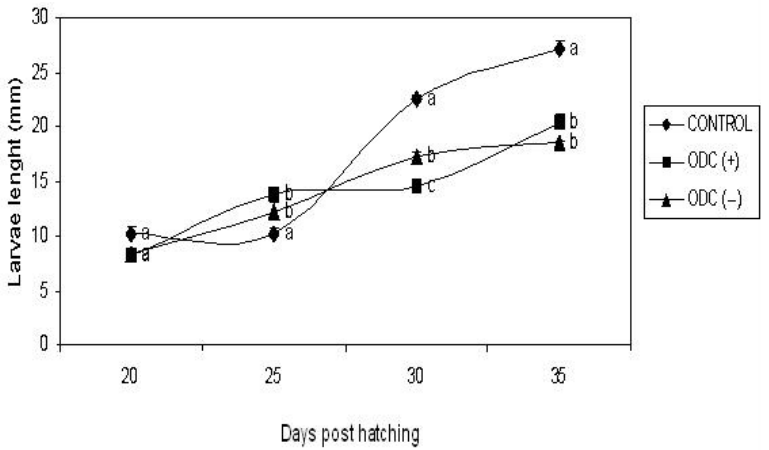

Fig. 1. Growth in length of *P. maculatofasciatus* larvae fed control diet and supplemented diets with either ODC+ or ODC- yeast. Means ± S.E. with different superscripts are significantly different ($P<0.05$).

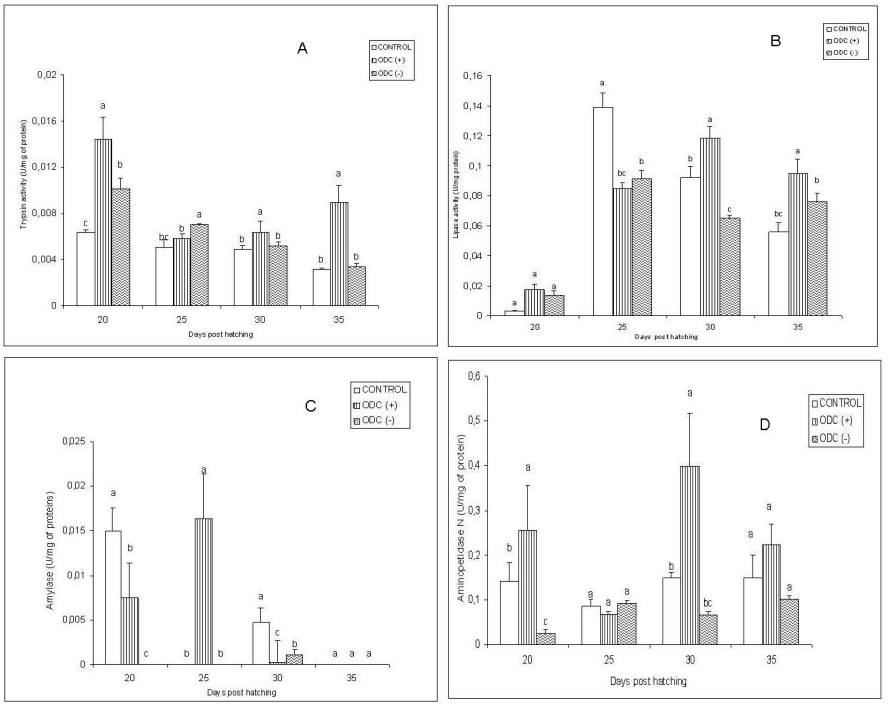

Fig. 2. Trypsin (A), lipase (B), amylase (C) and aminopeptidase N (D) activities in larvae fed control diet and supplemented diets with either ODC+ or ODC- yeast. Means ± S.E. with different superscripts are significantly different (P<0.05).

Conclusions

Our results suggest that a diet containing living polyamines secreting yeast can modify or induce important changes in the secretion pattern and digestive enzymatic activity, allowing intestinal and pancreatic maturation as seen previously in sea bass using molecular approaches (Tovar et al., 2004).

Acknowledgements

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EFFECTS OF FATTY ACID AND VITAMIN C ENRICHMENT ON THE NUTRITIONAL VALUE OF *ARTEMIA URMIANA* NAUPLII FOR PERSIAN STURGEON (*ACIPENSER PERSICUS*) LARVICULTURE

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Introduction

For restocking the sturgeon larvae (anadromus species) in the Caspian sea, the improvement of biochemical composition and vitamin C content of fish larvae is a current practice to evaluate its salinity tolerance under the sea water condition (~12ppt salinity). The present work intends to determine the effects of the bio encapsulation of *Artemia urmiana* nauplii with highly unsaturated fatty acids (HUFA) supplemented with vitamin C (AP) on larviculture of Persian Sturgeon *Acipenser persicus* larvae.

Materials and methods

The larvae were reared in a semi-closed circuit as described by Pousao-Ferreira and Silva (1989), and fed with *Artemia urmiana* nauplii unenriched from the first feeding stage to 5th day. The actual feeding experiments lasted from day 5 to day 20 when the larvae were fed with bio encapsulated *Artemia* nauplii. Two oil sources, *ICES30/4c* and sturgeon ovary oil three vitamin C levels (10, 20, and 30%) during two enrichment periods (12 and 24h), were tested.

At day 20 (after 1 day of starvation) the larvae were collected for chemical analysis and compared with the bioencapsulated *Artemia*. The HUFA were determined by the methods described by Bligh and Dyer (1959) and Metcalfe and Schmitz (1961) using liquid-gas chromatography. The salinity tolerance was measured in 6, 12, and 18ppt at 1, 2, 4, to 120h using % survival.

The experiments were carried out in three replicates and results analyzed by three factor factorial ANOVA. Data were normalized by an arc-sine%p transformation (Sokal and Rohlf, 1981) and significant differences determined by a Tukey multiple comparison tests, using SPSS, Ver. 14.

Results and discussion

The results show that the total amounts of (n-3) long chain fatty acids (C>20) were significantly different in all treatments mainly due to the DHA/EPA ratio (Fig. 1). The ration (n-3)/(n-6) was significantly different, with a higher level of (n-6) HUFA in the larvae fed the *ICES30/4c*-enriched treatment (Fig. 2).

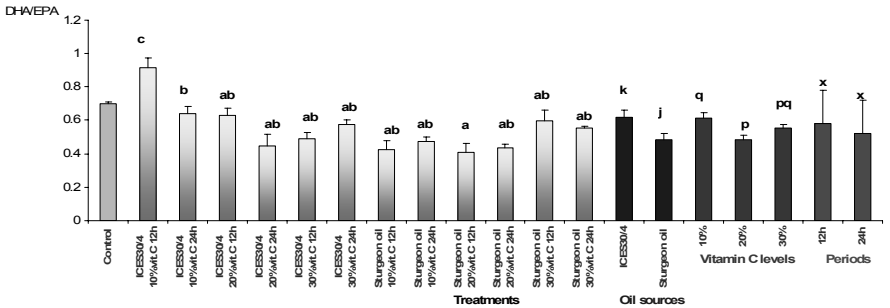

Fig. 1 DHA/EPA ratio of *Acipenser persicus* larvae enriched with different oils, vitamin C levels at 12, 24 h enrichment periods. Mean within the grouping followed by the different letters show statistically different (P<0.05).

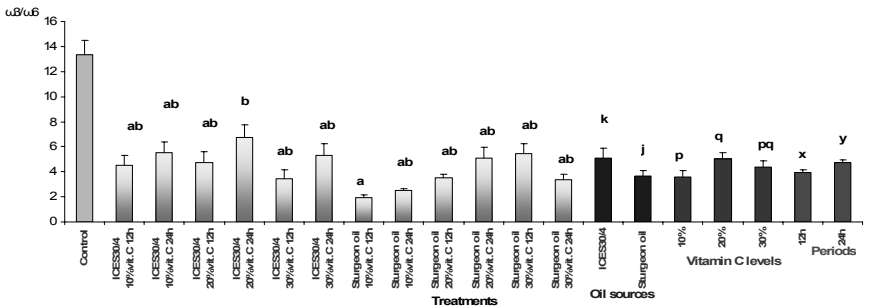

Fig. 2 ω-3/ ω-6 ratio of *Acipenser persicus* larvae enriched with different oils, vitamin C levels at 12, 24 h enrichment periods. Mean within the grouping followed by the different letters show statistically different (P<0.05).

Table I. Protein, lipid, vitamin C, and fatty acids compositions of Persian sturgeon larvae enriched with different oils, effects of different vitamin C levels (%), and enrichment periods. Values are the averages of three replications (SD).

	OIL SOURCES		VITAMIN C LEVELS			ENRICHMENT PERIODS	
	ICES30/4C	Sturgeon Oil	10%	20%	30%	12h	24h
Protein% DW	68.48(2.21)^{k}	64.89(1.56)^{j}	68.10(2.59)^{r}	67.20(2.52)^{q}	64.75(1.50)^{p}	66.09(3.00)^{x}	67.28(2.10)^{y}
Lipid%DW	16.08(2.02)^{k}	15.20(0.96)^{j}	16.38(2.37)^{q}	15.97(0.52)^{q}	14.56(0.73)^{p}	15.84(2.13)^{x}	15.44(0.88)^{x}
Vit. C µg/g DW	144.95(17.33)^{k}	80.76(17.01)^{j}	99.37(14.59)^{p}	123.52(14.86)^{q}	115.62(14.86)^{q}	98.03(18.39)^{x}	127.0(16.31)^{y}
Fatty acids mg g^{-1} DW							
C14:0	0.82(0.06)^{j}	0.90(0.16)^{k}	0.94(0.16)^{q}	0.86(0.09)	0.79(0.09)^{p}	0.85(0.13)	0.88(0.13)
C14:1n5	0.72(0.20)	0.67(0.15)	0.76(0.14)^{q}	0.77(0.19)^{q}	0.57(0.12)^{p}	0.78(0.18)^{y}	0.62(0.14)^{x}
C15:0	0.50(0.06)^{j}	0.75(0.34)^{k}	0.52(0.09)^{p}	0.83(0.39)^{q}	0.54(0.06)^{p}	0.61(0.26)	0.65(0.28)
C15:1	0.70(0.15)^{j}	1.00(0.28)^{k}	0.95(0.25)^{q}	0.97(0.28)^{q}	0.64(0.14)^{p}	0.81(0.23)^{x}	0.89(0.31)^{y}
C16:0	15.39(1.56)^{j}	16.08(0.80)^{k}	16.24(1.69)^{r}	15.86(0.69)^{q}	15.11(1.04)^{p}	15.09(0.78)^{x}	16.37(1.36)^{y}
C16:1n7	6.37(1.31)^{k}	5.52(1.09)^{j}	5.81(1.22)^{pq}	6.68(1.43)^{q}	5.35(0.73)^{p}	5.84(1.51)	6.06(0.99)
C17:0	1.34(0.90)	1.35(0.39)	1.73(0.90)^{r}	1.23(0.50)^{q}	1.07(0.40)^{p}	1.35(0.83)^{y}	1.34(0.52)^{x}
C17:1n7	1.90(0.75)^{k}	1.56(0.35)^{j}	1.74(1.01)	1.67(0.28)	1.79(0.20)	1.82(0.80)^{y}	1.65(0.30)^{x}
C18:0	8.74(2.35)	8.86(1.13)	8.69(2.23)^{q}	9.76(1.65)^{r}	7.67(0.67)^{p}	8.36(2.18)^{x}	9.05(1.34)^{y}
C18:1n9	13.54(3.17)^{j}	16.86(1.29)^{k}	14.07(4.84)^{p}	15.73(0.92)^{q}	15.82(0.92)^{q}	14.51(3.40)^{x}	15.90(2.24)^{y}
C18:1n7	11.31(1.73)^{j}	10.38(0.84)^{k}	9.89(1.51)^{p}	10.24(1.05)^{q}	10.91(1.33)^{r}	9.93(1.48)^{x}	10.77(1.07)^{y}
C18:2n6-ci	2.97(0.80)^{k}	1.56(0.52)^{j}	2.41(0.78)^{q}	2.60(1.30)^{r}	1.79(0.60)^{p}	2.24(1.25)	2.30(0.64)
C18:3n3	4.73(1.36)^{k}	3.93(1.19)^{j}	5.03(1.28)^{r}	4.12(0.82)^{q}	3.83(1.54)^{p}	4.22(1.50)^{x}	4.44(1.15)^{y}
C20:1n9	0.29(0.33)^{k}	0.00(0.00)^{j}	0.34(0.37)	0.10(0.19)^{q}	0.00(0.00)^{p}	0.20(0.32)^{x}	0.09(0.21)^{y}
C20:2n6	0.35(0.19)^{k}	0.26(0.03)^{j}	0.25(0.02)^{pq}	0.24(0.04)^{p}	0.29(0.06)^{q}	0.28(0.05)	0.28(0.04)
C20:3n3	0.29(0.01)^{j}	0.39(0.03)^{k}	0.50(0.03)	0.52(0.03)	0.51(0.05)	0.45(0.05)^{x}	0.54(0.04)^{y}
C20:4n6 (ARA)	1.41(0.35)^{k}	0.94(0.39)^{j}	1.34(0.16)^{q}	1.09(0.34)^{p}	1.09(0.64)^{p}	1.29(0.51)^{y}	1.06(0.32)^{x}
C20:5n3 (EPA)	4.24(0.86)^{k}	2.01(0.46)^{j}	2.85(0.93)^{p}	3.61(1.40)^{q}	2.91(1.53)^{p}	3.00(1.22)^{x}	3.25(1.44)^{x}
C22:6n3 (DHA)	2.52(0.40)^{k}	0.95(0.14)^{j}	1.84(0.98)^{p}	1.79(0.83)^{p}	1.58(0.76)^{p}	1.76(0.91)^{x}	1.71(0.80)^{x}
DHA/EPA	0.62(0.04)^{k}	0.49(0.03)^{j}	0.62(0.03)^{q}	0.49(0.03)^{p}	0.57(0.020)^{pq}	0.52(0.02)^{x}	0.53(0.20)^{x}
Σ Saturated	26.79(3.90)	27.76(1.96)	28.11(3.35)^{q}	28.53(1.84)^{q}	25.19(1.11)^{p}	26.26(2.16)^{x}	28.29(2.84)^{y}
Σ Monoens	33.84(3.90)^{j}	36.01(2.74)^{k}	33.56(5.31)^{p}	36.17(1.83)^{q}	35.06(1.92)^{pq}	33.88(3.66)^{x}	35.97(3.08)^{y}
Σ n-3 HUFA	6.75(0.86)^{k}	2.95(0.35)^{j}	4.68(0.37)^{p}	5.39(0.48)^{q}	4.47(0.36)^{p}	4.75(0.21)^{x}	4.94(0.21)^{x}
ω- 3/ ω- 6	5.06(0.82)^{k}	3.63(0.46)^{j}	3.61(0.50)^{p}	5.03(0.49)^{q}	4.40(0.46)^{pq}	3.93(0.23)^{x}	4.76(0.22)^{y}

Means in a row within the same group followed by the different letter are significantly differences ($P<0.05$)

According to New (1986), the (ω-3):(ω-6) ratio should be considered as the critical component in a diet, rather than each isolated HUFA level, and it can therefore be used as an indicator of possible HUFA deficiencies of the feeds.

The presence of 22:6n-3 in larvae fed the *ICES30/4c*-enriched *Artemia* seems to indicate that *Acipenser persicus* larvae, contrary to the majority of the marine fish, have the capacity to bioconvert the 18:3n-3 acid from 18:1n-9 since their feed, *Artemia* nauplii bioencapsulated with *ICES30/4c*, had a little 22:6n-3. The 18:1n-9 levels also decreased in larvae as compared to the levels in *Artemia*. The larvae could utilize the 18:1n-9 as a precursor of the 18:2n-6 and 18:3n-3. Likewise, the larvae showed an increase of 20:4n-6 levels as compared to their diet composition.

Conclusions

Persian sturgeon larvae seem to be able to tolerate 12-ppt seawater salinity if they are enriched with HUFA supplemented with 20% vitamin C. In addition, they can compensate HUFA deficiencies in their diet, especially 22:6n-3, by the bioconversion of precursor fatty acids; however, the low 22:6n-3 percentages found in the larvae do not allow for a positive conclusion about their bioconversion capacity. Further studies on the quantification of specific needs in (n-3) HUFA of *Acipenser persicus* during its larval development, using radioactive labelling methods, are therefore required.

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IMPACT OF NON GENETIC MATERNAL EFFECTS ON JUVENILE GROWTH IN RAINBOW TROUT *ONCORHYNCHUS MYKISS*

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The development of DNA fingerprints in aquaculture using microsatellites markers opened new perspectives for aquaculture breeding by the early mixing of the families and their a posteriori identification. This innovation could limit both the investment needed and the "tank" bias as candidates can be reared in the same tank since hatching. However, non-genetic maternal (NGM) effects (e.g., egg quality, egg size, hatching time, or age of the females) could bias estimates of genetic parameters and breeding values in such a type of selection program. The quantification of NGM effects is then needed to evaluate expected genetic gain in mass selection or family breeding schemes when families are reared together since hatching and pedigreed using DNA fingerprinting.

This study was designed to evaluate the genetic parameters and NGM effects for growth at 174 days post hatching (dph) in rainbow trout *Oncorhynchus mykiss* when families are reared together since eyed stage. In parallel, as it was foreseen that initial differences in egg weight could lower heritability estimates, an alternative strategy was tested on the same families to try to limit NGM effects.

600 full-sibs families were produced at the Aqualande breeding Center "Les Sources de l'Avance" (Pissos, France) by mating the same day 60 dams and 100 sires in a partial factorial design (each dam mated with 10 sires). At eyed stage, each spawn was subdivided into two groups: the NORM group, with maximal expected NGM effect, was composed by pooling 150 eyed eggs randomly chosen in each spawn; and the MIN group, with expected limited NGM effect, was composed in pooling the spawns in 12 sub groups of 5 spawns each with similar mean egg weight. The growth of the sub groups was managed to achieve the same mean body length (9.7cm) at 123dph. Then, 250 juveniles per sub group were sampled at random and pooled together.

At 174dph (69.1 ± 17.6g and 73.2 ± 17.6g for the NORM or MIN groups), body weight (BW) and body length (BL) were measured and the Fulton coefficient calculated ($BW*100*BL^{-3}$). Fin samples were collected in 95% ethanol (n=1000 and n=2000 for the NORM or MIN groups). Only fish unambiguously assigned to two parents using microsatellites were kept for statistical analysis.

Heritabilities, NGM effects, and genetic correlations were estimated within group with REML applied to an animal model using VCE 5.2.1. NGM effect was considered as a random dam effect. The significance of the NGM effects was evaluated by the comparison of the log-likelihoods (-2logL). Genetic correlation between groups was estimated considering the same trait (e.g. BW) as two different traits between the two groups (e.g., BW in NORM or MIN groups).

Success of single-pair parentage assignment (with one mismatch tolerated) was high: 100% in the NORM and 99.75% in the MIN groups. 79.3% (n=476) and 93.1% (n=559) of the full-sib families were represented in the NORM and MIN groups. The representation of the half sib families was not statistically different between the NORM and MIN groups either on half sib families from sires (χ^2=118.3; 99 d.f.; p>0.08) or dams (χ^2=74.4; 59 d.f.; p>0.08).

In the NORM group, a significant NGM effect was observed for BW (m = 0.08 ± 0.02) and BL (m = 0.07 ± 0.02) but not for K (m = 0.02 ± 0.02). When the NGM effect was introduced in the model, the heritability decreased from 0.34 ± 0.16 to 0.16 ± 0.04 for BW, from 0.30 ± 0.14 to 0.14 ± 0.14 for BL, but not for K (0.53 ± 0.06 and 0.49 ± 0.06). In the MIN group no significant NGM effect was observed for BW (m = 0.03 ± 0.02), BL (m = 0.03 ± 0.02) or K (m = 0.02 ± 0.02). Heritability estimates were moderately high for BW (0.42 ± 0.04), BL (0.37 ± 0.04) and K (0.30 ± 0.03).

Genetic correlations between the MIN (without NGM effect in the model) and the NORM (with NGM effect included in the model) groups for BW, BL and K were 0.94 ± 0.02, 0.96 ± 0.07 and 0.92 ± 0.04, showing that traits can be considered as the same traits in both groups.

This is the first study to estimate genetic parameters and NGM effects on early growth in a salmonid species when families are mixed together at eyed stage. It is concluded that NGM effects on early growth exist when families are mixed together at eyed stage, but not on the morphology. A proper management of egg size differences between spawns can almost eliminate NGM effects and dramatically increase the heritability for growth, leading to a doubling of the expected genetic gain by mass or family selection. Further experiments are needed to evaluate the persistence of such NGM effects on older fish. The study was supported by the French Ministry of Agriculture (Action Inno-vante n° 2005 PA01), the European Union (IFOP n°2005/159) and the CIPA.

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ANALYSIS OF SKELETAL DEFORMITY IN FISH USING VITAMIN A-INDUCED BONE DEFORMITY MODEL

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Annual aquaculture production of finfish in Japan is approximately 250,000 tons. To support increasing production of farmed fish, numbers of hatchery-raised juveniles are produced. Once the technique for mass production is established, the next step is to develop a technique to enhance the quality of the mass produced juveniles. Hatchery-raised juveniles however often show high percentage of skeletal deformity. Skeletal deformity is now a serious concern in modern aquaculture industry. It is thought that skeletal deformity is not curable after occurrence. Furthermore, skeletal deformity potentially occurs in all species and can be induced by various factors such as nutritional, environmental, behavioral and genetic factors and morphology of deformed skeleton resembles each other even if it is induced by different factors. Therefore, there are few examples of overcoming skeletal deformities in cultured fish that often occurred in hatchery-raised fish during history of juvenile production; the lordosis induced by unsuccessful inflation of swimbladder, the scoliosis induced by myxoporean parasite in the brain, and the vertebral fusion induced by oxygen deficiency during somitogenesis in several species. Unpredictability of occurrence and unknown etiology is major obstacles to study skeletal deformity in hatchery-raised fish. To study skeletal deformity in hatchery-raised juveniles, one effective approach is to establish model. Here we report several cases of our model study employing vitamin A inducing skeletal deformity in fish. This approach has potential to find out mechanism underlying skeletal deformity in hatchery-raised juveniles. We focused on jaw and vertebral deformity in Japanese flounder *Paralichthys olivaceus* induced by retinoic acid, active vitamin A compounds.

Retinoic acid (RA) is an active form of various vitamin A compounds. Vitamin A deficiency leads to growth retardation, skeletal deformity, skin dryness and inflammation, night blindness, and infertility. These pathologic conditions are recovered by supplementation of RA except for visual function. It is well accepted that vitamin A action is mediated by two classes of nuclear receptors; RA receptor and retinoid X receptor that have three subtypes such as α, β, and γ. Ac-

tivity of RARs/RXRs is inhibited by binding with repressors in ligand-free status in cell. These two receptors act as transcription factors upon binding ligands and forming complex with several coactivators. Several ligands are known to bind RAR and RXR (all-*trans* RA and 9*cis* RA for RARs, and 9*cis* RA, docosahexaenoic acid, and methoprene for RXR). It is suggested that numerous genes are directly/indirectly regulated under RARs/RXRs pathway. Although several downstream factors are proposed to mediate RARs/RXRs pathway to induce skeletal deformity in fish, clear mechanism has been unknown.

Japanese flounder *P. olivaceus* larvae were treated with various RA compounds that shows different affinity to RARs/RXRs. We examined three RA isomers and two types of synthetic RAR/RXR agonists (Am80 and methoprene acid) on the occurrence of jaw deformity in flounder larvae. It was found that RA isomers and synthetic RA that have higher affinity to RAR showed severer jaw deformity in flounder larvae. In addition, we observed that increased RAR expression in the jaw. In consistence with RAR upregulation and higher incidence of jaw deformity, it was found downregulation of *patch*, *vitamin D receptor*, and *pitx-2* in the jaw. These results suggested that jaw deformity was induced via activation of RAR pathway and in part, down regulation of these genes important for skeletal development in founder larvae. To study mechanism of vertebral fusion in fish induced by RA, we took advantage of use of *twiggy winkle hedgehog* (*twhh*)-green fluorescent protein (GFP) transgenic zebrafish which specifically express GFP in notochord and intervertebral disks. Detailed observation of GFP expression in this transgenic line revealed that first GFP starts to express in the notochord, but later on GFP specifically express in in tervertebral disks. We observed abnormal distribution of GFP expressing cells in the notochord by RA treatment in *twhh*-GFP transgenic line. Vertebral fusion in RA treated fish is clearly revealed by lacking GFP expression in fused centrum. It was observed that extensive calcification of notochordal sheath concomitant with upregulaion of osteopontin expression in zebrafish treated with RA. This suggests that RA induce premature ossification of notochordal sheath and eventually induce vertebral fusion in fish larvae. This study suggests usefulness of *twhh*-GFP transgenic zebrafish to study intervertebral disk development and possible causative genes responsible for skeletal deformity in fish.

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MASS CULTURE OF EURYHALINE CLADOCERAN *DIAPHANOSOMA CELEBENSIS* AND ITS FEEDING TO MARINE FISH LARVAE

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The supply of *Artemia* depends on natural resources, whose quantity and quality tend to be variable. It is therefore useful to explore and find other live food species which can be used as a supplementary or as a substitute of *Artemia*. Among other crustacean zooplankton, cladocerans are comparatively easy to mass culture at high density. Although most cladoceran species inhabit freshwater and are not appropriate for feeding marine fish larvae, there are several species that can be cultured in seawater. Among them is *Diaphanosoma celebensis* (Crustacea: Cladocera: Sididae), a euryhaline cladoceran with a wide but patchy distribution in tropical Asia.

Studies on *D. celebensis* have been conducted on its biology, ecology, and culture methods. It is characterized by parthenogenetic mode of reproduction, short generation time (4-5 days), and wide temperaturee (15-35°C) and salinity (7-32ppt) tolerance. The size distribution of *D. celebensis* (400-1100μm) covers that of newly born *Artemia* nauplii. Variability in sizes of *D. celebensis* in mass culture will give a better feeding regime to fish larvae that require appropriate size of food according to their development.

D. celebensis shows highest population growth when fed *N. oculata* at 12.5×10^6 cells.ml^{-1}. The population growth was further improved by co-feeding with *N. oculata* (2.5×10^6 cells.ml^{-1}) and *C. vulgaris* (1×10^6 cells.ml^{-1}) and the highest density in 2-l batch culture reached 37.7 ind.ml^{-1}. At this regime, longest life span (31.1 days) and highest fecundity (43.3 offspring) were also observed. Since *D. celebensis* shows strong phototaxis in response to light, higher population density is achieved under darkness. Optimal temperature and salinity for rearing were 30°C and 6ppt, respectively, and *D. celebensis* density reaches 90

ind.ml^{-1}. Renewal of culture water every 3 days resulted in significant increase in population growth compared to cultures without water exchange. By modifying the air supply equipment developed for freshwater cladocera *Moina macrocopa*, *D. celebensis* reached to a maximum density of 120 ind.ml^{-1}. The nutritional value of *D. celebensis* can be manipulated easily by enriching the culture with microalgae or commercial products.

The prey selectivity experiments using marine fish larvae, such as three-line grunt (*Parapristipoma trilineatum*) and devil stinger (*Inimicus japonicus*) showed that *P. trilineatum* with >11mm TL and *I. japonicus* with 4-7mm TL showed higher preference to *D. celebensis* than *Artemia franciscana*. Larval rearing trials of Japanese flounder (*Paralichthys olivaceus*) and kuruma prawn (*Penaeus japonicus*) using *D. celebensis* and *A. franciscana* showed similar results in terms of survival and growth. In the experiment using *Kryptolebias marmoratus*, jumping, escaping behavior of *D. celebensis* reduced feeding success of larval fish, but there was no difference in feeding amount. This gives us an insight that the development of skill in ingesting prey with active movement may enhance larval quality. The nutritional data and results of larval rearing trials suggest the potential and appropriateness of *D. celebensis* as live food.

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DIET AND MICROBIAL INTERACTIONS IN PALINURID LOBSTER LARVAE

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Marine lobsters represent some of the most valuable wild fisheries in the world and are either fully or over-exploited with limited yields. There is growing momentum to develop a closed-life cycle aquaculture sector for the Palinurid marine lobsters. The Palinuridae (spiny or rock lobsters) have a unique larval development as a zoeal form with a leaf-shaped carapace (known as a phyllosoma) with a larval phase that is one of the longest of any marine organism, from a minimum of 4-6 months up to 2 years in the wild. The phyllosomas pass through at least 11 morphological stages, often with supernumerary moults, each with an inter-moult period of a minimum of 7 days, increasing from an initial post-hatch total length of 1.5mm to a final larval size of 30mm. Due to their extended larval phase, and the 20-fold size increase during the hatchery phase, there are significant challenges for the development of a commercially viable hatchery technology.

Larval survival is based on an adequate nutrition supply and a high health status and these factors are closely associated. For instance, nutritionally compromised larvae are at increased risk from opportunistic and pathogenic microbes leading to increased moribundity, and a high degree of external fouling by filamentous bacteria may restrict the feeding capability of the larvae. In addition, the bacterial community associated with the feed itself can affect the microbial status of the phyllosoma. While the feed is a potential vector for opportunistic pathogens, it may also serve as a vector for bacteria that are beneficial for larval health.

The development of a diet suitable for phyllosoma rearing requires consideration of several aspects including identifying their nutritional requirements, as well as the consistency and size of feed particles that can be handled and consumed. Whereas larval fish gulp prey whole, phyllosoma larvae capture and gradually masticate prey before entry into the buccal cavity and digestive tract. Real-time

video experiments have been used to observe the feeding process of phyllosomas, including the flow of food particles through the buccal cavity and digestive tract. A series of mouthparts reduce food particles to specific sizes to pass from the foregut to the midgut gland. Fouling by filamentous bacteria identified as *Thiothrix* sp. has been observed to occur in particular around the mouthpart region, which is a surface exposed to high particulate and dissolve organic matter concentrations. The fouling is progressive, and can lead to complete cessation in the ability to feed with a concurrent decline in nutritional status opening the way for opportunistic microbial pathogens.

The phyllosoma itself is a habitat with a complex microbial community. Microbial analyses of wild phyllosoma captured in the Coral Sea (SW Pacific) and hatchery reared ones have revealed an extensive bacterial community in both as well as substantial differences between the two. In wild phyllosoma, 60% of the bacteria culturable on marine agar were members of *Alphaproteobacteria* or *Gammaproteobacteria*, but members of *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* were also represented. Clone libraries revealed that *Sulfitobacter,* the *Roseobacter* sp. clade, and *Epsilonproteobacteria* were relatively dominant groups in the wild phyllosoma. In contrast hatchery-reared phyllosoma had a higher proportion of known opportunistic pathogens and clone library analysis showed a higher relative abundance of *Thiothrix*, *Vibrio*, *Bacteroidetes*, and *Photobacterium*.

Dying and dead phyllosomas can be identified by their hepatopancreas turning white with subsequent spread to the rest of the body, followed by tissue degradation. Due to these visual characteristics, the syndrome is also known as "white gut disease". Several *Vibrio harveyi*-related strains isolated from moribund phyllosoma and biofilm forming on tank surfaces from larval rearing tanks have been showed to cause increased phyllosoma mortality rates in laboratory survival assays. In a typical assay, +90% of larvae die within 72 hours of the challenge, and the visual characteristics of the disease progress are similar to diseased animals in rearing tanks. One of the pathogenic strains has been selected for more detailed studies. The strain has been chromosomally labeled with green fluorescent protein, and microscopy-based studies of the infection route and progress in individual phyllosoma are discussed.

Bacterial strains from the Coral Sea samples have been screened for their ability to inhibit the growth of known phyllosoma pathogens and to degrade quorum sensing compounds utilized by *Vibrio harevyi*-related species. Current studies are investigating the potential of the most promising candidate strains to be used as probiotics in phyllosoma rearing. The potential to use enriched *Artemia* nauplii as a vector for probiont delivery has also been investigated, showing that *Artemia* can be a suitable vector for the tested probiont.

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DIETARY PHOSPHOLIPID EFFECTS ON HEPATIC METABOLISM IN PIKEPERCH (*SANDER LUCIOPERCA*) LARVAE

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Introduction

Pikeperch (*Sander lucioperca*) is one of the main valuable percid species that represents a great interest for aquaculture. The larval rearing remains a critical period and weaning using artificial diets poses a problem of satisfying the nutritional requirements. Phospholipids (PL) have been demonstrated to significantly affect survival, growth, and deformities in several fish species (Kanazawa, 1985; Gisbert et al., 2005). They play a major role in maintaining the structure and function of cellular membranes (Tocher, 2003). To date, little is known about their role in metabolic processes in fish species. Proteomics are mostly often used in environmental and medical sciences, where it focuses on the dynamics of the proteome of organisms in response to environmental changes or pathologies, respectively. Proteomics applied to nutritional research should contribute to the identification of bioactive food components, for assessing their biological efficacy (Kussmann et al., 2006). This study aimed to assess changes in the protein expression profile in the liver of pikeperch larvae to address the metabolic processes at a cellular level. 2D-DIGE (two dimensional differential in-gel electrophoresis) technique was used to analyze the effects of phospholipid supplementation in the diet on the liver proteome of 34-day-old pikeperch larvae.

Materials and methods

Pikeperch larvae fed from mouth opening (4 days post hatching, dph) ad libitum with newly hatched small *Artemia* nauplii. From 10-34dph, the larvae were fed with one of three isoproteic and isolipidic formulated microdiets (patent WO0064273). These diets contained modified levels of soybean lecithin and cod

liver oil to obtain increasing PL content: 1.5 (PL1), 4.7 (PL5), and 9.5% (PL9). For the proteomic analysis, 40 larvae per treatment were collected on day 34. After dissection (0°C), analysis was performed on liver homogenates.

Two-dimensional Differential IN Gel Electrophoresis (strips 24 cm; pH 4-7) technique was used to compare the protein expression in the three treatments with Cyanine Dyes (Cy2, Cy3, Cy5). Labelled proteins were visualized using a Typhoon 9400 Imager. Image analysis was performed using the DeCyder BVA 5.0 software. Protein spots with intensities analyzed as significantly different ($p<0.05$) using a Student's t-test for independent samples, were considered to correspond to proteins differentially expressed between PL1/PL5, PL1/PL9 and PL5/PL9 treatments. Peptides were analyzed using a nano-LC-ESI-MS/MS (Waters Ltd, USA) instrument.

Results and discussion

The PL9 diet clearly favouring the growth of the larvae. The growth rate of PL5 larvae was significantly higher ($p<0.01$) than that of PL1 group. Proteome analysis using the quantitative 2D-DIGE method revealed differential expression of 56 proteins. Among 23 excised spots, 13 proteins species were identified for a total of 10 different proteins. Changes in the protein expression pattern are presented in Table I.

Table I. Fold change in abundance for 13 proteins identified by MS according to the diet.

Spot	Protein	PL5vsPL1	*P*	PL9vsPL1	*P*	PL9vsPL5	*P*
1557	Aldolase B	-2.34	0.026	-3.60	0.006	-1.54	NS
816	Phosphoglucomutase	-1.60	0.041	-1.13	NS	+1.42	NS
193	Pyruvate carboxylase	-1.61	0.029	-1.21	NS	+1.33	NS
216	Pyruvate carboxylase	-1.45	NS	+1.16	NS	+1.68	0.006
225	Pyruvate carboxylase	-1.82	NS	+1.06	NS	+1.92	0.015
504	Pyruvate carboxylase	-1.32	NS	+1.21	NS	+1.60	0.008
596	Propionyl CoA carboxylase	+1.20	0.003	+1.37	0.015	+1.15	NS
1444	Methionine adenosyl transferase	+1.51	NS	+2.82	0.003	+1.86	NS
416	Sarcosine dehydrogenase	-1.08	NS	+1.25	0.015	+1.16	0.019
1824	Glutathione S transferase	-1.24	0.038	-1.17	0.009	+1.05	NS
633	HSP 9	-1.09	NS	-1.42	0.003	-1.31	0.017
1918	2-cys peroxiredoxin	+2.42	0.018	+1.49	0.02	-1.62	NS
518	Vinculin	-1.28	NS	+1.24	0.026	+1.58	0.005

Data were analysed by Student's t test. - and + indicate an under- and over-expression of the protein. NS : non significant

In the larvae fed with the highest dietary PL content (PL9), glycolytic enzymes (aldolase B, phosphoglucomutase PGM) were significantly under-expressed

whereas gluconeogenic enzymes (pyruvate carboxylase PC, propionyl coenzyme A carboxylase PCC) were over-expressed. In addition, a high PL content increased the expression of methionine adenosyl transferase MAT and sarcosine dehydrogenase SrDH, two enzymes involved in methionine metabolism, along with vinculin V, a structural protein. Moreover, several stress proteins (Glutathione S-transferase GST, Heat Shock Protein HSP and 2cys Peroxiredoxin 2cys Prx) were modulated in response to the dietary PL level and fatty acid composition.

In the liver of PL1 larvae, the glycolytic enzyme Aldolase B was over-expressed compared to PL5 and PL9 larvae. This result may reflect that the energy allocated to the primary metabolism was relatively more important in PL1 larvae, which were the smallest larvae. In a similar study, Martin et al. (2003) attributed the over-expression of aldolase in rainbow trout fed with a soyprotein rich diet to an increased metabolism as well as energy demand in these fish. The highest growth of PL9 larvae may reflect their ability to allocate a larger amount of nutrients and energy into tissues formation. Moreover, the highest levels of PUFA (n-6 and n-3) contained in PL5 and PL9 diets could have decreased glycolysis in the larvae of these treatments.

The under-expression of PC (gluconeogenic enzyme) in PL5 larvae might be explained by a decrease of gluconeogenesis in these larvae but it is difficult to conclude about PL role on this enzyme.

MAT was over-expressed in the liver of PL9 larvae compared to PL1 larvae, while there were no significant differences among the other treatments. PCC showed a moderate but significant over-expression in the liver of PL9 and PL5 larvae compared to PL1 group. The highest growth in PL9 larvae indicates a higher anabolism of proteins, thus a higher demand of amino acids and consequently a higher MAT and PCC activities.

Concerning the proteins related to the stress, the over-expression of GST in PL1 larvae could be due to its involvement in the detoxification of lipid peroxide products as it has been demonstrated for goldfish (Bagnyukova et al., 2006).

The over-expression of HSP in PL1 and PL5 larvae may point out an inverse relation between stress and PL level. Moreover, PL1 diet which is the richest in n-3 fatty acids may induce an oxidative stress in the liver of PL1 larvae. The over expression of 2-cys Prx in PL5 and PL9 larvae may be related to an enhanced protection against phospholipid peroxidation in the cell membrane.

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CONTROL OF LIGHT CONDITION AFFECTS FEEDING REGIME AND ENABLES SUCCESSFUL EYE MIGRATION IN ATLANTIC HALIBUT

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Incomplete eye migration is a major problem in intensive production of juvenile Atlantic halibut. Commonly, more than half of a juvenile population reared according to best practice suffers from this abnormality. Continuous light results in continuous feeding and high feed ingestion rates. Observations show that an empty gut is filled within 20min when food is present in adequate amounts. However, the halibut larvae will continue to feed even if the gut seemingly is packed. Changing continuous ingestion to well defined meals was therefore the goal of this experiment.

Control groups (triplicate design) were reared under continuous light conditions, whereas the experimental groups were given 7 hours of darkness and 17 hours of light, from 12 to 35dpf (days post first-feeding) at 12.5±0.3°C. Otherwise, both groups were reared under continuous light (300 µW/cm2 surface). During the experimental period, larvae were fed enriched Artemia (EG- type, Great Salt Lake UT, USA; MultiGain, DANA feed) supplied two times daily instead of continuous feeding. *Artemia*, was analysed for fatty acid composition. Larvae sampled at regular intervals, were measured for growth and analysed for fatty acids, outer ring deiodinase activity and deiodinase I, II, and III (DI, DII and DIII) expression. Observations of larval gut contents were done prior to and during feeding. Eye migration and skin pigmentation were analysed at 95dpf according to existing methodologies.

27±3% of the juveniles that had been reared under continuous light conditions had complete eye migration, whereas in juveniles reared under shifting light and darkness conditions, 85±7% had complete eye migration. The ratio of malpigmented juveniles was low; <1%, and similar in both groups.

There were no significant differences in growth and survival or in lipid content and fatty acid profiles between the two groups. ORD activity was lower in larvae reared in continuous light than in those exposed to a dark period on 25dpf

(p=0.049). The difference was not significant on 49dpf, two weeks after the treatment had been terminated. There were no differences in expression of DI and DII between the groups but a shift in relative gene expression was seen; where DI had the lowest relative expression compared to DII and DIII in 6dpf larvae while DI had the highest relative expression at 49dpf, regardless of treatment.

One possible hypothesis that explains the results in the present study is related to the digestive process and the gut transit time. It is evident that a continuously feeding larva presents a challenge to its own digestive system, particularly when the transit time is very short. Problems associated with rapid transit time in a continuously feeding larva may potentially include lowering luminal enzymatic activity due to overloading of the enzyme producing capacity in the exocrine pancreas and reduced levels of bile due to similar problems in the liver. Fast transit may also cause slowly digestible and essential compounds to be lost in the faeces while in larvae where feeding is halted, a longer transit time will enable these critical nutrients to be absorbed.

The hypothesis that energy limitation is the cause of incomplete eye migration in Atlantic halibut fed Artemia is weakened by the fact that no differences were detected in growth or larval lipid level between the two groups. However, increased absorption of other nutrients may have improved the nutritional balance of the larvae and therefore improved the eye migration. Alternation of light and darkness itself may have caused an improved development of the hypothalamus-pituitary-thyroid axis. Light is known to be a strong cue for hormonal regulation of many body functions. However, in a previous study, halibut larvae were fed either Artemia or zooplankton in an intensive system with natural light conditions (the rearing thanks were placed outdoors). In this experiment only 10% of the larvae fed Artemia had complete eye migration compared to 88% of the juveniles that had been fed zooplankton. This suggests that the dark period per se is of minor importance. These results represent a major improvement in production systems for Atlantic halibut juveniles and demonstrate that by controlling diurnal light and darkness periods together with a meal based feeding regime, incomplete eye migration can be dramatically reduced.

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SAFE AND EFFECTIVE MASCULINIZATION METHOD OF TIGER PUFFER, *TAKIFUGU RUBRIPES*, BY TEMPERATURE CONTROL DURING LARVAL AND JUVENILE STAGE

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Introduction

Tiger puffer, *Takifugu rubripes*, has an extremely high value in the Japanese market, and its culture has been well-practiced in Japan and in China with the production of 6000t. The market value of male tiger puffer is much higher than females because their testis is delicious and innoxious and the ovary is very toxic in contrast to it. We developed a safe and effective method of musculinization of artificially hatched tiger puffer by low temperature exposure of larval and juveniles. The new method can increase the male rate from 50% in case of no treatment to more than 80%. Our study gives a new knowledge on the mechanism of sex differentiation of teleosts which is less well understood and well demonstrates its practical application to the aquaculture industry.

Materials and methods

Experiment I: Elucidation of the body of the completion of sex differentiation that is verified by the histological method.
Fertilized eggs of tiger puffer obtained by artificial insemination were incubated at 16.2°C, and hatched larvae were reared at 17.3°C until 15 days post-hatch (dph). Thereafter, an aliquot of 1000 larvae were reared under low temperature condition below 16°C in a 1-m3 tank, and another aliquot of 1000 larvae were reared under natural temperature condition above 16°C. Fish were sampled periodically for histological analysis of the gonads.

Experiment II: Determination of the necessary period of low temperature rearing for the masculinization

Larvae were reared under low temperature condition below 16°C in a 50m3 volume tank. Aliquots of larvae were transferred to 30 m3 volume tanks on 45, 55, 70, 80, 100, and 110 dph, and were reared at the natural temperature above 16°C. Juveniles were sampled on 100-130 dph to examine the gonad histologically. Sex determination of the samples was done by the confirmation the presence and absence of ovarian cavity which is the specific structure of the female gonad.

Results

Experiment I: The minimum size at which the sex differentiation under temperature condition above and below 16°C was 18mm and 24mm, respectively. The ratio of males after the sex differentiation was 45% and 86% for the rearing under temperature condition of above and below 16°C, respectively.

Experiment II: The ratios of male juveniles reared for 45 to 80 days under temperature condition below 16°C were 38-46%. In contrast, they were above 80% for individuals reared for 100 and 110 days under such condition.

Discussion

Environmental factors influence sex determination and sex differentiation of fishes (Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002). This study elucidated that the low temperature rearing during larval stage increases the ratio of males in tiger puffer. This method to control sex ratio by rearing temperature is safe compared to the method using sex hormones and suitable for the practical use in aquaculture. However, the low temperature rearing decreases the speed of growth. Therefore, the reduction of period of low temperature rearing will be the issue resolved in the future.

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CO-ENRICHMENT OF *ARTEMIA* WITH WAX BEADS CONTAINING POTASSIUM IODIDE AND YTTRIUM OXIDE ADDED TO A COMMERCIAL ENRICHMENT DIET (ORI-GREEN)

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Introduction

Artemia sp., a common live prey organism used in larviculture, are often deficient in certain nutrients essential for the proper growth and development of fish larvae (Sorgeloos et al., 2001). Specifically, *Artemia* have been shown to be deficient in iodine when compared with wild-caught zooplankton (Moren et al., 2006; Solbakken et al., 2002). Delivery of water-soluble micronutrients to marine suspension-feeders has been problematic due to rapid leaching rates from suspended microparticles (Langdon, 2003). Wax spray beads (WSB) have been shown to effectively deliver the water-soluble antibiotic, oxytetracycline (OTC) to *Artemia* (Langdon et al., 2008) and may be useful in the delivery of water-soluble micronutrients. In this study, we investigated the ability of WSB to supplement brine shrimp *Artemia* sp. with a water-soluble trace mineral (iodine) to produce a more nutritionally complete *Artemia* for fish larvae.

Methods

Wax spray beads (WSB) containing either 10% (w/w) potassium iodide (KI) (Sigma-Aldrich MO, USA) and 2% (w/w) yttrium (III) oxide (Y) nanopowder (Aldrich MO, USA) or yttrium oxide only were prepared with beeswax according to the methods described by (Langdon et al., 2008). Yttrium oxide was used as an inert marker to determine ingestion and retention of WSB by *Artemia*. Particle size was analyzed by measuring >20 particles from digital images with Image-J software (NIMH MD, USA).

WSB were fed to 24hph *Artemia* metanauplii (GSL, INVE) at 200mg l^{-1} in combination with 800, 200, or 0mg l^{-1} Ori-green (Skretting, Norway), representing dietary treatments containing 25, 50, and 100% WSB. Each supplementation level was tested with either a) WSB containing 10% KI and 2% yttrium or b)

WSB containing 2% yttrium in combination with dissolved aqueous KI at a concentration of 20mg.l^{-1}; this latter concentration was equivalent to the total KI delivered in WSB treatments. A control of unenriched *Artemia* was included. All treatments were tested in triplicate. After a 12-h enrichment period, *Artemia* were collected and repeatedly washed in clean SW, frozen at -20°C and freeze-dried (Freezone®; Labconco, MO, USA). Total iodine and yttrium concentrations were determined by ICP/MS as described by Julshamn et al. (2001) and Otterå et al. (2003).

Statistical tests were performed with Statistica© 8.0 (Statsoft Inc., Tulsa, OK, USA). The assumption of equal variance was checked using Levene's test of homogeneity and normality was checked graphically. Multiple pair-wise comparisons were performed with Tukey's Honest Significant Difference (Tukey's HSD) test at a significance level of 0.05.

Results and discussion

WSB and Ori-green particles had average diameters of 8.0 and 9.4µm, respectively. Sixty percent of Ori-green particles and 61% of WSB were in the optimal size range (3-8µm) for ingestion by *Artemia* (Makridis and Vadstein, 1999). *Artemia* in all treatments had significantly higher iodine concentrations than unenriched *Artemia* (Tukey's HSD, threshold p=0.05) which had approximately 5µg.I.g^{-1}.DW. The elevated levels of iodine in the aqueous treatments suggest that *Artemia* were able to take up significant quantities of iodide directly from seawater by 'drinking' or being bound to the exoskeleton. Except in the 25% WSB treatment, *Artemia* enriched with WSB containing KI had higher concentrations of total iodine than *Artemia* exposed to an equivalent concentration of dissolved KI (Tukey's HSD, $p<0.05$). Total iodine concentrations in *Artemia* increased with increasing proportions of WSB in the diet (ANOVA, $p = 0.004$).

In our study, yttrium did not leak from WSB and did not appear to be absorbed into the tissues of *Artemia* (unpublished data). Therefore, yttrium was used to trace the amount of WSB retained in the digestive track of enriched *Artemia*. Yttrium concentrations in *Artemia* increased significantly as the proportion of WSB in the diet increased (ANOVA, $p = 0.0002$). After the 12h enrichment period, iodine to yttrium (I:Y) ratios in *Artemia* were 1.02, 0.50, and 0.45 for the 25, 50 and 100% WSB treatments, respectively. For comparison, the I:Y ratio in WSB after a 12-h leakage trial was 0.67 (unpublished data). The elevated I:Y ratio in the 25% WSB treatment was influenced by the iodine contribution *Artemia* received directly from seawater. These results suggest that much of the iodine measured in WSB-enriched *Artemia* was associated with WSB retained in the gut. It is possible that *Artemia* are able to regulate tissue iodine concentrations within a narrow range, limiting the effectiveness of WSB for iodine enrichment.

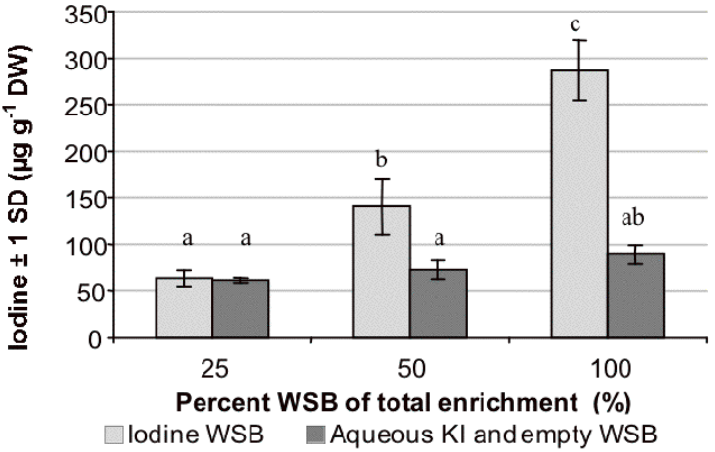

Fig.1. Total iodine concentrations in *Artemia* after 12h of enrichment. *Artemia* were fed either a) WSB containing KI and Y or b) WSB containing Y only and immersed in KI dissolved in seawater (aqueous KI) at concentrations equivalent with those delivered in WSB containing KI. All treatments received the same concentration of KI (either in WSB or in dissolved aqueous solution). Ori-green concentrations were adjusted to achieve the desired WSB enrichment levels. Different letters denote significant differences among groups (Tukey/Kramer test, $P<0.05$).

Conclusions

Our results indicate that WSB prepared with beeswax can be used to increase iodine concentrations in *Artemia*. We found that iodine uptake rates were significantly higher for *Artemia* fed on iodine-containing WSB compared to uptake from the aqueous phase. Based on previous research on *Artemia* fed on OTC-containing WSB (Langdon et al. 2008), *Artemia* are able to absorb OTC from WSB. Further experiments are required to determine if ingested iodine is similarly absorbed into the tissues of *Artemia* and if it is available to fish larvae feeding on the enriched *Artemia*.

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EFFECTS OF DIETARY N-3/N-6 RATIO ON THE BIOCHEMICAL COMPOSITION OF *PERCA FLUVIATILIS* SEMEN AND INDICATORS OF SPERM QUALITY

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Introduction

The quality of sperm is highly variable between species and individuals and depends on various external factors such as the feeding regime, the feed quality and the rearing conditions. In aquaculture, sperm quality has received less attention than egg quality, as far as fatty acids (FA) composition is concerned. Nevertheless, in mammals as well as in fish, several authors showed that the FA composition of the sperm is directly influenced by the composition of the diet (Bell et al., 1996; Wathes et al., 2007). In Eurasian perch (*Perca fluviatilis*), Wang et al. (2008) observed that males fed with either forage fish or commercial pellets produced milt of similar quality, but the diet and sperm FA composition was not assayed in this experiment. Therefore, our objectives were to study the effects of dietary n-3/n-6 ratio on some indicators of sperm quality in Eurasian perch, and to compare this quality with the lipid composition of the milt.

Materials and methods

Two diets were tested in duplicates from September 2007 to March 2008 on 40 fish (185g mean body weight) maintained in $1.2m^2.0.5m^3$ rectangular tanks, in an indoor recirculating system with natural photothermal cycle. The first experimental diet (D1) contained 12% of sardine oil, 3.2% of safflower oil, and 0.8% of vevodar oil (a synthetic oil enriched in AA (40%)), the second one (D2) contained 16% of menhaden oil. The n-3:n-6 ratio of D1 and D2 were 0.2 and 7.0, respectively. The FA and lipid class profiles of both diets are showed in Table I. Fish were fed daily to apparent satiation.

Table I. Fatty acid and lipid class composition (as percent of total fatty acids and total lipids, respectively) of experimental diets and semen at the end of the experiment. Significant differences are indicated with different letters

	D1 diet	D2 diet	D1 sperm	D2 sperm
Total lipid (% diet)	16	16	3.5 ± 2.3	4.9 ± 4.1
Saturated fatty acids	17.3	26.9	$28.4 \pm 1.8_b$	$30.8 \pm 2.1_a$
Monounsaturated fatty acids	22.7	26.2	14.9 ± 2.5	12.6 ± 6.0
Polyunsaturated fatty acids	60.0	46.9	56.7 ± 2.9	56.6 ± 4.3
18:2n-6	46.9	4.5	$11.0 \pm 2.0_a$	$5.0 \pm 1.0_b$
20:4n-6	3.0	1.1	$4.1 \pm 0.4_a$	$2.7 \pm 1.0_b$
18::3n-3	0.2	0.2	0.1 ± 0.1	0.2 ± 0.3
20:5n-3	3.1	10.5	8.1 ± 0.8	9.5 ± 2.2
22:6n-3	5.1	20.4	$31.7 \pm 4.0_b$	$36.8 \pm 3.9_a$
Total neutral lipid (NL)	96.7	93.7	$47.2 \pm 1.5_b$	$54.1 \pm 1.8_b$
Wax ester	14.1	67.2	1.7 ± 2.4	1.7 ± 2.3
Tryacylglycerol	68.7	21.6	$0.3 \pm 0.1_a$	$0.0 \pm 0.0_b$
Free fatty acid	6.6	1.3	9.4 ± 1.3	6.3 ± 2.8
Cholesterol	7.3	3.7	$35.8 \pm 0.3_b$	$46.2 \pm 2.3_a$
Total polar lipid (PL)	3.3	6.3	$52.8 \pm 0.1_a$	$45.9 \pm 2.0_b$
Phosphatidylethanolamine	0.3	0.0	27.3 ± 3.9	22.7 ± 0.3
Phosphatidylinositol	0.0	0.0	$10.0 \pm 0.0_a$	$5.7 \pm 4.3_b$
Phosphatidylcholine	2.9	6.2	11.6 ± 2.6	13.4 ± 4.6
sphyngomyeline	0.1	0.0	3.9 ± 1.3	4.2 ± 2.0
n-3/n-6	0.2	7.0	$2.8 \pm 0.8_b$	$6.9 \pm 2.9_a$

Semen samples were collected in the laboratory from 4 males per tank in March 2008. Sperm volume, density, osmolality, motility, and velocity were evaluated by the technique described in Alavi et al. (2007). Total lipid, FA and lipid classes were extracted from the semen and tested diets according to Bell et al. (1996). Data were compared by analysis of variance ANOVA1 (Statistica 5.5).

Results and discussion

The sperm indicators of quality measured in March 2008 are presented in Table II. Neither significant differences were reported between D1 and D2 concerning the relation between the production of sperm (around 0.37×10^9 spz.g^{-1} of male b.w.), the sperm density (between 46.1 ± 7.5 and 58.9 ± 2.1 $\times 10^9$ spz.ml^{-1}), motility and velocity after 15 or 30s post activation (around 94% and 70%, respectively), nor for the sperm osmolality (between 293.6 ± 2.9 and 299.0 ± 2.4 mOsm.kg^{-1}). Motility duration of perch spermatozoa was very short, and the velocity decreased significantly after the activation with the activation solution. Results on sperm density, motility, velocity, and osmolality are at least similar or higher than results obtained by other authors for Eurasian perch (Lahnsteiner et al., 1995; Wirtz et al., 2006; Alavi et al., 2007). From this we can assume that the sperm is of high quality, despite the fact that we cannot prove this by the fertilizing ability of the spermatozoa.

Table II. Sperm quality indicators of Eurasian perch breeders after the experiment of nutrition with D1 or D2.

	D1	D2
Males body weight (g)	171.4 ± 42.1	180.4 ± 24.1
Volume of sperm (ml)	1.44 ± 1.20	1.03 ± 0.03
Sperm density ($x10^9$ spz ml^{-1})	46.1 ± 7.5	58.9 ± 2.1
Spermatic index (%)	0.8 ± 0.4	0.6 ± 0.1
Sperm number (10^9) per g of male b.w.	0.40 ± 0.37	0.34 ± 0.07
Motility after 15 s post activation (%)	93.8 ± 0.4	93.9 ± 0.1
Motility after 30 s post activation (%)	74.5 ± 12.2	63.8 ± 0.8
Velocity after 15 s post activation (μm s^{-1})	164.8 ± 0.7	159.0 ± 9.1
Velocity after 30 s post activation (μm s^{-1})	35.0 ± 2.2	29.2 ± 2.9
Osmolality (mOsm kg^{-1})	293.6 ± 2.9	299.0 ± 2.4

This study was the first to investigate the FA and lipid class profile of perch semen in relation to the dietary composition. The FA composition and the lipid class profile of the semen were both correlated to the tested diet ($P<0.05$). Semen of perch is characterized by high levels of 14:0, 18:0, 18:1 n-9, 18:2 n-6, 20:5 n-3 (EPA), and 22:6 n-3 (DHA). Only traces of 18:3 n-3, 18:4 n-3, and 20:4 n-3 were measured in the sperm, whatever the tested diets. The FA profile of semen produced by fish fed D1 contained high levels of n-6 polyunsaturated fatty acids (PUFA), 18:2 n-6 and 20:4 n-6 (AA) ($P<0.05$), while for D2, there were higher levels of saturated FA, mainly due to the high dietary concentrations of 14:0 and 16:0, and n-3 PUFA, such as 22:5 n-3 and DHA, as expected. By consequence, the EPA:AA and n-3:n-6 ratios of semen from fish fed D1 were significantly lower (Table I). When we compare the FA composition of the semen with eggs of females for the same tested diets (Henrotte et al., 2008), we observe that semen of perch contains more saturated FA than eggs, and more EPA and DHA. Spermatozoa need energy to achieve their movement, in order to fertilize the oocytes, so the saturated FA are very good energy resources for this. Moreover, EPA and DHA are major components of membrane phospholipids and they contribute to facilitate the mobility of spermatozoa.

We can observe that the lipid classes in sperm were characterized by approximately the same level of neutral (NL) and polar lipids (PL), while in the tested diets, between 94 and 97% of lipid classes were constituted with NL (Table I). NL were mainly constituted by cholesterol, then free FA. Only traces of wax esters and triacylglycerol (TAG) were found in the semen, while both were predominant in the diets. PL were characterized by high levels of phosphatidylethanolamine (PE), followed by phosphatidylcholine (PC) and phosphatidylinositol (PI). Semen produced by fish fed D1 contained higher levels of TAG and higher levels of PL, mainly due to PI concentrations. D2 increased the level of NL in the semen, due to high cholesterol content. Sperm contained higher levels of PL than what was observed for eggs, oocytes being enriched in NL in order to cover the energy required for the embryonic development and first larval developmen-

tal stages. Furthermore, membrane phase behaviour of spermatozoa, which is determined by the PL composition, may be important in controlling fusion of the sperm plasma membrane (Bell et al., 1996). Interestingly, we found more PI concomitantly with high levels of AA in the semen. In female gonads, AA is often associated to the PI class. Apparently, a similar observation could be made for male gonads.

Conclusions

The two tested diets had no impact on the reproductive capacities of the perch males, as far as sperm production, density, velocity, motility and osmolality are concerned, despite the fact that FA and lipid class compositions of the semen were directly correlated to the lipid and FA composition of the diets provided to the breeders.

Acknowledgements

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ADVANCES IN REARING TECHNIQUES AND ANALYSIS OF NUTRITIONAL QUALITY OF TWO MYSID SPECIES PRESENT IN GRAN CANARIA

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Introduction

Studies on the relationship between mysids and fish indicate that mysids are one of the most important foods for fish, especially in coastal environments, where they are abundant (Murano, 1999). In aquaculture mysids have to be a very good quality food for the juvenile stages of cuttlefish *Sepia officinalis* (Domínguez et al., 2001), and adult seahorse *Hippocampus hippocampus* (Otero et al., 2007). Traditionally two types of live food are used in culture of fish larvae: *Artemia* and rotifers, and this reduction in the range of food available can lead to nutritional imbalances (Izquierdo, 1996). The aim of this paper is to study the survival and production of *Leptomysis lingvura* and *Paramysis nouveli* under the conditions of our facilities, to determine the nutritional quality of these species, and to determine if they could be reared as live prey in our aquaculture program. As a first step in our research, we present their lipid and protein composition.

Materials and methods

In the area of Risco Verde, Gran Canaria (Spain) samples of both species were captured at depths between 5 and 15m with a 500-µm hand net**.** After an acclimation period of 2 days, 10 males and 10 females of each species were then placed in small 1-l farrowing containers that were placed in larger 14-l tanks with filtered seawater at a salinity of 37‰. The seawater, common to the farrowing containers and the 14-l tanks, was maintained at 18.2±0.4ºC, renewed ever 12 hours, and monitored for pH, oxygen, ammonium, nitrate, and nitrite. pH was maintained at 8.2± 0.1, O_2 at 7.1±0.1mg.l^{-1}, and NH_4^+, NO_3^-, and NO_2^- at concentrations below 0.2, 1, and 0.02mg.l^{-1}, respectively. The photoperiod was 14h:10h light and dark. Mysids were fed twice daily using 100 enriched (Easy –DHA,

INVE, Belgium) *Artemia* (EG type, INVE aquaculture, Dendermonde, Belgium) nauplii per mysid.

Adults and young were counted daily. The survival rate was expressed as a percentage. Every day the count was made of the offspring and production was estimated by dividing the number of hatchlings per day by the number of females alive. Production rates were expressed as young.female^{-1}. The experiments were carried out in three replicates. Mann-Whitney non-parametric test with significance $P<0.05$ was used to determine statistical differences in the survival and production of each species. The results were processed using a SPSS Statistical Software System version 14.0 (SPSS Chicago, Illinois,1999).

Samples for lipid and protein analysis were collected in Risco Verde between March and April 2009. The mysids captured were separated by species and after an acclimatization period of 2 days were maintained in three tanks. The culture conditions were identical to those used in the survival and production experiments. Proteins are calculated from total nitrogen in the samples as determined by Kjeldhal technique (AOAC, 1995). Crude lipids (% wet weight) were extracted following the method of Folch et al. (1957). Fatty acid methyl esters from total lipids were prepared by transmethylation as described by Christie (1982), separated and quantified by Gas-Liquid chromatography. Proteins, lipids, ash and moisture were expressed as percentage of dry weight. Fatty acids are expressed as percentage of total them.

Results

At the end of experiment the average survival for *L. lingvura* was 65±8.7% and for *P. nouveli* 16.7±5.8% and the total hatchling production was 166±2 and 45±7 respectively. The relative production (young.female^{-1}) and survival was significantly higher ($P<0.05$) in *L. linguvura* that *P. nouveli*, from day 9. Not hatchlings of *P. nouveli* were found from day 12 of the experiment.

Lipid and protein analysis was the first step in determining the nutritional quality of these mysids. The proteins and lipids as a % of dry weight, for *P. nouveli* were 73.38±1.77% and 15.01±1.12% and for *L. lingvura,* 74.19±5.22% and 14.79±2.66% (Table I). The most abundant fatty acids in both species were oleic acid 18:1n-9, 16:00, eicosapentaenoic acid (EPA) 20:5n-3, docosahexaenoic acid (DHA) 22:6n-3, α-linoleic acid (ALA)18:3n-3, and linolenic acid (LA) 18:2n-6 (Table I). The omega-3 (n-3) and the omega-6 (n-6) polyunsaturated fatty acids (PUFA), in *P. nouveli* and *L. lingvura* accounted for 39.44±0,94% and 8.43±0,42%, and 42.4±0,8% and 8.4±0,15% of the total lipids, respectively (Table I). The ratio DHA:EPA was 0.85 and 0.89, DHA: arachidonic acid (AA) 6.26 and 4.74, and EPA:AA 7.32 and 5.32, respectively (Table I). The PUFA, DHA, EPA and AA are required for normal growth and development of fish,

they satisfy a fundamental role in the structure and function of integral cell-membrane and as precursors of a group of highly active hormones known as eicosanoids (Izquierdo, 1996; Sargent, 1999; Roo et al., 2008). The EPA:AA ratio is also important in the formation of these hormones (Sargent et al., 1999). Both mysids have a high DHA content. However, current evidence suggests that not only the content of DHA and the n-3 highly unsaturated fatty acids (HUFA) are important for normal growth and development of the larvae, but also that a balanced dietary ratio of DHA:EPA:AA it necessary (Izquierdo, 1996; Sargent et al., 1999). The composition of DHA, EPA and AA in our mysids is higher than that reported by Roo et al. (2009) for rotifers and *Artemia* enriched with Selco® (INVE, Belgium) (Table I).

Table I. Lipids, proteins and ash composition (% dry weight) and fatty acids (% total fatty acids) of *Paramysis nouveli*, *Leptomysis lingvura*, and two live prey used frequently in aquaculture (rotifers and *Artemia*) reported by Roo et al. (2009). Values (mean±SD).

		Paramysis nouveli	***Leptomysis lingvura***	**Rotifers enriched with Selco®**	***Artemia* enriched with Selco®**
% Lipids (dw)		15.01±1.12	14.79±2.66	22.05±3.84	26.04±0.41
% Proteins (dw)		73.38±1.77	74.19±5.22	54.28±4.57	56.39±4.84
% Ash (dw)		2.99±0.07	3.63±0.21	1.48±0.5	0.75±0.02
16:00	Palmitic acid	16.94±0.62	15.48±0.23	13.00±2.48	15.22±3.8
18:00	Stearic acid	4.01±0.1	3.64±0.04	4.73±1.21	4.42±0.37
18:1n9	Oleic acid	19.11±0.39	17.9±0.23	20.1±1.72	20.36±7.38
18:2n6	Linolenic acid	4.79±0.24	4.76±0.02	8.14±1.31	3.78±2.61
18:3n3	α linoleic acid	8.22±0.19	14.18±0.26	1.62±0.11	10.81±4.23
20:5n3 EPA	Eicosapentaenoic acid	14.78±0.2	12.45±0.15	6.51±0.62	11.10±4.27
22:6n3 DHA	Docosahexaenoic acid	12.63±0.37	11.10±0.2	9.68±0.93	4.47±1.43
20:4n6 AA	Arachidonic acid	2.02±0.06	2.34±0.09	1.46±0.73	1.49±0.37
∑PUFA n-3		39.44±0.94	42.40±0.8	21.12±0.48	31.14±11.43
∑PUFA n-6		8.43±0.42	8.40±0.15	10.77±2.11	7.03±3.73
DHA/EPA		0.85	0.89	1.49±0.01	0.4±0.34
DHA/AA		6.26	4.74	8.10±4.45	2.99±3.87
EPA/AA		7.32	5.32	5.45±2.99	7.43±11.53

Conclusions

The study of lipid and protein composition reveals that both species have a high potential as live food in aquaculture. The levels of lipids, proteins, and fatty acids in *P. nouveli* and *L. lingvura* meet the food requirements for fish and crustacean farming according to FAO recommendations (Tacon, 1989). Furthermore the composition in DHA, EPA, and AA is higher than that present in rotifers and *Artemia,* organisms commonly used as live prey in aquaculture.

According to the preliminary results of our survival and production experiments, *Leptomysis lingvura* appears to be the most suitable, of the two mysid species, for cultivation under the conditions given.

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CULTURING OF EMBRYONIC STEM CELLS ISOLATED FROM BLASTULA STAGE EGGS OF ATLANTIC COD, *GADUS MORHUA*

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Introduction

Following fertilization, all vertebrate embryos consists of pluripotent cells. Pluripotency is defined as the embryonic stem cells (ES cells) ability to generate all cell types of the embryo; endoderm, ectoderm and mesoderm (Niwa, 2007). To understand the molecular interactions underlying cod ES cell development it is necessary to search for and identify stage specific development markers as well as markers that identify cod cell specific lineages. This is mandatory for establishing cellular differentiation models or stage specific in vitro systems with a broad range of applications.

Materials and Methods

Cod eggs were prepared for cell culture within 24-26 h (dpf 1-1,5) following fertilization. The eggs were crushed and cells were filtered through a nylon mesh before washing. The cells were seeded on gelatin coated cell culture slides and incubated in a normal atmosphere incubator at 10°C. The culture medium was DMEM with high glucose and HEPES supplemented with FBS, glutamax, MEM Sodium Selenite, Sodium puryvate solution and mercaptoethanol (Holen and Hamre, 2004). In an approach of inducing ES cell differentiation the cells were cultured on fibronectin coated cell culture slides in medium enriched with insulin like growth factor II, platelet derived growth factor, nerve growth factor epidermal growth factor, N_2-supplementation and B27.

Cod ES cells were also cultured on low adherence dishes in the presence of medium and all-trans retinoic acid in an approach to form embryonic bodies, following a protocol described for mouse ES cells.

Results

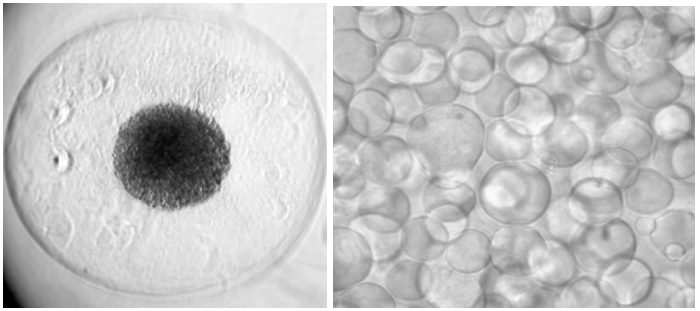

Fig. 1. Atlantic cod egg (left, 1mm) in blastula stage. Freshly isolated embryonic stem cells (right; 1.5 dpf) cultured in cell culture clusters (40×10×)

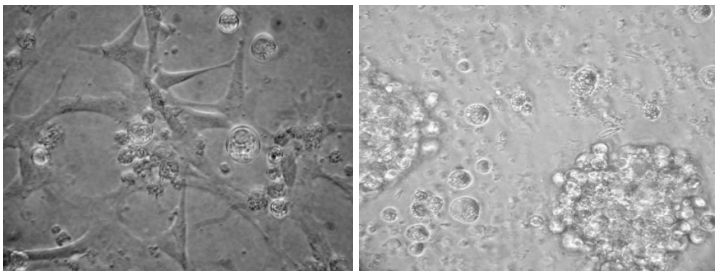

Fig. 2. (Left) Unidentified (due to lack of functioning markers) spontaneously differentiated cod ES cells, fibroblast like cell type (20×10×) (Right) Embryonic bodies following retinoic acid treatment (20×10×)

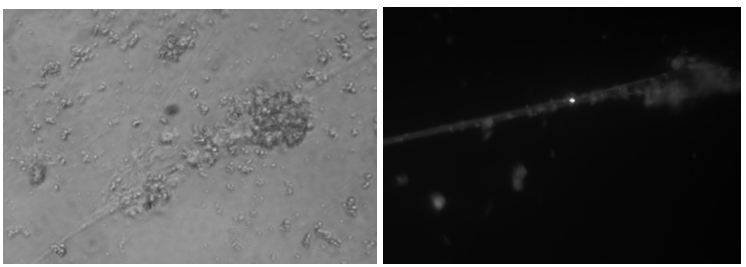

Fig. 3. Neuronal-like cells (left, 20×10×) were stained with anti-neuronal class III beta tubulin antibody raised in mice coupled to Texas red dye secondary antibody (right).

Conclusions

Cod ES cells in vitro show common features for all ES cells: spontaneous differentiation and the ability to form embryonic bodies following retinoic acid treatment. The ES cells could be directed to differentiate upon relevant treatment which is promising for the evaluation of optimal protocols for cell differentiation

models. We need to identify several cod specific markers as a tool for further development of cod ES model systems.

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THE USE OF PROBIOTIC BACILLUS SPORES FOR ENHANCEMENT OF GROWTH PARAMETERS IN SILVER CARP (*HYPOPHTHALMICHTHYS MOLITRIX*) LARVAE VIA BIOENCAPSULATION OF *ARTEMIA URMIANA*

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Introduction

Probiotics are usually defined as live microbial feed supplements, which are administrated in such a way as to enter the gastrointestinal tract and to be kept alive; beneficially affecting the host by improving its intestinal microbial balance. As an inoculative pathway for probiotic delivery to the digestive tract of larvae, live prey may be bioencapsulating with probiotics. The objective of this study was to investigate the effects of probiotic bacillus and yeast on the growth parameters of Silver carp (*Hypophthalmichthys molitrix*) larvae.

Materials and methods

The spores of *Bacillus circulans*, *B. lichenifirmis* from Iran-Nikotak Co., and *Saccharomyces cerevisiae* from Doxal-Italy Co. were used. The decapsulated cysts of *Artemia urmiana* (from Artemia center of Urmia-Iran) were incubated at 30°C with constant illumination (2000Lux), 30ppt salinity, and oxygenated through air pump. After 24h. instar 1 nauplii were collected and incubated at the density of 2g per liter at 30°C, illumination (2000lux), salinity of 30ppt, and aeration (Gomez-Gil et al., 1998). The initial body weight of trout fry was 10.3±1.0mg. Fifty acclimated silver carp (*H. molitrix*) larvae were randomly allocated into each of fifteen circular fiberglass tanks (3.5 l).

The *A. urmiana* nauplii were bioencapsulated by blends of yeast and bacterial suspension in concentration of 1×10^8, 2×10^8, 3×10^8, and 4×10^8 CFU l^{-1} for 10h. The bioencapsulated *Artemia* nauplii were fed by silver carp larvae in experimental treatments on the base of the 10 percent of their body weight a day. The control was fed on unbioencapsulated *Artemia* nauplii. The experiment was conducted in a completely randomized design with four treatments (trial 1-4 and control).

At the end of the experiment (30 days), all fish were seined and the total weight, length of fish larvae was measured and their growth parameters were calculated.

Results

The effects of probiotics on the growth parameters of Silver carp (*H. molitrix*) larvae are presented in Table I.

Table I. Growth performance of Silver carp larvae by using bioencapsulated *Artemia* nauplii with probiotic in experimental treatments and control.

Parameter / Trial	Control	Silver 1 1×10^8.l^{-1}	Silver 2 2×10^8.l^{-1}	Silver 3 3×10^8.l^{-1}	Silver 4 4×10^8.l^{-1}
Initial weight (mg)	10.3± 1.0	10.3± 1.03	10.3± 1.03	10.3± 1.03	10.3± 1.03
Final body weight (mm)	122.1 ±41.0^b	160.6 ±47.2^a	144.6 ±38.4^a	162.0 ±51.6^a	122.1 ±35.1^b
Final body length (mm)	26.2 ±1.1^b	28.2 ±0.5^a	27.6 ±0.6^a	27.7 ±0.6^a	27.8±0.7^a
FCR[1]	4.29 ±0.57^a	3.20 ±0.96^b	3.49 ±0.52^b	3.17±0.19^b	4.13±0.17^a
FCE[2]	26.3 ±6.0^b	34.4 ±2.1^a	30.9 ±1.6^a	34.7 ±2.5^a	26.3±1.3^a
SGR[3] (mg)	12.89 ±0.59^b	13.64 ±0.16^a	13.36 ±0.13^a	13.66 ±0.19^a	12.94±0.14^a
CF[4]	0.68 ±0.08^{ab}	0.72 ±0.04^a	0.69±0.02^a	0.76 ±0.02^a	0.57 ±0.07^b
CER[5]	102.4 ±28.2^b	140.7±10.1^a	124.1 ±7.6^{ab}	142.2 ±12.3^a	102.1 ±6.6^b
DG[6]	4.09 ±0.93^b	5.36±0.32^a	4.82±0.24^a	5.40 ±0.39^a	4.14 ±0.20^b

[1] Food conversion ratio (FCR) = food intake (g)/ living weight gain (g);
[2] Food conversion efficiency (FCE) = living weight gain (g)/ food intake (g);
[3] Specific growth rate (SGR) = 100× [ln final weight of fish-ln initial weight of fish)]/days of feeding;
[4] Condition factor (CF) = 100× [(g final weight of fish)/ (total length of fish- cm)^3];
[5] Conversion efficiency ratio (CER %) = 100 × [Wet weight instant growth rate/ Daily food intake;
[6] Daily growth (DG) = [g final weight of fish- g ln initial weight of fish] / days of feeding.

The probiotic significantly increased the growth parameters of silver carp larvae in comparison with the control. The best body weight (162.00mg) was obtained in Silver 3 (silver carp larvae fed on bioencapsulated nauplii in 3×10^8 CFU l^{-1}) while in control was 122.1mg. The Specific growth rate (SGR %) and food conversion efficiency (FCE %) in comparison with the control significantly increased ($p<0.05$). While the food conversion ration (FCR) significantly decreased ($p<0.05$). The maximum of FCE (34.7%) and the best FCR (3.17) obtained in treatment of Silver 3 that the silver carp larvae were fed on with bioencapsulated *A. urmiana* in suspension of 3×10^8 CFU l^{-1}.

The daily growth rate (DG) and Conversion efficiency ratio (CER) in experimental treatments were significantly promoted in comparison with control ($p<0.05$) while in treatment of Silver 4 these parameters were significantly decreased and hadn't significantly difference with control ($p>0.05$). There was no

significant difference in Condition factor of Siver carp larvae in experimental treatments and control ($p>0.05$).

Discussion

In the present study *A. urmiana* was used as a vector to carry yeast to the digestive system of Silver carp larvae. The highest growth performance was obtained in larvae fed on bioencapsulated *Artemia* nauplii. This resulted in better growth results in the trial Silver 3 (fed on bioencapsulated *Artemia* nauplii with 3×10^8 yeast and bacillus pre litre). Similar effects had been reported for other fishes with the use of probiotic in the diet (Tovar et al., 2004). Jafaryan et al. (2007) indicated that providing bioencapsulated *S. cerevisiae* yeast in *Daphnia magna* increased the growth parameters and survival rate in *Acipenser persicus*. Also similar effects reported for *A. nudiventris* via bioencapsulation of *Artemia urmiana* with probiotic bacillus (Jafaryan et al., 2008)

However, in trial Silver 3, the silver carp larvae fed bioencapsulated *A. urmiana* at 3×10^8 l^{-1} obtained the best growth performance while increasing the concentration of probiotics decreased the growth parameters. This study showed that the different concentrations of these probiotics could cause different effects on growth parameters and had the highest ability to increase the growth parameters in Silver carp (*H. molitrix*) larvae.

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EFFECTS OF DIETARY VITAMIN B_1 (THIAMINE) AND MAGNESIUM ON SURVIVAL, GROWTH, AND HISTOLOGICAL INDICATORS IN LAKE TROUT (*SALVELINUS NAMAYCUSH*) JUVENILES

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Introduction

Early mortality syndrome (EMS) has been described in salmonid populations in the Great Lakes of North America and in the Baltic Sea (M74 syndrome) (Honeyfield et al., 2005). It is associated with low concentrations of thiamine in eggs that results in high mortality of offspring at yolksac and swim-up stages. The effect in offspring has been linked to deficiency of thiamine in the diets of lake trout females (Brown et al., 2005). However, the role of magnesium (Mg) has been completely ignored in the studies of EMS and M74.

It has been observed in mammals that thiamine and Mg are metabolized interdependently. In rats combined thiamine/Mg deficiency resulted in enhanced depression of growth in comparison to either nutrient alone. Thiamine participates in energy metabolism as a coenzyme with Mg as a cofactor. Thus, it has been suggested that Mg deficiency inhibits the mitochondrial thiamine-related enzyme system.

The purpose of this study was to investigate the effects of dietary thiamine and Mg levels in the first feeding of lake trout alevins to better understand the mechanism leading to the EMS syndrome.

Materials and methods

Experimental fish and feeding trial
In the experiment we used progenies from 3 lake trout females collected in Lake Michigan. Thiamine levels in the unfertilized eggs were 6.4±2.8nmol.g^{-1}. Eggs

were sampled after ovulation, fertilized individually with the sperm of several males, and the embryos transported to the Columbus laboratory and incubated in a semi-recirculation system at 5-9°C. Embryos from all 3 females were mixed in equal proportion for the experiment. Lake trout alevins were distributed in glass tanks at a density of 99 fish per tank (four replicates per treatment).

Experimental diets

Semi-purified diets were prepared to accomplish 2×2 factorial design (thiamine×Mg), with (+) or without (-) thiamine (T) and Mg (M): treatments TM++, TM+-, TM-+, and TM--, respectively. Casein and gelatin were used as a source of protein (47%) and cod liver oil and soy-lecithin were used as lipid sources (20%). The basal diet had 19.4MJ.kg^{-1} diet. An amino acid mixture was added to increase the palatability of these semi-purified diets and the quantities of amino acids used were based on attractant strength for this species. Vitamin sources were derived from the Rovimix series, a gift from Aquaculture Research Group, DSM Nutritional Products France, (Saint-Louis, France). Four semi-purified diets that were isonitrogenous (47%) and isolipidic (20%) were mixed, pelletized, and freeze-dried. The desired pellet sizes (0.5 to 1.5mm) were sieved and gradually increased over the course of the trial as the fish grew. A commercial diet (CD) (AgloNorse, Stavanger, Norway) was used as a reference diet.

Biochemical analysis

Sample preparation of the fish and the experimental diets for vitamin analysis followed the method described by Brown et al. (1998). High performance liquid chromatography (HPLC) analysis of free thiamine and its phosphated derivatives (mono- and di-phosphate) was carried out (Lee et al., 2009; Jaroszewska et al., 2009). The HPLC system consisted of a delivery system pump (Beckman Instruments Inc.) equipped with a 20-µl injection loop connected to a 4.6 × 250mm i.d. (Waters 5µm) column coupled with a NH2 packed guard column. The fluorescence detector (FP-920, JASCO Co., Japan) was set at 375nm for excitation and 430nm for emission

Histological studies

For the histological studies 28 individuals from all groups (4 in one group) were randomly sampled at the 7th and 10th week of the feeding experiment. Light microscopy methods were used to examine the central nervous system, liver and muscles. Sections were stained using three methods, with Mayer's haematoxylin and eosin (H+E) for topographic histological analysis; with AB/PAS (pH 2.5; counterstained with Gill's hematoxylin) to identify glycogen in the liver and muscles, and with cresyl fast violet (Nissl staining at pH 2.5) for brain and eyes.

Results and discussion

At the 7th week, the TM-- group exhibited increased mortality (65%) in comparison to other groups fed the semi-purified (34-37%) and commercial (4%) diets. At the 10th week, the TM-+ group showed the lowest mean weight and the highest mortality among treatments (Table I). Thiamine concentration in trunk muscle and Mg levels of whole body were correlated with dietary concentration of two nutrients. Thiamine concentration in the tissue was dominated by thiamine in its pyrophosphate form. Dietary thiamine deficiency resulted in severe mortality of lake trout alevins, whereas dietary Mg supplementation did not affect growth or survival. Mg concentrations in the fish body showed little variation and were unlikely (Shearer and Åsgård, 1992) to affect fish performance in the present study.

Table I. Weight gain (%) and cumulative mortality (%) of lake trout alevins fed one of four experimental diets containing or missing thiamine (T) and/or magnesium (M) for 7 and 10 weeks (n=4). Significant differences at $p<0.05$.

Treatment	Weight gain (%)		Cumulative mortality (%)	
	7th week	10th week	7th week	10th week
TM++	128±5[a]	207±21	34.6±3.8[b]	49.7±4.8[c]
TM+-	170±25[b]	200±18	35.1±2.9[b]	40.9±2.4[b]
TM-+	132±10[a]	167±47	37.6±2.5[b]	80.1±5.5[d]
TM--	117±33[a]	discontinued	65.4±4.9[c]	100*
CD	118±8[a]	207±38	3.5±3.7[a]	5.3±4.1[a]

*predicted based on mortality curve

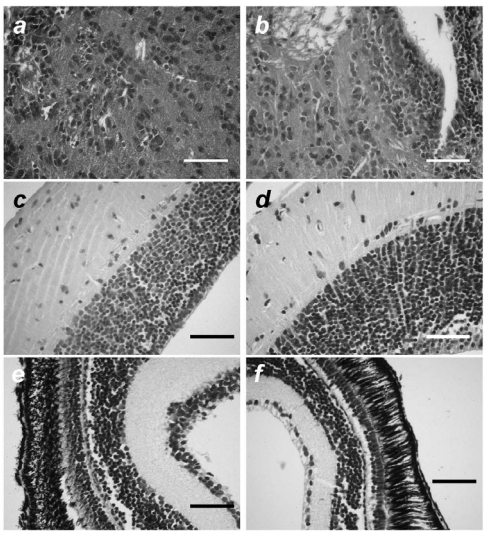

Fig. 1. Cross sections of diffuse nucleus of the inferior hypothalamic lobe (a, b); optic tectum (c, d), and longitudinal section of retina (e, f) reveal no lesions (necrotic cells) in neural tissue of central nervous system and eyes of individual from group TM++ and TM--, respectively. Hematoxylin and eosin staining. Scale bars indicate 50μm.

No pathological changes were observed in the brain, and retina in lake trout juveniles in either the TM++ or TM-- groups at the 7th week. No necrotic cells,

having the form of rounded, shrunken neurons with abnormal distribution of the Nissl granules and with large interstitial spaces between them, which would indicate edema, were found in these organs. Examinations were carried out on the telencephalon, optic tectum, diencephalon, mesencephalon, cerebellum, medula oblongata and spinal cord. These parts of the central nervous system showed well-defined structures with unchanged cells in all strata (Fig. 1a-d). The retina of the eye, surrounded by a pigment layer, had normal structure with no lesions inside the inner nuclear layer (Fig. 1e-f). No differences in glycogen density in the liver between different individual fish were detected in any of the juveniles after 7 weeks of feeding.

Conclusions

Our experiment showed that low dietary Mg worsened overt symptoms of thiamine deficiency in lake trout leading to significantly higher mortality of fish when both nutrients were missing during 7 weeks. The fish fed thiamine devoid and Mg supplemented diet survived longer (week 10) than the TM-- fish, suggesting that Mg enhanced utilization of the thiamine remaining in the body. At the 7^{th} week of the feeding trial we did not observe histopathological changes in the brain and liver tissues that were earlier demonstrated in alevins at the swim-up stage (Lee et al., 2009).

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OUTDOOR LARVAL REARING OF FRINGE-LIPPED CARP, *LABEO FIMBRIATUS* (BLOCH) WITH PROVISION OF DIFFERENT INPUTS

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Introduction

The fringe-lipped carp, *Labeo fimbriatus* (Bloch), an endemic Indian carp, has been identified as a potential candidate due to its high growth potential and consumers' preference. The species has been categorized under potentially endangered group due to its dwindling distribution in most of its parental riverine systems (Gopalakrishnan et al., 1994). While inclusion of the species in freshwater aquaculture systems in the country has been attempted only recently, non-availability of quality seed in adequate quantity is posing major hurdle for its large-scale adoption. Poor survival and growth during larval stages has been one of the primary issues for seed availability. The production performance by and large being the function of input provision (Jena et al., 1998), an outdoor field trial was attempted to evaluate the role of inputs like fertilizers, supplementary feed and aeration during nursery rearing of the species.

Materials and methods

The outdoor seed rearing experiment was carried out in 12 concrete tanks (50m^2 each provided with 15-cm soil base) with rearing of *L. fimbriatus* spawn (6 mm, 1.68 mg) at 10 million/ha stocking density for 30 days. Provision of fertilizers (T1), fertilizers + supplementary feed (T2) and fertilizer + supplementary feed + aeration (T3) were the three treatments evaluated, each with four replications. The tanks were filled with filtered pond water up to 1m level one week before stocking, and subsequently the water loss due to evaporation was compensated at weekly intervals. Standard protocol of phased fertilization with mixture of groundnut oil cake, cow-dung and single super phosphate at split doses (Sahu et al., 2007) were followed for sustaining the production of plankton. Powdered supplementary feed comprising the conventional mixture of rice bran and groundnut oil cake (1:1 ratio) was provided to the larvae with daily ration at three times of the stocked biomass for the first week which increased to six times in second week and eight times thereafter, in two split doses. The growth

increment was assessed through intermittent sampling at 5 days intervals through measurement of length and weight of twenty individuals from each tank. Selected physicochemical water quality parameters of the experimental tanks were monitored weekly following standard procedures.

Enzyme activities were measured from fish intestine and hepatopancreas after termination of the experiment. The estimated data were subjected to analysis of variance (ANOVA) using PC-SAS Programme for Windows Release V6.12.

Results and discussion

The specific growth rates (SGR) of the fry in T2 (16.43%.day^{-1}) and T3 (16.73%.day^{-1}) were almost similar ($p>0.05$) and were significantly higher ($p<0.05$) than that of T1 (10.58% day^{-1}) (Table I). However, survival levels showed a marked difference among the three treatments. The highest survival of 61.7% in T3, followed by 46.9% and 39.8% in T2 and T1, respectively, showed contribution of every additional input viz., feed and aeration in such high-density larval rearing phase. Gross and net biomass also followed the same trend as survival. No provision of supplementary feed in T1 at such high stocking density might have led to inadequacy in natural food for the fry leading to such lower growth and survival levels (Biswas et al., 2006). Whereas in T2 and T3, adequate supplementary feed along with natural food ensured proper nourishment of the fry and improved their production performance. Most of the water quality parameters in the treatment tanks were within favourable ranges (Sahu et al., 2007) for the carp fry rearing. Water temperature remained within 28.0-32.0°C. Comparatively, higher values of total ammoniacal nitrogen was recorded in T2 with provision of fertilizers and feed than other two treatment, attributed to the higher organic load due to accumulation of metabolites and uneaten feed with progress of culture (Sahu et al., 2007). While higher oxygen availability through aeration in T3 group despite the higher biomass helped in better mineralization process thus decreasing ammonia load and prevalence of better environmental condition resulting in the improved survival as well as growth.

Table I. Larval rearing of fringe-lipped carp, *Labeo fimbriatus* with provision of different inputs

Treatment	Initial length/ wt. (mm.mg^{-1})	Final weight (mg)	Final length (mm)	Survival (%)	SGR (%.day^{-1})
T-1	6.0/1.68	42.32±16.3^{b}	16.87±2.06^{b}	39.83±3.95^{b}	10.58±1.21^{b}
T-2	6.0/1.68	233.7±27.1^{a}	28.3±1.06^{a}	46.95±4.10^{b}	16.43±0.38^{a}
T-3	6.0/1.68	258.8±51.1^{a}	28.97±2.37^{a}	61.67±8.51^{a}	16.73±0.68^{a}

Values (mean±SD) with same superscript in a column do not differ significantly ($P>0.05$, $n=4$)

Non-availability of supplementary feed and inadequacy in the dietary energy supplemented through natural plankton for the densely stocked fry in T1 might have led to starvation. Under starving condition, there tends to be an increase in the protein catabolism in fish aiding gluconeogenesis through the transamination of glucogenic aminoacids (Kim et al., 2004) where in protein is used to meet the energy demand rather than utilisation for growth. In the present study, activities of the two aminotransferase enzymes ASAT, ALAT studied in both hepatopancreas and intestine of the fry showed higher elevation in T1 compared to the other two groups (Fig. 1) and indicated such protein catabolism due to starvation leading to the poor fry growth. LDH activity also followed the same trend as ASAT and ALAT in the order as T1>T2>T3 which might be attributed to the higher conversion of pyruvate to lactate for regenerating NAD^+ required to sustain glycolysis, and associated ATP production to meet the energy need during food deprivation (Das et al., 2004). Alkaline phosphatase activity was observed to be higher in intestine than hepatopancreas in T1 group whereas in T2 and T3, it was lower in the intestine than hepatopancreas. The exact reason for such alteration of ALP is unclear. Further study in this regard might give an insight into the matter.

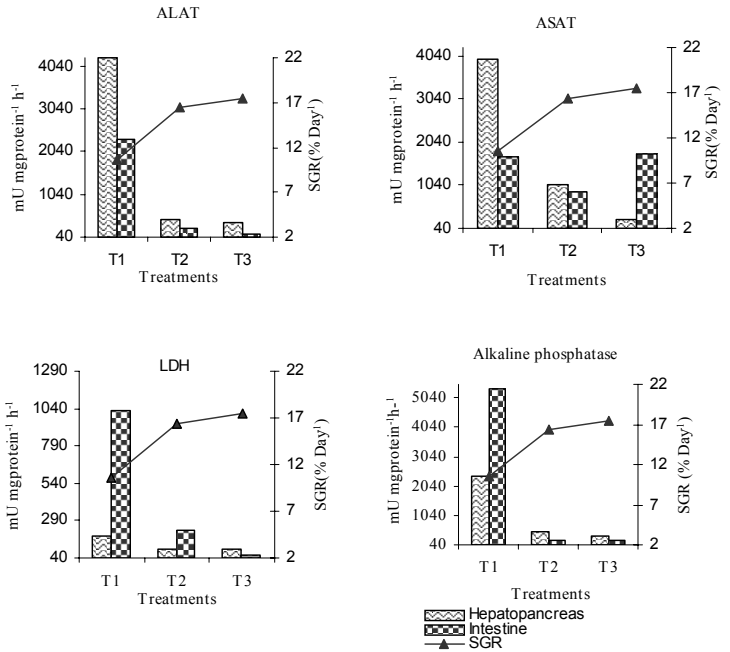

Fig. 1. Relation between different enzyme activities in the hepatopancreas and intestine at 30th day and specific growth rate of *L. fimbriatus* fry from different treatments.

Conclusion

The study revealed that absence of supplementary feeding in the high density nursery rearing leads to starvation stress and cause poor fry performance in terms of growth and survival. It further revealed that additional inputs in the form of supplementary feeding and aeration reduce such stress and ensure better environmental conditions and fry growth performance.

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DEVELOPMENT OF DIGESTIVE ENZYMES IN COMMON SNOOK *CENTROPOMUS UNDECIMALIS*

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Introduction

The common snook (*Centropomus undecimalis*) is a species with important value in Mexico (commercial) and the United States (recreational). Some studied have been conducted to develop its culture; however, fry production is still a bottleneck due to the need of live foods, which are neither necessary nor adequate. To understand the digestive physiology of fish during early ontogeny, several species have been studied allowing strategies for early weaning, but little is known regarding snooks. The objective of this research was to evaluate the development of digestive enzymes using biochemical and electrophoretic techniques during early ontogeny of *C. undecimalis*.

Materials and methods

Embryos were obtained from an induced spawning (Avaplant) of broodstock maintained in 13-m^3 circular tanks in the UMDI-UNAM in Sisal, Merida, Mexico. Embryos hatched thirty-two hours after spawning and the larvae were collected and placed in one 400-l cylindroconical tank with constant water exchange and continuous aeration until yolk absorption (24h later). Larvae were fed using the microalgae *Nannochloropsis sp* and S-type rotifers *Brachionus rotundiformis* (R) from mouth opening (day 1 after hatching, dah) until 10dah. Rotifers were mixed with newly hatched *Artemia* nauplii (AN) until day 25 after

hatching. Finally, from day 25 to 36dah lipid-enriched (SELCO) *Artemia* metanauplii (EAMN) were supplied to the larvae.

Several numbers of larvae were collected at 0, 1, 3, 5, 7, 12, 25, 34, and 36dah. Three pooled samples of fed larvae taken on each day were homogenized in cold 50mmol l^{-1} Tris–HCl 20mmol l^{-1} $CaCl_2$ buffer, pH 7.5. The supernatant obtained after centrifugation (16 000g for 15min at 5ºC) was stored at -20ºC for enzyme analysis. Digestive enzyme activity was expressed as U $mg.protein^{-1}$ and $U.larvae^{-1}$. Enzyme activities measured were: total alkaline proteases, acid proteases, chymotrypsin, trypsin, leucine aminopeptidase, carboxypeptidase A, α-amylase, lipase, acid and alkaline phosphatases.

Alkaline protease isoforms were revealed using SDS-PAGE and acid protease isoforms were revealed using PAGE. A low-range molecular weight marker was applied to each SDS-PAGE to determine the molecular weight of each isoform and calculated with Quality One V. 4.6.5 software. A Kruskal-Wallis test was used to compare the enzyme activity between ages for each activity. A nonparametric Nemenyi test was used when significant differences were detected. All tests were carried out with Statistica v7.0.

Results and discussion

For all specific and individual digestive enzyme activities, statistical differences were detected ($P<0.05$). The specific alkaline protease was detected at hatching (1dah) and increased at 3dah, reaching the maximum value at 12dah (Fig. 1a). Specific acid protease activity was detected with low levels from 7dah, and increasing gradually from 12 until 36dah (Fig 1c). Specific trypsin activity showed two maximum peaks of activity at 7 and 12dah, while after this, the activity decreased rapidly (Fig. 1e). Chymotrypsin specific activity started from 5dah to the maximum activity at 25dah, decreasing from this day onwards (Fig. 1g). Carboxypeptidase A specific activity was low during the first days, increasing rapidly at 7 and 12dah; then, reduced at 25dah and increasing again at 36dah (Fig. 1i). Specific leucine aminopeptidase was first detected at 1dah, and reaching the maximum peak of activity at 12dah decreasing from this day until the end of the culture (Fig. 1k). For individual alkaline protease (Fig. 1b), acid proteases (Fig. 1d), trypsin (Fig. 1f), and carboxypeptidase A (Fig. 1j) showed low activity during first days, reaching the maximum peak at 36dah. For chymotrypsin individual activity, the maximum peak was detected at 32dah, decreasing at 36dah (Fig. 1h). Leucine aminopeptidase individual activity showed no activity from hatch until 5dah increasing at 7dah, reaching the maximum peak at 12dah (Fig. 1i).

Specific lipase activity showed a slightly increment at 3dah, increasing at 36dah (Fig. 2a). The individual lipase activity gradually increased its activity reaching the maximum value at 32dah and decrease at 36dah (Fig. 2b). Specific α-

amylase activity is present from 1dah, increasing to reach the maximum peak at 7dah, and suddenly decreased from 25dah onwards (Fig. 2c). Individual α-amylase showed two peaks at 7 and 36dah (Fig. 2d). Acid phosphatase specific activity, low values were detected until 12dah; increasing at 25dah, and reaching the maximum peak at 34dah (Fig. 2e). The individual acid phosphatase activity showed a similar tendency, having maximum peaks at 25 and 36dah (Fig. 2f). The specific and individual alkaline phosphatase activities had the same pattern with low values until 12dah, increasing rapidly at 25dah and decreasing at 36dah (Figs. 2g and h).

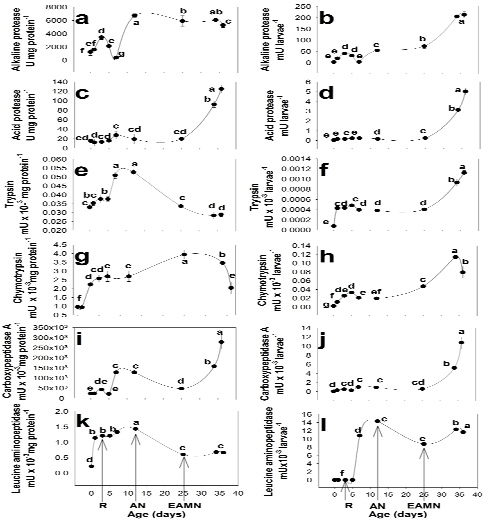

Fig. 1. Digestive proteases activities during common snook larviculture (mean ± SD, n = 3 pooled larvae). R: rotifers, AN: *Artemia* nauplii, EAMN: enriched *Artemia* meta-nauplii. Mean values with different letters show significant differences ($p < 0.05$)

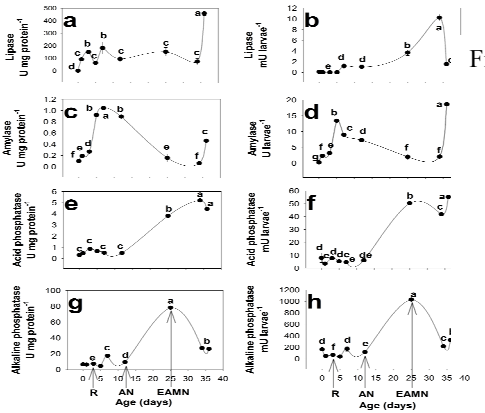

Fig. 2. Digestive lipase, amylase, and phosphatase activities during common snook larviculture (mean ± SD, n = 3 pooled larvae). R: rotifers, AN: *Artemia* nauplii, EAMN: enriched *Artemia* meta-nauplii. Mean values with different letters show significant differences ($p < 0.05$)

The zymogram for acid proteases showed two different types of bands, the first band had a Rf of 0.32, and the second a Rf of 0.51. Both bands were observed

for days 34 and 36 after hatching (Fig. 3a). On the other hand, the zymogram for alkaline proteases showed only one band for days 25 (26.1 kDa) and 34 (26.3 kDa) after hatching; for day 36 after hatching two bands were detected the first band of 28.4 kDa, which could correspond to the bands observed for days 25 and 34 after hatching, and the appearance of one additional band with higher molecular mass (51.6 kDa) (Fig. 3b). Our results agree with those obtained for other species where the changes in activities are related with morphophysiological changes in the larvae gut; when this organ differentiates to hind, mid and foregut, the maturation of microvilli in the enterocytes, as well as in live or artificial food changes during larval growth (Moyano et al., 1996; Zambonino-Infante and Cahu, 2007).

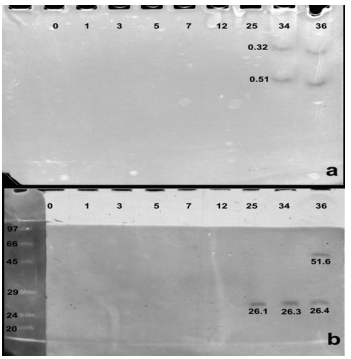

Fig. 3. Zymograms of (a) acid and (b) alkaline protease digestive activities during the development of Common snook larvae.

Conclusions

C. undecimalis larvae have the classic digestive enzyme development as other marine fish. We propose the weaning for this species from 34dah onwards.

Acknowledgements

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DEVELOPMENTAL EXPRESSION OF GLUCOCORTICOID RECEPTOR DURING EARLY ONTOGENY IN GILTHEAD SEA BREAM, *SPARUS AURATA*, AND EUROPEAN SEA BASS, *DICENTRARCHUS LABRAX*

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Understanding the onset of the corticosteroid stress axis and the neuro-endocrine regulation of the stress response of an aquaculture fish species, is pivotal to get a better knowledge in the process of adaptation to physical, chemical, biological and husbandry changes and/or disturbances during early ontogeny. Cortisol is the key corticosteroid hormone in teleosts. It is secreted from the interrenal cells in response to noxius stimuli and plays a major role in the organism response to cope with stressors. Cortisol is not just a stress hormone but it is also implicated in a variety of essential biological functions and processes such as development, carbohydrate, protein and lipid metabolism, energy allocation, immune response, bone metabolism, gastric secretion, cardiovascular regulation, learning and memory, and hydromineral regulation in the marine environment.

Cellular responses to glucocorticoids are attributed to their binding to specific glucocorticoid receptor(s) (GR or NR3C1). The GR is a ligand-activated transcription factor that binds with high affinity to cortisol and other glucocorticoids. In the absence of cortisol, unbound GR resides within the cytoplasm in an inactive complex with some regulatory proteins such as the heat shock proteins. Cortisol binding to the GR results in the activation of the receptor that, in turn translocates to the nucleus modulating gene expression. Modulation of gene transcription is operated by the active GR through two major mechanisms of actions: direct gene transactivation and non genomic transrepression.

Two different genes that encode GR protein have been identified in a number of teleost fish, such as the rainbow trout (*Oncorhynchus mykiss*), the common carp (*Cyprinus carpio*), the puffer fishes (*Takifugu rubripes* and *Tetraodon nigroviri-*

dis), and a cichlid (*Haplochromis burtoni*). However, in the zebrafish (*Danio rerio*) only one GR was identified. In the European sea bass (*Dicentrarhus labrax*) sequences of two genes have been published (NCBI Gene Bank accession no AY549305 and AY619996) encoding two GR proteins named GR (GR54) and DIGR1 (GR61). These proteins display high amino acid sequence identity, but no significant sequence similarity has been found at their transcription activation domains. No data are available on the quantification of GR in the gilt-head sea bream, *Sparus aurata*.

While several studies have been conducted on the role of cortisol and GRs in the stress response of adult fish of economical importance for aquaculture, little is known on the development of the corticoid system during early ontogeny. To get a better understanding on the corticosteroid signaling pathway, we investigated, using real-time qPCR, gene expression of GR in gilt-head sea bream and of the two GR isoforms (GR61 & GR54) in European sea bass, at specific phases during early ontogeny.

RNA was extracted using liquid nitrogen and RNeasy Plus Mini Kit by QIAGEN (Valencia, USA). RT reactions were performed with MMLV reverse transcriptase by Finnzymes. SYBR Green I and Opticon monitor system by MJ Research were used for real-time qPCR reactions. Primers used for RT-qPCR reactions were: Sea bass target gene AY619996: 5'-GACGCAGACCTCCAC TACATTC–3' (forward) and 5'-GCCGTTCATACTCTCAACCAC-3' (reverse); Sea bass target gene AY549305: 5'-GAGATTTGGCAAGAC CTTGACC-3' (forward) and 5'-ACCACACCAGGCGTACTGA-3' (reverse); Sea bream target gene: 5'-GCGGATGAAGTTGCCCTACAT-3' (forward) and 5'-TGCACAAATACTCGTCGTGGGA-3' (reverse). Housekeeping gene 18S: Sea bass, 5'-TCAAGAACGAAAGTCGGAGG-3' (forward) and 5'-GGACATC TAAGGGCATCACA-3' (reverse); Sea bream 5'-AGGGTGTTGGCAG ACGTTAC-3' (forward) and 5'-CTTCTGCCTGTTGAGG AACC-3' (reverse).

Our data showed expression of GR in embryos and hatched eggs of both species. In gilt-head sea bream a clear up-regulation of GR was observed at first-feeding, while in sea bass, GR61 and GR54 showed different expression patterns indicating possible.

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RAPID DETECTION AND ENUMERATION OF *VIBRIO* SPP. FROM SHRIMP HATCHERY ENVIRONMENTS USING ENZYME-LABELLED PROBES

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Introduction

The members of the genus *Vibrio* are widespread in the aquatic environment and constitute the natural microbial flora of that environment as well as the animals being cultured therein. *Vibrio* species such as *Vibrio harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, and *V. anguillarum* can sometimes become pathogenic and are common disease agents that affect animals in the shrimp hatchery and aquaculture ponds (Ruangpan and Kitao, 1991; Nash et al., 1992). The disease caused by *Vibrio harveyi* has been the most studied since it has been responsible for severe mortalities in shrimp hatcheries worldwide (Karunasagar et al., 1994; Karunasagar et al., 1996)

Classically, *Vibrio* isolation and enumeration is done on thiosulphate citrate bile salt sucrose (TCBS) agar. However, some workers have reported the enumeration of vibrios on (TCBS) agar and compared the inhibitory effect of the medium to non-selective media like trypticase soya agar (Nicholls, 1976; Roberts and Seidler, 1984; Raghunath, 2007). Therefore, when there is a mixture of organisms, alternative methods are necessary to enumerate the levels of *Vibrio* spp. in shrimp aquaculture environments. Several investigators have described the usefulness of *gyrB* gene sequence for discrimination between closely related bacteria (Dauga, 2002). On the other hand, hemolysin gene has been well characterized in various *Vibrio* spp. including *V. harveyi* for specific detection (Zhang and Austin, 2000). Therefore, we considered the possibility of using enzyme-labelled probe targeting these genes for detection by dot blot and enumeration by colony hybridization of *Vibrio* load as well as *V. harveyi* in the aquaculture environments.

Materials and methods

Various *Vibrio* isolates belonging to different species from our laboratory stock culture that included *V. parahaemolyticus* (AQ4037), *V. vulnificus* (ATCC27562), *V. cholerae* (ATCC39315), and *V. harveyi* (LMG07890) were used for the study. A set of other non-vibrios such as *Edwardsiella tarda* (DSM30052), *Salmonella typhimurium* (ATCC14028), *Escherichia coli* (ATCC25922.), *Klebsiella pneumoniae* (MTCC109), *Enterobacter aerogenes, Proteus* sp., *Pseudomonas aeruginosa, Listeria monocytogenes, Acinetobacter baumanni,* and *Citrobacter* sp. were also included as negative control. The total genomic DNA was isolated according to the method of Ausubel et al. (1995). Concentration and purity was measured in a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA).

The genus-specific alkaline phosphatase (AP) labeled probe targeting the *gyrB* gene and digoxigenin-labelled species specific probe targeting *vhh* of *V. harveyi* was used. The vibrios were initially confirmed by performing PCR for the genes to be used for total vibrios as well as for *V. harveyi.* The detection was carried out by dot blot hybridization (Dyson, 1995) which was performed with the genomic DNA samples of different *Vibrio* species upon a pre-treated nylon membrane (BioTrace™ NT; Pall Corp., USA). Enumeration by colony hybridization was done using the respective labeled probes. For colony hybridization, isolates were spot-inoculated on T_1N_1 agar plates and incubated at 30°C overnight. Colony lifts were performed onto nylon membrane and hybridization carried out as described in the FDA manual (FDA, 2004), with minor modification.

Results and discussion

The specificity of the probe was evaluated using various *Vibrio* spp. and other vibrios. *gyrB* probe reacted only with vibrios and with none of the other bacteria used in this study. The total *Vibrio* counts using the probe was much higher than the counts obtained on TCBS agar plates. All *V. harveyi* isolates showed the presence of *vhh* gene by PCR whereas other vibrios and gram negative bacteria did not amplify the gene (Fig. 1). Further, the isolates could be detected by dot blots (Fig. 2) and enumerated by colony hybridization. Sensitivity of the probe when assessed by using shrimp homogenates spiked with known concentration of *V. harveyi* cells and comparing the counts with that obtained on TCBS agar and non selective medium TSA with 1% salt confirmed that TCBS is highly selective and inhibits the growth of vibrios. It has been found that some non-vibrios also may be able to grow on TCBS. Therefore, there is a need for reliable and alternative methods to overcome these shortcomings. Methods based on enzyme labeled probe is a good alternative for quantification of total *Vibrio* and *V. harveyi* in aquaculture systems. The results indicate that dot blotting is rapid and useful for the direct detection of vibrios and colony hybridization is an efficient

method of enumerating total *Vibrio* spp. in aquaculture systems which is a prime need in hatchery systems. Since these techniques do not require any expensive equipments or special laboratory facilities, they would be useful in hatchery settings.

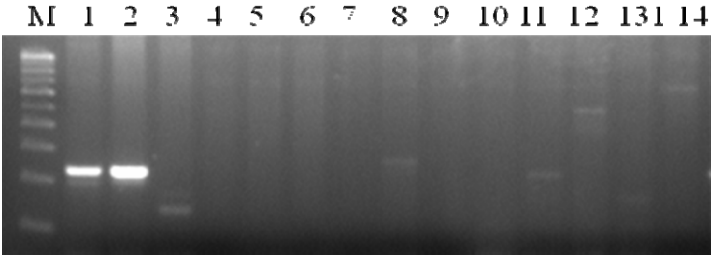

Fig. 1. Representative photograph of PCR amplification of *vhh* gene. Lane M, DNA marker- 100 bp. Lanes 1 and 2, environmental and standard strain (LMG07890) of *V. harveyi.* Lanes 3-14, other gram negative bacteria.

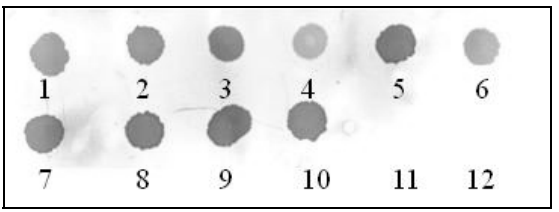

Fig. 2. Representative photograph of Dot blot analysis of *V. harveyi* strains using *vhh* gene portion as probe. Lanes 1-10, different *V. harveyi* isolates. Lanes 11 & 12, negative controls

Conclusion

In conclusion, the method based on enzyme labelled probes was found to be highly specific, sensitive and reliable which would be very useful for the rapid confirmed detection and enumeration of *Vibrio* spp. from shrimp hatchery environments.

Acknowledgments

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MARINE FISH LARVAE MICRODIET – BEYOND NUTRITION

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During the past three decades, extensive efforts have been made to develop microdiets (MD) as a complete or partial substitution for both rotifers and *Artemia*. The majority of the focus has concentrated on the larvae's nutritional requirements. Despite substantial achievements, complete replacement of live feeds for most marine species is still not feasible.

In addition to MD quality, important physical characteristics of MD particles within the water column such as sinking rates, nutritional stability, leaching rates and leachate profiles all contribute to the MDs attractiveness. This influences ingestion and digestion rates and can greatly influence larvae growth; however these factors have received very little attention.

The first contact between larvae and MD occurs in the water column, when the particle is recognized as food and accepted, or rejected. Therefore, it is essential that this interaction be optimised to achieve a maximum likelihood that this feeding process is successful. There are many factors affecting this process including particle/organisms concentration, frequency and duration of interactions, and chemical and physical recognition.

Various substances, such as free amino acids, nucleotides, nucleosides, and ammonium bases, are known to be 'feed attractants' for fish larvae. A practical way to increase the ingestion rates of MD would be to incorporate or coat the diet particles with extracts or hydrolysates of marine organisms such as fish, krill or squid.

One of the challenges in manufacturing MD particles is to optimize the leaching rates and amino acid profile in the leachate. Studies have reported amino acid leaching ratios and leaching rates resulting from different manufacture techniques, ranging from 4% to almost 100% after 5 minutes of immersion in water. Although some amino acid leaching is necessary for food particle recognition, a balance between rates of leaching, stability of MD particles in water and the digestibility of the diet need to be met.

MD is expensive (up to 200€/kg) and is likely to remain so, due to expensive raw ingredients, difficult manufacturing techniques and relatively small-scale production leading to high fixed-cost contributions. Therefore, it is necessary to optimize yield from such a product. Food conversion ration (FCR) should be used as an indicator to help hatchery managers improve their methods and efficiency when substituting MD for live feed. However, FCR figures for larvae feed, live or formulated do not exist, and estimating FCR with larvae is extremely difficult. Actual MD FCR (ingested MD / weight gain) could be as low as 0.6:1 due to the high digestibility of the MD, however this is rarely achieved.

One of the most significant problems with MD particles is their negatively buoyant inert state, contrary to the movement of live zooplankton. Movement acts as a visual stimulus for increased feeding activity. Furthermore MD can lead to bacterial proliferation and deterioration of water quality if the particles sink to the bottom of the tank and accumulate there. Different attempts have been made to increase MD particle buoyancy by varying manufacturing methods as well as using rearing systems with up welling currents. Knowledge of sinking and leaching rates of MD can and should be used to optimise feeding procedures. Ultimately, using the right feeding system to reach a precise and controlled distribution of MD improves FCR values considerably. Some European hatcheries quoted 20-40% less feed being used when shifting from hand distribution to automation, with additional benefits of increased larvae survival and growth.

Larvae feeding methods and feeding systems play a crucial role towards the way MD is received by the larvae. The best MD is only as good as the method used to dispense it into the larvae tank. However, this area of research is undeveloped compared to feeding systems and methods for on-growing fish. Continuous availability of MD is optimal for larvae and is best achieved by dispenser automation. Only a handful of automated MD feeding systems currently exist including, belt feeders, horizontal drums with pneumatic pistons, rotating disks and more recently, dynamic solenoid operated slotted-plates. While some were specifically designed for MD, others were adopted from large pellet feeders with varying degrees of success.

Although larvae nutritional requirements are now recognized, MD properties and feeding technologies are neglected. Therefore a more integrated approach to microdiet development is needed, which takes into account the effect of physical and chemical properties, manufacturing techniques, feeding systems, tank hydrodynamics and water quality's influence on ingestion, digestion, and assimilation of the diet.

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ENVIRONMENTAL FACTORS AND NUTRITIONAL REQUIREMENTS AFFECTING *SERIOLA* SP. LARVICULTURE

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Introduction

Seriola sp. (*S. lalandi, S. dumerili, S. quinqueradiata*) are an emerging species for aquaculture around the world. Currently, they are cultured and/or under different stages of research and development in Japan, Australia, New Zealand, Mediterranean countries, Mexico, Chile, and US. Although *Seriola* sp. are very robust and have one of the fastest growth rates amongst temperate fish species, the larvae culture is more difficult than with other temperate species such as gilthead sea bream and European sea bass. The larvae are very susceptible to bacterial infection at different stages and have very high rates of deformities. These deformities are related to genetics, broodstock and larval nutrition, and stress due to environmental conditions as well as handling.

In recent years numerous R&D centers have conducted substantial work on rearing protocols as well as the effect of environmental and nutritional factors on *Seriola* sp. larvae.

Rearing environments for larviculture

Physiological and ecological features of *Seriola* sp. larvae demonstrate the importance of optimizing the rearing environments for larviculture. Larvae of this species have negative buoyancy and sink to the bottom of the rearing tank at nighttime resulting in mass mortality, namely 'sinking syndrome' (Teruya et al., 2009). To prevent sinking syndrome, excessive aeration is required, however this decreases feeding activity and survival of larvae.

Juveniles of *Seriola* sp. (*S. dumerili* and *quinqueradiata*) often associate with drifting seaweeds, which is coincided with the drastic changes in phototaxis from strong light intensity in larval stage to dim light in juvenile stage (Sakakura et al., 1999). Therefore, in order to maximize the larval feeding activity together

with minimizing sinking syndrome, optimizing the physical environment, such as water flow and lighting should be carefully designed for *Seriola* sp. larvae.

Tank design

Hatching tank
Traditional hatching tank design comprised of a conical bottomed tank with surface water inlet as well as outlet and central aeration from the base, resulted in relatively poor hatching success, unrelated to the egg quality. It is known that *Seriola* larvae sink to the bottom immediately after hatching. Therefore, improvements to the hatching tank that encourage hydrodynamics that emphasize homogenous mixing of the hatching media were necessary. The modified design is based on an up-welling water inlet at the apex of the bottom cone directed outwards towards an aeration ring around the base of the tank wall, while the screened outlet remained at the surface. This design ensured that hatched larvae remained in the water column and resulted in significant increases in hatching rates and survival of larvae (Kolkovski et al., 2007, Kolkovski and Sakakura, 2007).

Rearing tank
Seriola sp. and specifically *S. lalandi* larvae are susceptible to high rates of deformities. Observations have revealed a tendency of larvae to 'bang' their heads on the tank wall, which is understood to cause some skeletal deformities. Therefore, an aeration ring was installed around the base of the tank wall, which creates a gentle curtain of bubbles that prevents the larvae from approaching the wall.

In order to reduce 'sinking syndrome' and to encourage a gentle homogenous mixing of the culture media, a bottom centralized water inlet directed outwards towards the base of the tank wall was designed to complement the currents created by the air curtain. Coupled with this, live food and microalgae is dosed into the main water inlet line resulting in an even distribution of resources. These hydrodynamics and methods of feeding reduce competition for food and prevent 'dead spots' in the water column (Kolkovski et al., 2007).

Nutritional requirements

Due to the very fast growth rate of *Seriola*, any deficiency in the larvae nutrition can result in low growth and survival. Moreover, these larvae are susceptible to stress resulting in low survival.

Vitamins
The effect of a 'mega' dose of vitamins E and C on yellowtail kingfish (*S. lalandi*) larvae growth was determined. The vitamins were added to the enrichment of rotifers and/or *Artemia*. The results determined that the effect of the vi-

tamins was significant at the *Artemia* stage while no effect was found at the rotifer stage (Kolkovski and Sakakura, 2007).

Feeding activity of *Seriola* sp. larvae are quite unique among the marine teleosts. Although body size at the first feeding is relatively large (~4mm), larvae prefer smaller sized rotifers and this preference lasts until flexion stage (~7mm) with increased consumption (Akazawa et al., 2008; Hamasaki et al., 2009).

Remarkable findings for incorporation of DHA on the development of *S. quinqueradiata* were reported. It is essential not only for the activity and quality of the fish but also for the development of schooling behavior in the juvenile stage (Masuda et al., 1998, 1999; Ishizaki et al., 2001). Once rotifers were enriched with HUFA, their HUFA contents did not decrease in the semi-static rearing tank with micro algae (Yamamoto et al., 2009), suggesting that enrichment of live feeds should be done once before feeding.

Broodstock supplemented with high levels and specific ratios of EPA : DHA (different than sea bream and sea bass) resulted in higher quality eggs and larvae (S. Kolkovski, personal communication).

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MODULATION OF HORMONAL REGULATION AND DIGESTIVE CAPACITY IN ATLANTIC COD LARVAE (*GADUS MORHUA*) AS INFLUENCED BY PREY

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As a result of the postulated decrease in wild stocks, interest in the intensive production of Atlantic cod (*Gadus morhua*) has increased markedly over the past couple of years. However, a major challenge in intensive cod larval rearing is the development of optimal nutrition feeding protocols. The natural feed for pelagic fish larvae such as cod are copepods, which are regarded as better quality feed organisms than cultivated live feed (e.g., *Artemia*). Consequently, cod larvae fed cultivated live feed and formulated diets by intensive methods generally show lower growth rates and higher deformity frequencies than larvae fed copepods in extensive and semi-extensive systems. It has been demonstrated that larvae having an early onset of growth generally maintain this lead throughout the first feeding phase until metamorphosis. Additionally, they seem to perform better in terms of survival and viability, emphasizing the importance of optimal nutrition during the first feeding stage.

The ontogeny of gastrointestinal tract has been described in numerous marine fish species. But, relatively few studies have employed molecular biological techniques to study the ontogeny of digestive capability and nutritional effects on growth and appetite in marine fish larvae. Feeding and digestion are complex processes controlled by the nervous and endocrine systems, involving an array of neurotransmitters, hormones, paracrine-signal transduction, and transcription factors. Previous studies have demonstrated that there is a close relationship between larval nutrition at the first feeding stage and larval growth and development. For example, the brain produces key factors that stimulate or inhibit food intake, and these factors may in turn regulate pituitary hormone secretion via the growth hormone-, thyroid and reproductive axis. Thus, by manipulating larval first feeding protocols, one may influence the ontogenic development of digestive capability and endocrine functions that may ultimately lead to numerous physiological effects, such as enhanced somatic growth and overt development.

In this study, Atlantic cod larvae were fed either *Acartia tonsa* nauplii or enriched rotifers and *Artemia* nauplii, before weaning to formulated diet up to 38 days post hatch (dph). Larvae were sampled at 5, 8, 16, 29 and 38 dph. Larval growth and development were correlated to gene expression patterns of classical digestive enzymes such as trypsin, amylase, bile-salt dependent lipase (BAL), phospholipase A2 (PLA2) and Acyl CoA dehydrogenase (ACADM) and factors potentially controlling appetite and somatic growth; cholecystokinin (CCK), neuropeptide Y (NPY), thyroid receptors (TRα and β) and growth hormone (GH), determined by quantitative PCR. Gene expression profiles were also correlated to activity levels of digestive enzymes and nutritional content of prey and larvae.

Larvae fed copepod nauplii showed enhanced growth compared to other larval groups. Differential gene expression patterns that were dependent on diet and/or larval age were observed. To our knowledge, most of these mRNA expression profiles have not been described previously for Atlantic cod during larval development. Significant transcript levels of all genes investigated were detected at the onset of first feeding (at 5dph), but these were not diet-specific at this time point. However, genes involved in appetite regulation and digestion showed differential expression profiles after different feeding protocols at more advanced larval stages (i.e., at 8, 16 and 29dph). Interestingly, we observed some correlated transcription profiles for genes coding for digestive enzymes and genes coding for neural controlling factors. Finally, this correlation was also partly reflected in digestive enzyme activity levels and larval specific growth rates.

In conclusion, this study provides novel information on some candidate genes suited for characterizing ontogeny of digestive capability and fitness in early larval stages. The consistency in the expression patterns observed for digestive enzymes and neural growth- and digestive controlling factors suggest that these processes are closely linked at the molecular and cellular levels of biological organization. Furthermore, functional genomics focusing on the patterns of gene regulation typical for early onset of growth and digestive capability may tremendously increase our understanding of the basis for the observed problems, and form a basis for generation of hypotheses on critical factors for functional development of fish larvae.

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DIEL FEEDING RHYTHM OF FINFISH LARVAE AND ESTABLISHMENT OF APPROPRIATE FEEDING SCHEDULE

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The feeding regime and schedule of finfish larviculture is usually determined separately in each hatchery and there are many variations among hatcheries even if fish species are same. There are no biological and technological bases of the determination. Or sometimes it is determined by the convenience of manipulator. It is important for successful larviculture that the feeding on larvae at earlier stages and the failure of intake by irrelevant feeding sometimes results in serious problems, such as high mortality and decline of activity. So, feeding schedule has important role to avoid such serious problems. On the other hands, since fishes have species-specific diel feeding rhythm, the feeding schedule should not be determined uniformly across some species. It is necessary to establish the feeding schedule corresponding to the diel feeding rhythm of larval fishes in order to improve the larval health and quality.

The feeding rhythm of fishes is said to relate to the circadian rhythm. Therefore, in the early stage in which the eyes of larvae do not develop so sufficiently that they can recognize the illumination, definite feeding rhythm cannot be observed in each fish species. After their photosensitive organisms start to develop, most fish species have some peaks of feeding in daytime, especially in dusk and dawn, such as flat fishes, groupers, sparids, devil stinger, and ayu. These fishes do not take food in night time. Larvae have higher peak of feeding in dawn than in dusk. This trend does not change between rotifer and *Artemia* nauplii feeding periods. Although larval ocellate puffer also has the peaks of feed intake in daytime, they kept feeding in night time. While they did not get in midnight, they started getting in dusk.

Moreover, the timing of first feeding is also important. It has been already clarified that delayed first feeding also results in serious problems. In many cases, the first feeding is performed just after mouth opening in order to prevent delayed one. However, just after mouth opening, larvae do not have well-developed function of jaws to ingest the feed. Then live feeds, rotifers or *Artemia* nauplii, remain in rearing water without ingested. They metabolize the enriched nutrition

by themselves and it results in the deterioration of nutritional quality of residual live feed. Therefore too early first feeding also should be avoided.

When the feeding schedule is determined, it should be taken into account when larvae of cultivated fish species have the peaks of food intake within a day. If the feeding is late for the peaks, live feeds remain in rearing water without ingested and it results in the nutritional deterioration of residual live feed. Therefore the feeding schedule should be set before larvae have the peak of feed intake or, at least, around the peak.

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FEEDING STRATEGIES FOR EARLY LIFE STAGES OF RED KING CRAB (*PARALITHODES CAMTSCHATICUS*) AND GIANT FRESHWATER PRAWN (*MACROBRACHIUM ROSENBERGII*) UNDER ARTIFICIAL CONDITIONS

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In recent years, the cultivation of crustaceans is an important direction for aquaculture in East and Central Europe, and particularly Russia. This is important both for preservation of species variety, maintenance of natural population size of commercially valuable species (red king crab), and increase of production and diversity of delicacy hydrobionts (giant freshwater prawn).

The most vulnerable stages of life cycle of crustaceans are larval stages. It is generally accepted that there is extensive mortality in the early stages of organisms which produce large number of planktonic larvae endowed with little stored energy. Thus, for cultivation of the red king crab and giant freshwater prawn it is important to study different aspects of feeding of larvae, as food availability and quality are main limiting factors in their survival.

This paper describes the main feeding acceptance in larval rearing and provides a basis for development of cultivation practices under artificial conditions for two crustacean species.

The experiments were held (2002-2008) in the Laboratory of Crustacean Reproduction (VNIRO, Moscow) in 200-300-l recirculating rearing tanks.

Red king crab larval culture. Red king crab larvae pass through a series of developmental stages: a brief prezoea stage and four zoea stages (zoeae I-IV). Prezoeae is a non-feeding, non-active stage. Water temperature was maintained at 7-8ºC, water salinity at 32g.l^{-1}. Larvae were fed with *Artemia* nauplii 2 times a day. Daily food intakes were determined for each instar (zoeae I-IV). We have calculated experimentally maximal daily rations and optimum initial concentration of nauplii for zoea stages. Optimal initial concentrations of *Artemia* nauplii for feeding zoeae I-IV thus constitue 400-600, 600-800, 800-1000 and 1000-1200 nauplii.l^{-1}, respectively.

Red king crab larvae, as well as most planktonic crustaceans, can capture food objects dispersed in the water only up to certain concentration which is not equal to zero. For the whole larval period (zoeae I-IV) the average value of these minimum non-consumable food concentrations is 160 nauplii.l^{-1} per larvae.

In the process of revealing of effective larval feeds we have tested artificial starting mixed feed for sea organisms Start and Wean-Ex (DANA FEED, Denmark), Micron (Sera, Germany) and Troco (Coppens, Netherlands).

Giant freshwater prawn larval culture. Larvae pass through eleven stages (I-IX.) Water temperature was maintained at 28-31ºC, water salinity at 12g.l^{-1}. Larvae were fed with *Artemia* nauplii and egg mix 4 times a day. The size of particles of the egg mix and number of *Artemia* has been chosen depending on the stage of development and the size of larvae.

Optimal initial *Artemia* nauplii concentrations for feeding larvae of II stage constitute 40-60, III-V stage - 90-100, VI-VIII stage - 150-170 and IX-postlarvae - 200-220 nauplii.ind.$^{-1}$, respectively.

The survival rate of individuals of the red king crab up to hatchling of the first stage has made 30-35%, which is tens times higher in comparison with the survival rate in the natural environment and surpasses results of all experiments which have been carried out earlier. Significant reduction of duration of the larval period of development (from 65-80 days in natural conditions to 32-38 days) has been also achieved.

During the larval period, the survival rate of giant freshwater prawn in controlled conditions of the recycling water system at planting density of 100 pieces/l has made on the average 52% (45-60%), larvae (XI) length 7.9 (7.2-8.5) mm and larvae (XI) weight 7.1 (6.6-7.4) mg. The duration of the larval period has varied within the limits of 28-36 days.

The feeding strategies developed by us (differentiated feeding depending on the stage of development) in the process of cultivation of red king crab and giant freshwater prawn, in complex with other technological methods, have become the basis for creation of the biotechnical requirements for the red king crab and giant freshwater prawn artificial reproduction and cultivation by mass-culture method in Russia.

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THE EFFECT OF LARVAL REARING ON JUVENILE QUALITY

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It is well documented that providing the correct nutrition and environment during the larval rearing of commercial teleosts improves growth and survival, which ultimately determines production levels. However, specific environmental factors such as temperature, salinity and current speed as well as dietary components that include vitamin A, iodine and lipid class can also have far reaching effects on the quality of juveniles produced, in terms of percent deformity, metamorphic success, sex ratio and growth.

Larval rearing temperature and current speed are main environmental factors influencing skeletal deformity in juvenile fish. The confounding effect of temperature during early ontogeny on swimming-induced lordosis was tested in European sea bass (*Dicentrarchus labrax*), previously reared as larvae at 15 or 20°C, and then grown under different water current speeds. Fish reared at cooler temperatures grew significantly better and exhibited less severe lordosis than their cohorts raised at 20°C. Lordotic vertebrae, as a function of increased swimming effort, have larger bone volume, flattened dorsal zygapophyses and extra lateral ridges which decrease the strain from faster tail beating. Although lordosis may be primarily caused by swimming intensity, temperature exacerbates the situation by causing differential growth of muscles and bone involved in skeletal development. In the absence of increased swimming activity, lordosis can also be associated with non-inflation of the swim bladder. In gilthead sea bream (*Sparus aurata*) 20-dph larvae reared in 40‰ seawater demonstrated significantly lower swim bladder inflation and markedly higher levels of juvenile skeletal deformities compared to fish reared in 25‰ seawater. In contrast, these studies found no correlation with skeletal deformity and temperature which may be tied to brood stock selection from regional warmer seawater and the progeny exhibiting lower levels of abnormality.

Studies on European sea bass have focused on the effect of temperature during specific and labile ontogenetic periods, which precede any sensitivity to exogenous steroids, on the sex ratio in the population. As female fish generally grow

faster than males, this approach would generate considerable interest in the aquaculture industry. Studies found that rearing eggs and larvae of European sea bass at higher temperatures skewed the sex ratio towards males while lower temperatures increased the female: male ratio. These findings were supported by other workers who found temperature sex determination (TSD) strain dependent but effective in both larval and nursery rearing periods while it was also demonstrated that TSD can occur much earlier during the first nine days of life.

Studies on various flatfish species have improved metamorphic pigmentation, eye migration and general development, through feeding copepods within a critical developmental window. Copepods are richer in PUFA, carotenoids and iodine compared to Artemia. Immersion of flounder larvae in retinoic acid have stimulated the formation of adult type chromatophores possibly through enhanced rhodopsin synthesis. Although thyroid hormone (TH) is critical for metamorphic development, the effectiveness of increasing the TH precursor iodine in the diet has not been demonstrated. Workers have also focused on the essential fatty acids eicosapentaenoic acid and arachidonic acid, the precursors of eicosanoids that may be involved in pigmentation. In other teleosts such as grouper species (*Epinephelus coiodes*, *E. aeneus*), non-synchronous metamorphosis can produce great size variability leading to cannibalism, poorly developed juveniles and low production. It has been shown that immersing premetamorphosing grouper in various concentrations of T_4 and T_3 resulted in the synchronization and shortening of metamorphic duration in a dose dependent manner and independent of larval size.

Studies on the dietary effects of vitamins and lipids on skeletal deformities are currently a major focus. It has been shown that gilthead sea bream larvae were very sensitive to enhanced vitamin A during rotifer feeding which lead to various types of skeletal deformity such as cranial, vertebral and the caudal fin complex. Other workers found a vitamin A dose response in 4-20dph sea bream larvae on juvenile cranium malformation while increasing levels of this vitamin in 20-34dph larvae resulted in higher skeletal deformity in later development. A recent study tested the effect of feeding ratios of phosphatidylcholine (PC) and phosphatidylinositol (PI) to 20-34dph gilthead sea bream larvae on performance in 141dph juveniles. Increasing the PC/PI ratio increased jaw deformity in 67dph fish, which may have interfered with feeding on a dry, hard starter feed resulting in significantly reduced growth. In addition, osteocalcin mRNA levels showed a strong correlation with development in fish fed the high PI diet that conceivably contributed to normal bone growth.

These selected studies have justifiably focused on larval ontogeny as a major genesis of skeletal deformity that appears later on in development. Moreover, they form the basis for future research avenues which will lead to increased productivity and efficiency of the aquaculture industry.

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COMPARATIVE REARING OF THREE LARVAL LABYRINTH FISHES (*BETTA SPLENDENS*, *COLISA LALIA*, AND *MACROPODUS OPERCULARIS*) UNDER CONTROLLED CONDITIONS

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Introduction

The world ornamental fish trade is conducive to global proliferation of pathogens (Whittington and Chong, 2007). Labyrinth fish are known as hosts for danger pathogens like iridovirus (DGIV) (Gibson-Kueh et al., 2003), and *Mycobacterium marinum*, which may cause also human infection (Decostere et al., 2004). Promoting local breeding based on modern reproduction methods could limit the mass import of exotics from Asia and reduce the risk of spreading of transboundary diseases. The aim of the work was the attempt to optimize the rearing of larval labyrinth fish species: dwarf gourami (*Colisa lalia*), paradise fish (*Macropodus opercularis*), and Siamese fighting fish (*Betta splendens*) under controlled conditions.

Materials and methods

The reared larvae were obtained from natural spawning in aquaria (three pairs of each species). The rearing was carried out in three replicates in densities of 50, 100, and 150.l^{-1}. Due to the time differences between individual pairs reproducing, the larvae acquired from them were reared separately, in appropriate density. The rearing of the larvae was conducted in a device designed specifically for this purpose. The device consisted of one glass 50-l tank, acting as a water bath, as well as smaller 1-l tanks. In each of the smaller tanks half of one of the wall's length was replaced with flour mesh with the mesh's side length of 200µm, which allowed constant circulation of water between the water bath and the smaller tanks. Water temperature was identical (27±0.1°C) for all reared larvae. Amount of the dissolved oxygen in the water oscillated between 61.0 and 80.0%, the concentration of ammonium nitrogen was between 0.01 and 0.05mg.l^{-1}, and the pH was between 8.30 and 8.65. Larvae were fed four times a day ad libitum with live *Artemia* nauplii. The photoperiod was set to 12 hours of lighting and 12 hours of darkness per day.

The rearing began with the active swimming (3rd day from hatching) and lasted to the disappearance of the finfold. The duration of the experiment included whole larval development (Krejszeff, 2008), and lasted 17 days for paradise fish and Siamese fighting fish, and 27 days for dwarf gourami.

The initial and final total lengths of 10 live fish from each tank were measured. The acquired data were used to calculate the specific growth rates (SGR) (Brown, 1957), the relative growth rates (RGR) (Myszkowski, 1997), and the increments in total length (ITL) (Penáz et al., 1989). Significant differences in the total length of the larvae at the last day of rearing were determined by post-hoc analysis (Tukey test). Differences in survival rate of the larvae at the last day of the rearing were established by a two-proportion's test.

Results and discussion

Larvae of dwarf gourami, reached the maximal total length at the lowest density of $50.l^{-1}$. With increased density, the larval size decreased. The average length of larvae (13.8±2.1mm) reared at the density of $100.l^{-1}$ did not significantly differed from the average length of fish from densities of 50 and 150 specimens per dm^3 (14.9±1.4mm and 13.5±2.2mm, respectively). However, the average lengths of the larvae from extreme densities of 50 and $150.l^{-1}$ were significantly different (Table I). The highest survival rate (44.0±8.2%) was at the highest density. Rearing of the larvae in smaller densities resulted in the decrease of the survival rate to 35.7±6.4% at a density of $100.l^{-1}$, and to 30.0±6.0% at a density of $50.l^{-1}$. But these values were not significantly different among densities (Table I). The best values for ITL and RGR were obtained for rearing at the density of $50.l^{-1}$ (0.43 and 5.99, respectively) (Table I).

In paradise fish, the biggest total length of larvae (15.6±1.0mm) was also observed at the density of $50.l^{-1}$. There was not significant difference between size of larvae from densities of 100 and $150.l^{-1}$ (14.3±1.6mm and 13.8±1.4mm, respectively) (Table I). The results of the survival rate were reverse, as in dwarf gourami. The highest survival rate (97.3±3.1%) was observed in the larvae reared at the lowest density. Rearing of the larvae in higher densities resulted in the decrease of the survival rate to 96.7±1.5% in the density of 100 specimens per dm^3, and to 96.7±2.0% in the density of 150 specimens per dm^3. These values were not significantly different between densities (Table I). Similarly as in dwarf gourami, the best values for ITL and RGR were observed in larvae from density of $50.l^{-1}$ (0.71 and 9.27, respectively) (Table I).

The total length of Siamese fighting fish larvae were similar to these observed in paradise fish. The maximal length (15.0±1.4mm) reached larvae reared in the lowest density. This size was significantly different from the lengths observed in densities of 100 and $150.l^{-1}$ (13.3±1.2mm and 13.2±1.7mm, respectively) (Table

I). Survival rates at the end of rearing were not significantly different (Table I). Similarly to the other two species, the highest values for ITL and RGR were observed in larvae reared at density of 50.l^{-1} (0.64 and 7.82, respectively) (Table I).

Table I. The results of rearing of three labyrinth fish larvae in different densities (mean ± SD). Data in rows marked with the same letter did not differ significantly.

Parameter	Density (specimens per l)		
	50	100	150
Dwarf gourami			
Initial TL [mm]	3.09±0.06^{a}	3.09±0.06^{a}	3.09±0.06^{a}
Final TL [mm]	14.87±1.39^{a}	13.82±2.15^{ab}	13.50±2.17^{b}
Survival [%]	30.0±6.0^{a}	35.7±6.4^{a}	44.0±8.2^{a}
ITL [mm · d^{-1}]	0.43	0.40	0.39
RGR [% · d^{-1}]	5.99	5.70	5.58
Paradise fish			
Initial TL [mm]	3.46±0.11^{a}	3.46±0.11^{a}	3.46±0.11^{a}
Final TL [mm]	15.63±0.99^{a}	14.31±1.65^{b}	13.77±1.37^{b}
Survival [%]	97.3±3.1^{a}	96.7±1.5^{a}	96.7±2.0^{a}
ITL [mm · d^{-1}]	0.71	0.64	0.61
RGR [% · d^{-1}]	9.27	8.70	8.45
Siamese fighting fish			
Initial TL [mm]	4.16±0.14^{a}	4.16±0.14^{a}	4.16±0.14^{a}
Final TL [mm]	14.99±1.45^{a}	13.35±1.24^{b}	13.23±1.71^{b}
Survival [%]	92.0±8.0^{a}	91.7±8.0^{a}	89.5±6.3^{a}
ITL [mm · d^{-1}]	0.64	0.54	0.54
RGR [% · d^{-1}]	7.82	7.10	7.04

Conclusions

The highest growth rate among the larvae of the tested labyrinth fish species was obtained for the density of 50.l^{-1}. Such density also resulted in the best survival rate for paradise fish and Siamese fighting fish, and thus can be recommended as the most effective in the case of mass rearing of larvae of those species.

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THE INFLUENCE OF STOCKING DENSITY ON SURVIVAL AND GROWTH OF DACE *LEUCISCUS LEUCISCUS* (L.) LARVAE REARED UNDER LABORATORY CONDITIONS

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Introduction

Dace *Leuciscus leuciscus* (L.) is characteristic ichthyofaunal cyprinid species of the rivers across Europe. With other cyprinids included in the group of rheophilic fish, they are one of the most sensitive to changes in the environment that stem primarily from constructions on rivers and pollution. In case of intensive rearing of larvae and production of stocking material, density is one of the key factors influencing effectiveness of rearing, particularly in case when water recirculation systems are used. This study aimed at determining the effect of the stocking density on the growth rate and survival of dace during initial rearing under controlled conditions.

Materials and methods

Experimental larval dace were obtained from artificial reproduction conducted at the hatchery of the Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn (Kujawa 2004).

The rearing of the larvae was carried out in a customized system consisting of one large glass 50-l tank functioning as the water bath with 16 smaller 1-l tanks each submerged in it. The bath was equipped with a controllable heater adjusting water temperature with an accuracy of up to ±0.1°C, fluorescent lighting, and an aeration system. To ensure the most effective water exchange and prevent food leakage in each of the small tanks, one of the walls was substituted with fine (200μm) mesh. Additionally, each of the small tanks was equipped with top inflow (6-10 $l.h^{-1}$) of filtered water. Biological and mechanical filtration for the entire system was provided by an external filter (Fluval 400) and 30% of water was replaced at the same temperature daily.

The experiment started when the fish had resorbed 2/3 of the yolk sac and started intake of exogenous food and it ended after 21 days of rearing. Water temperature during rearing was constant at 25.0°C. The dissolved oxygen content did not drop below 85% saturation, ammonia was not detected, and the pH ranged between 7.9 and 8.5. The photoperiod during the entire rearing period was constant (12L:12D). The larvae were fed 3 times a day (8.00; 12.00 and 16.00) ad libitum with live *Artemia* nauplii. Rearing of the larvae was conducted at eight densities of 50; 100; 150; 200, 250; 300; 350 and 400 individuals per l (L50, L100, L50, L200, L250, L300, L350, and L400, respectively). The experiments were carried out in duplicate.

Larvae samples were collected every 7 days and the total length of 30 random selected individuals from each experimental variant was measured (±0.1mm). Fishes for measurement were anaesthetized in 2-phenoxyethanol ($0.4g.l^{-1}$). They were then transferred back to the tanks from which they were collected. The weight of individuals (±0.1mg) was determined at the beginning and at the end of the experiment. Photographic documentation and length measurements conducted on its base were done on the basis of computer software Dp-Soft (Olympus, Japan). The measurements were used for calculations of the relative individual growth rate in weight (RGR_W) and the length growth in the time unit (ITL). Statistical differences between groups in length and weight of larvae were analyzed by applying the variance analysis (ANOVA) and Tukey's post hoc test (α=0.05). Survival rate of the larvae observed on the last day of the rearing was established by two-proportion test.

Results and discussion

Significant differences between groups in the average total length of the fishes were observed after the first week of rearing, when individuals in group L50 until the end of the experiment were characterized by a significantly larger total length than those from the other experimental groups (Fig. 1). The same correlation was observed frequently during rearing of numerous other species (Kupren et al. 2008; Żarski et al. 2009). The average final weight of fishes in that group was 79.26mg, with the average length of 23.27mm. The sizes of fishes originating from the groups L200-L400 were very similar and did not show statistical significance. At the end of the experiment their total length was within the range of 16.18-17.43mm, and the weight 23.76-33.53mg. The relative weight growth rate ranged from 12.91 (L400), to 19.58 (L50) (Table I). During the experiment the larvae survival rate was very high and similar in all the experimental groups ranging from 89.0 to 93.6%. The highest larvae mortality was recorded during the two initial weeks of rearing (Fig. 2).

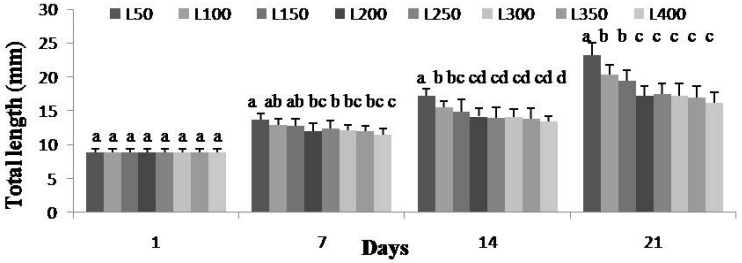

Fig. 1. Mean (± SE) length of larval dace, *Leuciscus leuciscus* (L.) during rearing. Data with the same letter index do not differ significantly (P<0.05).

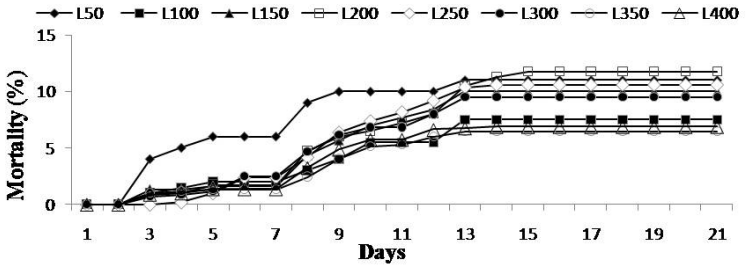

Fig. 2. Cumulative mortality of larval dace, *Leuciscus leuciscus* (L.) during rearing under controlled conditions.

Table I. Initial and final characteristics of the rearing of dace, *Leuciscus leuciscus* (L.) (mean±SD)

Parameter	Stocking density (ind.dm^{-3})							
	50	100	150	200	250	300	350	400
Mean initial weight (mg)*	1.85 ± 0.38 ^{a}	1.85 ± 0.38 ^{a}	1.85 ± 0.38 ^{a}	1.85 ± 0.38 ^{a}	1.85 ± 0.38 ^{a}	1.85 ± 0.38 ^{a}	1.85 ± 0.38 ^{a}	1.85 ± 0.38 ^{a}
Mean final weight (mg)*	79.26 ± 17.66 ^{a}	52.60 ± 13.44 ^{b}	44.43 ± 12.26 ^{bc}	33.23± 9.70 ^{cd}	31.77 ± 10.91 ^{d}	33.53 ± 13.99 ^{cd}	29.78 ± 9.60 ^{d}	23.76 ± 8.70 ^{cd}
Mean initial length (mm)*	8.83 ± 0.56 ^{a}	8.83 ± 0.56 ^{a}	8.83 ± 0.56 ^{a}	8.83 ± 0.56 ^{a}	8.83 ± 0.56 ^{a}	8.83 ± 0.56 ^{a}	8.83 ± 0.56 ^{a}	8.83 ± 0.56 ^{a}
Mean final length (mm)*	23.27 ± 1.71 ^{a}	20.33 ± 1.40 ^{b}	19.42 ± 1.61 ^{b}	17.17 ± 1.53 ^{c}	17.43 ± 1.62 ^{c}	17.18 ± 1.89 ^{c}	16.95 ± 1.67 ^{c}	16.18 ± 1.61 ^{c}
Survival (%)*	89.0± 1.0 ^{ab}	92.5± 1.5 ^{ab}	90.0± 2.0 ^{ab}	88.3± 1.8 ^{b}	89.4± 1.4 ^{ab}	90.5± 1.2 ^{ab}	93.6± 0.1 ^{a}	93.1± 0.9 ^{ab}
Index of incremental total length (ITL) (mm.d^{-1})	0.69± 0.01	0.55 ± 0.00	0.50± 0.00	0.40± 0.00	0.41± 0.01	0.40± 0.01	0.39± 0.00	0.35± 0.00
Relative growth rate in weight (RGR_W) (%.d^{-1})	19.58± 0.09	17.26± 0.23	16.32± 0.30	14.73± 0.19	14.49± 0.03	14.78± 0.04	14.13± 0.08	12.91± 0.11
Biomass (g.dm^{-3})	3.53± 0.10	4.86 ± 0.12	6.01 ± 0.46	5.86 ± 0.08	7.10 ± 0.15	9.10 ± 0.19	9.75 ± 0.12	8.85 ± 0.27

*Data in the same row with different superscripts are significantly different (P<0.05)

The accumulation of mortality at a similar time i.e. during the initial days of life was probably caused by mortality of larvae that did not start exogenous feeding. Similar observations were made by Kujawa (2004) and Kwiatkowski et al. (2008). ITL ranged from 0.35mm (L400) to 0.69mm (L50). After three weeks of dace larvae rearing, in group L350 the highest biomass was obtained (9.75g.l^{-1}), which was almost 3 times higher than in group L50 (3.53g.l^{-1}) (Table I).

Conclusion

The results show that if optimal environmental conditions and satisfactory food are available, rearing of dace can be successfully carried out at a density reaching 400 individuals.l^{-1}. High densities had no significant influence on survival and obtaining a larger number of larvae from one volume unit compensates for a slower growth rate.

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APPLICATION OF NATURAL STABLE ISOTOPES IN LARVAL NUTRITION STUDIES

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Stable isotopes are non-hazardous, non-invasive markers that can be used to determine the contribution of dietary sources to growth in individuals or at the population level. Where different food items have different isotopic signatures and different isotopes of the same element are incorporated in tissues at different rates, they can be used to infer trophic linkages, providing an integration of feeding over time and mixing and mass balance models can be used to estimate the relative contribution of different food sources. The use of bulk stable isotope ratios, particularly for C and N, as natural tracers has been a widely-used and powerful tool in assessing energy flow within aquatic ecosystems. In hatchery systems, which represent highly-controlled mesocosms with a limited number of food sources and short planktonic food chains, naturally occurring stable isotopes in live plankton have been used to investigate the sources and fate of dietary nutrients and to assess tissue carbon and nitrogen turnover rates. This has been also proved a useful approach to investigation of utilisation of inert diets in co-feeding and early weaning studies. After a consuming organism has reached isotopic equilibrium with its diet, a difference in isotope values between diet and consumer is frequently observed. Nitrogen discrimination factors ($\Delta^{15}N$) have been linked to formulated diet quality and protein content.

A relatively few studies have attempted to use diet-consumer relationships in terms of naturally-occurring stable isotopes to investigate the relative utilisation of components of formulated compound diets; for example, recent work has indicated a relatively low contribution of soy-protein to tissue growth in shrimp post-larvae when used to replace fishmeal. However, investigation of mechanisms underlying such observations requires application of techniques for tracing routing of dietary sources of carbon and nitrogen into individual amino-acids. Very high ranges of up to 20‰ have been observed for both $\delta^{15}N$ and $\delta^{13}C$ in amino-acids in a wide range of plant, algal and animal species. Separation of amino-acids and analysis of their individual stable isotope ratios has been applied in experimental ecological studies, with differences in $\Delta^{15}N$ and $\Delta^{13}C$ for individual amino acids being observed. This suggests that the historical use of

bulk isotope ratio analysis has tended to mask more informative effects at the single amino acid level. In the few aquatic examples to date, data for zooplankton suggests that those amino acids which show large $\Delta^{15}N$ from diet to consumer may be better indicators of trophic level than bulk values, while those which are more conservative may provide insight into dietary sources. In insects, differences in $\Delta^{13}C$ between amino-acids has been used to indicate sources of carbon, with putative essential amino-acids in consumers remaining close to dietary amino acid values, while putative non-essential amino acids reflect the $\delta^{13}C$ values of dietary carbohydrate sources. Thus compound specific stable isotopic composition of diet and consumer amino-acids has potential to be a useful tracer for investigation of dietary requirements for amino-acids in aquatic organisms, evaluation of dietary ingredients and routing of nutrients from different components of the diet.

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IMPORTANCE OF BROODSTOCK DIET COMPOSITION ON THE SPAWNING RESULT IN ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS* L.)

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Introduction

The Atlantic halibut (*Hippoglossus hippoglossus* L.) live for decades in the wild and mature halibut spawn repeatedly for many years. During each spawning season the halibut undergo a natural fasting period of 1-2 months. During this period body reserves are utilized for maintenance and for final production of eggs and sperm. To maintain their fecundity and the eggs and milt quality the fish need to restore the body reserves that are lost during each spawning season. In captive brood stocks it has been observed that only a few females produce high quality eggs. Although there are no individual recordings of feed intake in commercial stocks, a large variation in appetite has been observed indicating that the nutritional status of individual fish may influence the fecundity and egg quality. The main purpose of this study was to determine the effect of feed composition with regard to macro nutrients and energy content on the spawning performance.

To establish the reduction in body burden of different nutrients during the fasting period, the loss in energy, protein, lipids and fatty acids in different organs of mature Atlantic halibut males were recorded during a fasting period of two months. The energy and protein loss during these 2 months was 24.7% and 14.3% of initial content respectively. There was considerable reduction in content of the fatty acids DHA (43.5%) and EPA (39.9%) during the same period.

Material and methods

The results from the fasting experiment were used in the design of three experimental brood stock diets containing 15 (LL), 25 (ML), and 35(HL) % of lipids respectively (Table I). These diets were tested over a period of 3.5 years covering 3 spawning seasons. Halibut brood stock (5 females and 3 males per tank) was reared in three 10M diameter outdoor concrete tanks covered by tents. The water temperature was maintained at 6°C from 2 months prior to spawning and during the spawning period. Between the spawning seasons the temperatures were kept <10°C except for the first 5 months when the temperature occasion-

ally reached 13°C. The fish were held under artificial light at a natural light cycle. The experimental diets were prepared by mixing a dry premix with water and a high quality fish oil rich in n-3. The final mix was prepared as sausages which were hand fed to individually tagged brood fish.

Table I. Feed composition

Content per kg DW	Energy (MJ)	Protein (g)	Lipid (g)	n-3 (g)	Starch (g)	Protein/ energy
Feed 1	22.9	639	157	18	100	27.9
Feed 2	24.5	539	250	37	90	22
Feed 3	26.8	455	356	61	75	17

Results and discussion

The fish were weighed at initiation of the project in July 2005 and thereafter twice each year.

Recordings of the feed intake showed that the energy intake was similar in all experimental groups regardless of energy content in the feed. The weight increase during the experimental period was also similar in all groups. Because the fish fed to cover their energy needs, there might be an unbalance in other nutrient. The protein intake exceeded the estimated needs in all groups, especially in the group fed the low lipid diet (LL). The intake of n-3 fatty acids was lower than the estimated needs in fish fed diet LL and exceeded the estimated needs in the HL group.

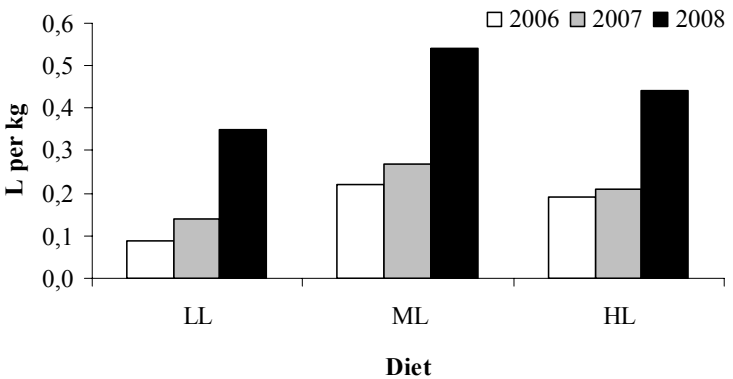

Fig. 1. Increase in production of eggs (L) per kg fish in for halibut fed experimental brood stock diets containing 15 (LL), 25 (ML) or 35 (ML) % of lipids respectively during a 3 year experimental period.

During the first spawning season in 2006 the fecundity of the fish in all the three groups was low compared to commercial production. This could be due to the

fact that the fish were fed a commercial growth diet until July 2005 and therefore did not get sufficient supply of nutrients for optimal egg production. The egg production increased in all the three experimental groups of the following two spawning seasons. The trend was that the production of eggs per kg fish was much higher in fish fed diets ML or HL. In the LL group one or two fish did not spawn at all each season (Table II). In addition the number of egg batches per spawning season and the size of each batch was smaller in the LL group compared to the two other groups. The fish fed diet ML produced more eggs per unit protein and energy eaten compared to fish fed diets LL and HL.

Table II. Mean spawning success for females over a period of 3 years (2006-2008).

	Feed 1	**Feed 2**	**Feed 3**	**ANOVA p-value**
Amount of eggs (l per fish)	4.87 ± 2.3	7.85 ± 2.7	6.67 ± 2.3	0.70
Numbers of spawnings	4.70 ± 0.5 (ab)	9.50 ± 1.7 (a)	6.60 ± 1.0 (b)	0.06
l of eggs per kg fish	0.25 ± 0.1	0.38 ± 0.1	0.31 ± 0.1	0.56
kg (MJ) eaten per l eggs *				
Dry matter	2.93 ±1.6	1.22 ± 0.3	1.53 ± 0.5	0.51
Protein	1.78 ± 0.9	0.66 ± 0.2	0.71 ± 0.3	0.41
Energy	0.07 ± 0.04	0.03 ± 0.01	0.04 ± 0.01	0.56

* Data from 2007 and 2008 which had a full year of feed intake before the spawning season.

The chemical content of the eggs were not significantly affected by diet. Chemical analysis of eggs after 3 years of feeding on the three experimental diets showed very small differences in energy (MJ) or protein (%). The relative egg content of total n-3 fatty acids and DHA and EPA was also similar for all experimental groups.

Conclusions

Feed composition affected amount of eggs produced per kg fish, and number and size of batches per female per season. The chemical content of eggs was not affected by diet composition. The fish fed to cover their energy needs which might result in an unbalance in other nutrients.

Acknowledgements

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INFLUENCE OF INITIAL FEEDING ON MUSCLE GROWTH AND THE EXPRESSION OF MYOGENIC REGULATORY FACTORS IN PACU *PIARACTUS MESOPOTAMICUS* LARVAE

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Teleosts are found in various environments that generally offer a wide range of ambient conditions. Individually and as a set, these variables affect fish physiology and growth. Several studies have shown that the growth and development of tropical fish larvae are affected by diet quality and quantity. The reduction of fish growth as a function of feeding strategy and food quality implies a reduction in the striated skeletal muscle that accounts for at least 60% of body mass in the majority of teleosts. This abundant muscular mass, beyond representing important tissue for adaptation in the aquatic environment, is also the most valued portion of the fish for human consumption.

Muscle growth is positively and negatively regulated by a variety of transcription and growth factors. The myogenic regulatory factors (MRFs) are transcription factors that share a highly conserved domain, *basic-helix-loop-helix* (bHLH). The MRFs recognize, through their basic domain, a consensus sequence in DNA known as E-box, which is found in the majority of skeletal muscle-specific genes. The HLH region of MRFs is the domain linking of this molecule with E proteins. The link of MRF-protein E to the E-box sequence activates the transcription of muscle-specific genes, inducing their expression. Moreover, this interaction can initiate the transcription of the MRF genes during muscle growth. Among the MRFs, MyoD and Myf5 are primary factors and are expressed in myoblasts during the cellular proliferation phase; in contrast, Myogenin and MRF4 are expressed in cells during the fusion and differentiation phases.

Post-embryonic muscular growth involves myogenic progenitors cells (MPCs). These cells are directly affected by abiotic factors (e.g., temperature, photoperiod, hypoxia) and biotic factors (e.g., food availability and parasitic infections). These factors affect the regulation of proliferation and differentiation, protein degradation and synthesis, and gene expression. Temperature has been demonstrated to affect MyoD and Myogenin expression as well as the number and size

of muscular fibers, whereas feeding can affect the proliferation of these cells. Diets supplemented with ascorbic acid increase MPC proliferation and differentiation, whereas MPCs extracted from starved fish were different in terms of size, morphology, and proliferation rate in comparison to those from fed fish. Other variables, such as exercise, can influence MRF expression in fish.

MRF expression levels are related to myoblast hyperplasia and hypertrophy during growth. Knowledge regarding MRF expression can indicate strategies that benefit the development and muscular growth of fish species that are commercially cultured. Thus, clarification of the mechanisms that control muscular growth is important to aquaculture. The hypothesis of this study was that the mechanisms of hyperplasic and hypertrophic growth of muscle fibers and the expression of MRFs (MyoD and Myogenin) in pacu *Piaractus mesopotamicus* larvae are influenced by feeding, because larval growth is affected by the diet quality or feeding strategy that they receive during the initial feeding.

Pacu larvae (4dph) were fed *Artemia* nauplii or formulated diets as a total or partial substitute for *Artemia* nauplii in the first weeks of life. Performance (weight, length, and survival) and muscular growth (morphometry of skeletal muscle fibers) were evaluated. In the muscle of pacu larvae fed *Artemia* nauplii or formulated diets as a partial substitute for *Artemia* nauplii, we also evaluated the expression of the MRFs MyoD and Myogenin. The results of these analyses were compared with hyperplasic and hypertrophic muscle growth.

Artemia nauplii improve pacu larvae growth and produce bigger fish upon completion of the experiment. However, the morphologic, morphometric, and molecular results showed that the substitution of *Artemia* nauplii for formulated diets did not negatively affect the muscle development due to intense muscular hyperplasia verified in the larvae of these groups. These results open perspectives of investigation regarding both posterior compensatory growth and the use of more efficient and economic feeding strategies in fish commercial production.

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CLOSING THE LIFE CYCLE OF *OCTOPUS VULGARIS* IN CAPTIVITY

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A lot of interest is going nowadays to the attempt to reproduce *Octopus vulgaris* under captive conditions, as a new species for aquaculture. Known as a fast growing species and much appreciated for human consumption, makes the Octopus an ideal candidate for research with the aim of closing the complete life cycle under controlled rearing conditions. The recruitment of juveniles from the wild and their on-growing in captivity up to maturation, already reached a good and well known standard, as well as the maturation, the reproduction and the deposition of eggs. It is not difficult anymore to obtain a lot of viable paralarvae, hatched out at a high hatching rate (>98%) of numerous eggs (30 000 to 200 000 eggs per female). For a long time, the longest life span of an Octopus reared under captive conditions from hatch remained around 60-70 days, even if high survival rates were already obtained until 40 days post hatch (>30%). Only lately, a few individuals survived for more than 3 months and 1 individual arrived at 5 months, when the juveniles were demonstrating the feeding and swimming behaviour comparable to adults. This work presents the different steps and milestones achieved in the Octopus research work that has been carried out at Maricoltura di Rosignano Solvay, Italy between 2002 and 2008, and co-financed by ARSIA (Regional Agency for Development in Agriculture).

The establishment of a numerous and good quality broodstock is the basis for any further research on paralarval and larval rearing of the Octopus. For this research, collaboration was done with professional fishermen whom provided Octopus juveniles of similar size (few hundred grams) during fall. The broodstock was kept during winter season, following the natural photo-thermo period in order to obtain fecundation and afterwards deposition of egg masses by the end of spring time, start of summer.

Paralarval rearing has been evaluated in different rearing volumes ranging between 100 and 6000 l. Different stocking densities of paralarvae were compared, varying between 3 and 35ind.l^{-1}. During the first 20 days, the survival was not influenced by the stocking density, later on differences were observed. The best survival rates were obtained in the larger volumes (1000 and 6000-l tanks).

Multiple trials have proven the superior growth and survival using green water technique (use of micro-algae) compared to clear water. The role of the algae could be due to a combined bacteriostatic effect and the diffusion of light, next to a nutritional role (direct and indirect). Two types of algae were compared (*Isochrysis* spp. and *Nannochloropsis* spp.) obtaining similar results.

Many feed trials were performed including rotifers, *Artemia* nauplii, meta-nauplii and adults, mysids, and natural plankton (copepods). Feeding a combination of different stages of *Artemia***,** all enriched in HUFA, vitamins and minerals (INVE enrichment products) seems to be the most appropriate live prey for the paralarvae. No increase in survival was obtained including wild zooplankton as live prey. Different artificial diets (moist, semi-moist and dry) were supplied but a clear ingestion (microscopically observed) was never observed, even if the paralarvae were attracted to the artificial diets.

The survival during the planktonic stage has increased considerably compared to the start of the research, when no individuals were obtained older than 25 days. A first big improvement increased the survival rate to around 30% at 40 days post hatch, with few survivors reaching the age of 70-75 days. During the last year of research, for the first time several individuals reached the benthic stage and started to demonstrate the adult behaviour. These individuals were also weaned on frozen crab and mussels. The oldest individual reached 5.5 months and died most probably due to high stress conditions.

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METAMORPHOSIS AND SURVIVAL OF TRIPLOID SHRIMP LARVAE AND ITS POTENTIAL RELATIONS TO THE EXPRESSION OF AMP AND STRESS-RELATED PROTEIN GENES

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Introduction

Triploid induction is an alternative way to improve the production of cultured animals. Triploid Chinese shrimp *Fennerapenaeus chinensis* was successfully produced by heat shock. The present work intended to compare the survival rates at larval stages between triploids and diploids, and the response of juvenile shrimp to high temperature and salinity variation. The expression profiles of antimicrobial peptides (AMP) and stress related genes were analyzed and tried to find the relations between the survival and the expression of these genes.

Materials and methods

Triploids were produced and reared in the aquarium of our institute as described previously (Li et al., 2006). Ploidy detection was performed by flow cytometry techniques at embryo stages, larval stages and juvenile stages according to the method described by Li et al. (2003).

Triploid and diploid juvenile shrimp with body length of 4.8±0.5cm were separated from one triploid population by flow cytometry. Four groups include diploids at normal temperature (20-23ºC) (2nNT), triploids at normal temperature (20-23ºC) (3nNT), diploids at high temperature (32ºC) (2nNT), and triploids at high temperature (32ºC) (3nNT) were set after they were acclimatized for 7 days and their mortality rates were recorded.

Triploid and diploid shrimp with body length of 6.4±0.8cm were used for salinity experiments. Four groups, 2n-NS (diploid at normal salinity 32ppt), 3n-NS (triploid at normal salinity 32ppt), 2n-LS(diploid at low salinity 10ppt) and 3n-LS (triploid at low salinity 10ppt) were set. The mortality rates in these four groups were recorded.

Real time RT-PCR was used to analyze the transcription levels of AMPs and stress related genes at embryo and larval stages of triploids and diploids. The detailed methods and data analysis are as the same as those described previously (Li et al., 2009).

Results and discussions

At the larval stage from nauplii to post-larvae, heat shocks lowered the survival at larval stages even if the ploidy was not changed. Ploidy did not affect the shrimp larvae survival.

Comparison of survival rates between triploids and diploids under different stresses were performed, and the data showed that the survival rate of diploid shrimp at high temperature is significantly higher than that of the diploids cultured at natural temperature (20-23ºC), but no difference was observed for triploids. So we inferred that triploids have the high ability to endure chronic high temperature (Fig. 1).

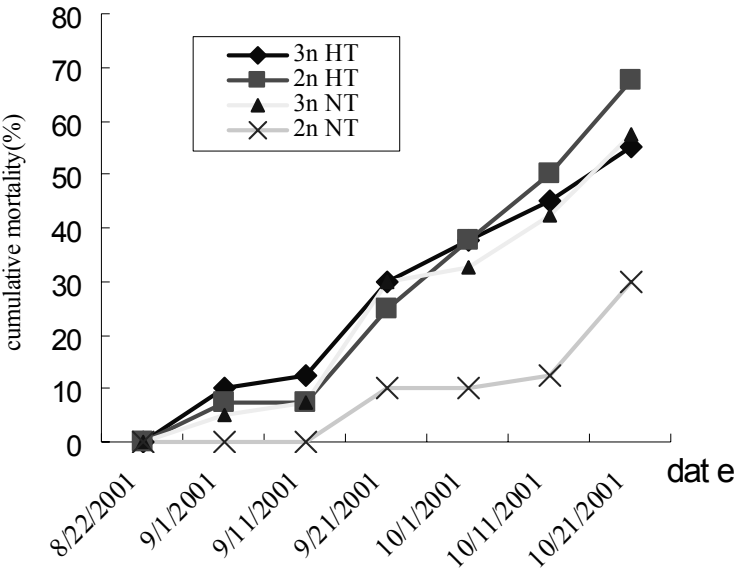

Fig. 1. Comparison of the cumulative mortality rates between triploid and diploid juvenile shrimp under chronic high temperature.

When the shrimp experienced abrupt salinity variations from 32 ppt to 10 ppt, the survival rate of triploid shrimp was not greatly affected, while that of diploid shrimp decreased.

Expression of AMP genes (Penaeidin, crustin) and some stress related genes (HSC70, HSP70, HSP90, Catalase, mMnSOD, cMnSOD) in different embryo stages and larvae stages were analyzed. The data showed that some genes showed higher expression level in triploid embryos or larvae than those in diploid embryos or larvae (Fig. 2). The different expression of these genes might contribute to different survival rates between triploid and diploid shrimp response to temperature and salinity stress.

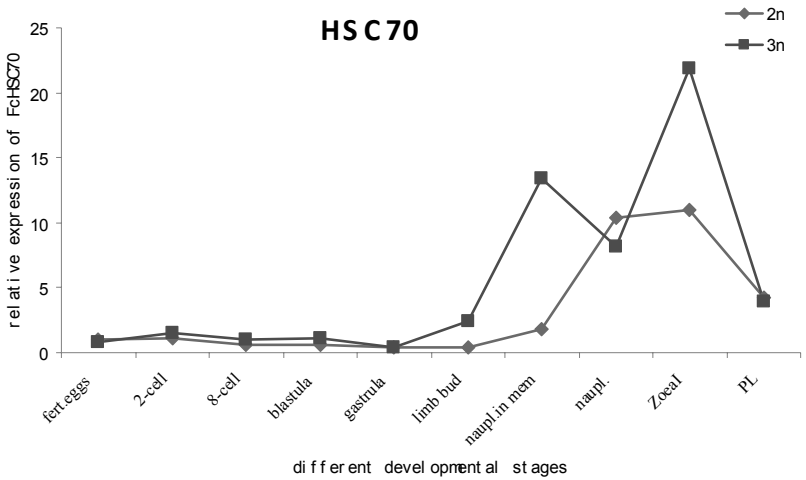

Fig. 2. Relative expression of HSC70 genes at different developmental stages.

Conclusions

Triploid Chinese shrimp larvae showed similar survival rate with their diploid siblings experienced the same treatment. Triploid juvenile shrimp had higher endurance to the chronic high temperature and salinity variations than diploids. The difference between triploid and diploids might be related to the different expressions of some AMP and some stress related genes.

Acknowlegements

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JAW DEFORMITIES OF CULTURED ORANGE-SPOTTED GROUPER (*EPINEPHELUS COIOIDES*) LARVAE IN RESPONSE TO DIFFERENTLY ENRICHED *ARTEMIA FRANCISCANA*

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Introduction

Many different aquaculture fish species were found to have skeletal deformities during larval stages in artificial rearing environment; the causative factors have been reported to be physical such as improper water temperature (Georgakopoulou et al., 2007) or biological including initial stocking density or rearing methodologies (Gimenez and Estevez, 2008; Boglione et al., 2009), the nutritional factors also played an important role in skeletal metabolism (Lall and Lewis-McCrea, 2007). The orange-spotted grouper, *Epinephelus coioides*, is one of the most important economic fish species in Taiwan, and the larval stage skeletal deformities will affect the value dramatically, in this study, the grouper larvae were fed with differently enriched *Artemia*, then the jaw deformities incidence were analyzed 40 days post hatching.

Materials and methods

Larva rearing

The fertilized orange-spotted grouper eggs were first disinfected with 20ppm iodine for 20 minutes, then transfer to hatching tanks filled with filtered seawater until 90% of the eggs were hatched, and then transferred to 1-m^3 circular fibreglass tanks at an initial density of 6 larvae.l^{-1}. During the rearing period, the water was treated by recirculation system and temperature maintained between 27-28°C; from day 4 to 25, *Nannochloropsis oculata* was added to the rearing tanks everyday to keep the cell density around 2 to 2.5×10^5 cells.ml^{-1}. Larvae were fed SS-type rotifers during day 4 to 7 at a density of 3 to 5 individuals.ml^{-1} and S-type rotifers during day 6 to 24 at a density of 3 to 7.5 individuals.ml^{-1}. From day 14 to 40, larvae were co-fed with newly hatched *Artemia* instar I nauplii and enriched instar II *Artemia*. Two different commercial enrichment products including (1) A1 DHA SELCO® or (2) SERRANI SELCO® (INVE, Ghent, Bel-

gium) were used to enrich *Artemia* according to the manufacturer's instruction. After 40 days, larvae were transferred to nursing tanks and fed by artificial feed.

Sample analysis

Larvae were observed and sampled every day, five standard measurement parameters including standard length, head length, snot length, upper jaw length, maxilla width and two additional measurement including length of eye to maxilla (from lower edge of the eye to the lower edge of maxilla) and length of lower jaw tip to upper jaw tip were measured under a binocular microscope for further analysis, deformity rate were recorded at 40 days post hatching.

Table I. Incidence of jaw deformity of orange-spotted grouper larvae fed with differently enriched *Artemia*.

	A1 DHA SELCO®	**SERRANI SELCO®**
Trial 1	0%	64.7%
Trial 2	0.1%	40.2%

Results

Two independent trials were performed under the same condition, at 19-20DPH, the mouthpart skeleton malformation was first observed on grouper larvae fed by *Artemia* nauplii which enriched with SERRANI SELCO®. At 40DPH, the frequency of larval jaw deformities of grouper larvae fed by *Artemia* nauplii which enriched with SERRANI SELCO® were 64.7% and 40.2%, compared to the A1 DHA SELCO® were 0% and 0.1% (Table I); most of the larval jaw malformations were caused by an abnormal maxilla curvature (Fig. 1), resulted in severe mouthpart deformities including downward curving of maxilla and elongated lower jaw compared to normal larvae at 40DPH (Fig. 2), these data showed that the nutrient contents in the SERRANI SELCO® may affect the larval skeleton development.

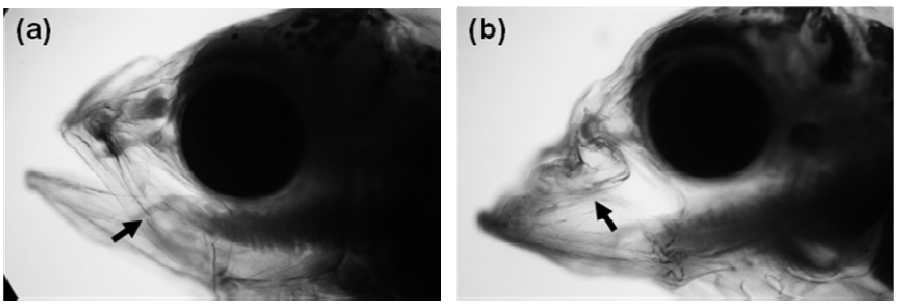

Fig. 1. Upper jaw skeleton deformity of 24-DPH orange-spotted grouper larvae fed with differently enriched *Artemia*. The maxilla (arrow) of SERRANI SELCO® group (b) showed abnormal curvature compare to A1 DHA SELCO® (a).

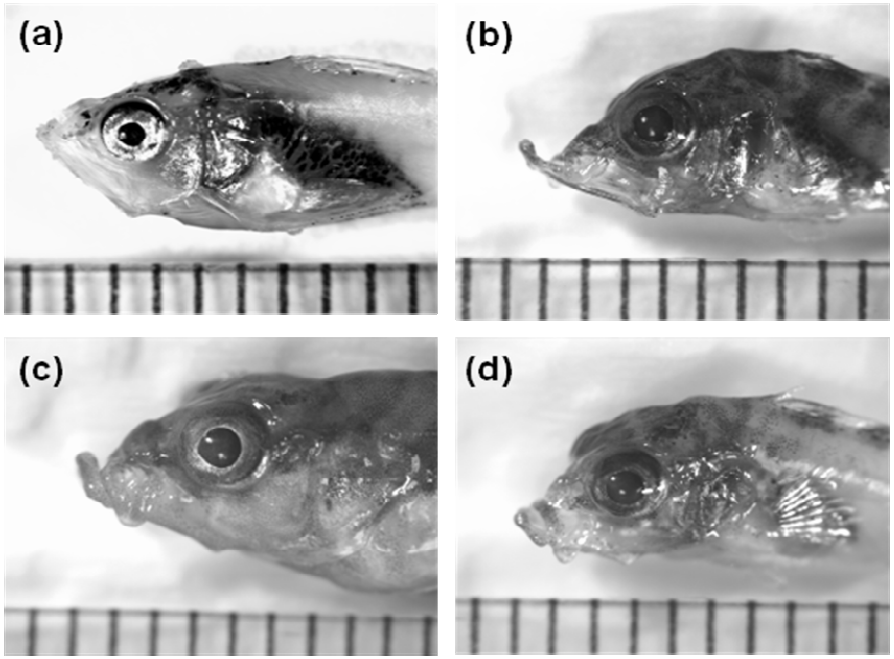

Fig. 2. Mouthpart deformities of 40 DPH orange-spotted grouper larvae fed with differently enriched *Artemia*. (a) A1 DHA SELCO® group, (b) SERRANI SELCO® group showed severe mouthpart deformities including downward curving of maxilla and elongated lower jaw. (Scale unit=1mm).

Discussion

Nagano et al. (2007) reported similar jaw deformities of larvae of seven-band grouper (*Epinephelus septemfasciatus*), and concluded several possible factors including different algae species used for greenwater, nutritional enrichment of live feeds and water temperature, in this study, both environmental and biological factors were well maintained, indicated that the nutrient contents or composition of SERRANI SELCO® may affect the larval skeleton development, a similar result was also observed in giant grouper (*E. lanceolatus*) suggested that the same mechanism could be found in other species of grouper (unpublished data). The further examination of actual causative factor is proceeding.

Acknowledgements

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UPTAKE AND METABOLISM OF PREDIGESTED LIPID IN THE ATLANTIC COD, *GADUS MORHUA*

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Introduction

Production of good quality juveniles depends on high quality diets. To achieve this, detailed studies of digestive efficiency, absorption, and metabolism are needed. In the present work we study absorption rates, digestive efficiency, and metabolism of intact and predigested lipids in larval Atlantic cod, *Gadus morhua* to see if cod has similar limitation in lipid digestion as Atlantic halibut (Mollan et al., 2008). This was done by quantification of differences in absorption of intact and pre-digested lipid after feeding diets containing ^{14}C-labeled lipids.

Materials and methods

The lipid free fraction of all diets was identical. Each diet was added 15% lipid, with a ratio of PC:TAG of 40:60 (Table I). The diets were added intact or hydrolyzed TAG or PC according to Table I. All the lipids in the diets, both labeled and unlabeled, contained only oleic acid (OA).

Table I. Lipid composition of the 4 diets.

Diet	TAG (60 %)	PC (40 %)
HTAG	Hydrolyzed ^{14}C	Intact
HPL	Intact	Hydrolyzed ^{14}C
TAG	Intact ^{14}C	Intact
PL	Intact	Intact ^{14}C

Commercial enzymes (Lipozyme and Phospholipase A_2) were used to hydrolyze TAG and PC.

Atlantic cod larvae 40 days post hatch (9.75±0.2mm and 4.53±0.323mg, length and weight, respectively) originating from Austevoll Aquaculture Research Sta-

tion, Storebø, Norway were acclimatized in a cold room at 10°C by feeding a non radioactive feed for two days prior to the experiment. The fish larvae were fed the radioactive labeled diets for 30min and then washed briefly in pure sea water, before transfer to an incubation chamber. Incubation and analysis of larvae were done in a HOT chase experimental set up according to Rønnestad et al. (2001) with replacement of tube feeding with natural feeding. The larvae were incubated for 5h. The fractions sampled and counted for radioactivity were: the incubation water, CO_2, the digestive tract, and the carcass. The relative (%) tracer distribution in each compartment was calculated from the count per min (cpm) for each compartment and the summed cpm.

Results and discussion

The results obtained from the present study show that after 5h incubation, cod larvae had digested and absorbed 70-80% of the dietary lipids. Fish fed the PL diet metabolized significantly lower fraction of the labeled lipid to CO_2 (70.9%) than groups fed HTAG, HPL, and TAG diets (80.5, 77.5, and 80.2% ^{14}C, respectively) (Fig. 1A). Gastrointestinal tract (Fig. 1B) and carcass (Fig. 1C) ^{14}C activity were significantly lower in fish fed HTAG than in groups fed the PL diet. The fecal evacuation (activity in the incubation water; Fig. 1D) was not affected by dietary treatment and ranged from 11.8% to 14.2%.

The highest net absorption (CO_2 trap and carcass, Fig. 2) was observed in the fish group fed with intact TAG (86.3%), which was significantly different from the fish fed the diet with intact PL (79.3%).

Lowest metabolized CO_2 and highest activity of ^{14}C in gastrointestinal tract and in carcass of the fish fed intact PL agreed with Krogdahl (2001), who states that PL is essential for chylomicon formation. PL may accumulate in the intestinal tract for use in chylomicron formation and further transport of TAG into the circulatory system. PL acts as natural emulsifier which helps in dispersion of lipid molecules in the gut and assists the larval digestion of lipids (Koven et al., 2001). The lower CO_2 production from the ^{14}C label in fish fed intact PL may be due to the fact that PC or polar lipid are less responsible for the production of energy, when compared to TAG. A possible explanation for better absorbed and faster absorption by HPL from gastrointestinal tract than intact PL might be associated with the presence of lyso-phospholipid and free fatty acid in HPL which may be absorbed rapidly than the normal intact PL, due to limited need for digestion.

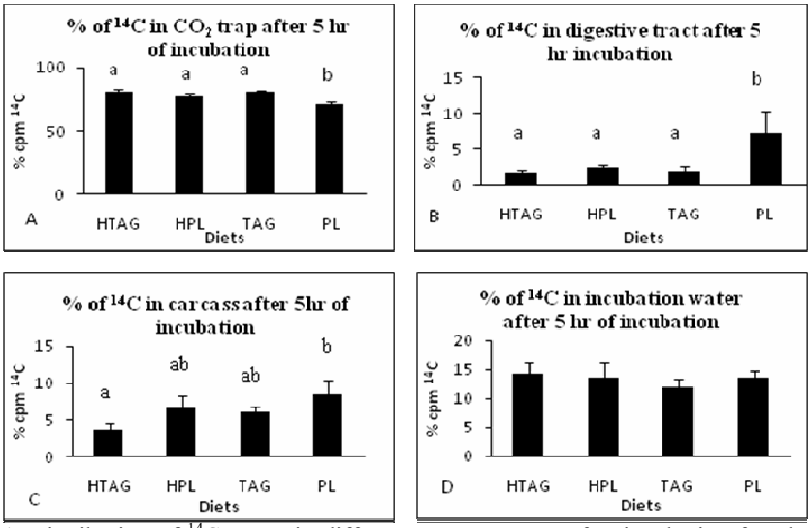

Fig. 1. Distribution of ^{14}C tracer in different compartments after incubation for 5h with 40 days post hatching Atlantic cod *Gadus morhua*. The different compartments were (A) CO_2 trap, (B) intestinal tract, (C) carcass, and (D) H_2O. All the values are average of 10 individual's larvae. $^{a-b}$ mean values with different superscript letters are significantly different ($P<0.05$).

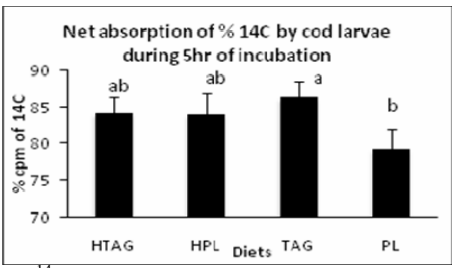

Fig. 2. Net absorption of ^{14}C by cod larvae after 5hr incubation. All the values are average of 10 individual's larvae. Treatment having the same letter(s) are not significantly different ($P<0.05$).

In the present study, there was a better digestion and absorption of lipid when compared with studies made with Atlantic halibut (*Hippoglossus hippoglossus*) (Mollan et al., 2008). In the previous work, a tube feeding was used to administer radio-labelled lipid MAG, DAG, TAG, PC, separately. The diets did not contain PL, which may be the limiting factor of lipoprotein synthesis, affecting the transport of labeled lipids from the gut and into the circulatory system, as hypothesized before in marine fish larvae (Haldas et al., 2003; Koven et al., 2001). This may be one reason for better absorption in this study than in Mollan et al. (2008). Other explanations may be species differences and that cod larvae in the present study were offered a complete diet under "natural" feeding conditions.

Conclusions

Cod larvae digested and absorbed 70-80% of dietary lipids 5 hours after ingestion whether hydrolyzed or intact when presented in a natural diet with 15% total lipid in the ratio of 40:60 between the PL and TAG. Most of the lipid was metabolized into CO_2, regardless of diet. The present study demonstrated that lipid is well digested and absorbed in cod larvae.

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INFLUENCE OF DIETARY ARACHIDONIC ACID COMBINED WITH LIGHT INTENSITY AND TANK BACKGROUND COLOUR ON PIGMENTATION OF COMMON SOLE (*SOLEA SOLEA* L.) LARVAE

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Introduction

The presence of abnormally pigmented fishes in flatfish culture is an unsolved problem. However, the mechanisms of colour anomaly in relation to external or internal processes are unknown.

Both nutritional as well as environmental factors may cause pigment defects in flatfish larvae and genetics may determine the larval sensitivity. High dietary arachidonic acid levels (20:4n-6, ARA) have been implicated in malpigmentation of various flat fishes. A hypothesis linked to ARA is that it causes suboptimal biochemical composition of membranes of neural tissues involved in the control of metamorphosis, melanin (black pigment) synthesis and chromatophore differentiation during early development.

Light has shown to play a significant role for the development of pigmentation in flatfishes (Denson and Smith, 1998). Inappropriate lighting such as complete darkness or strong lighting may be responsible for pigment abnormalities. The colour of the background may be another determining factor for the intensity or pattern of skin pigmentation (Van der Salm et al., 2004).

The aim of present study was to investigate possible physical and environmental pathway interactions on larval pigmentation by studying the combined effect of dietary arachidonic acid, light intensity as well as background colour.

Materials and methods

The experiment was performed in triplicate Plexiglas (acrylic) tanks randomly located in lightproof enclosures. (Table II). Ambient lighting was provided by overhead fluorescent lights (36W Lum Lux Plus cool white tubes) 24h.day^{-1} for 35 days. Maximal and minimum light intensities (lux) were recorded at the water surface by a digital illuminator. To obtain different tank background half the

plexiglass tanks were completely covered with an adhesive black foil (referred to as 'bl') and half of the tanks were left transparent (referred to as 'wh'). 12 tanks were kept at a low light intensity (max. 80-100lux surface illumination) of these 2 × 3 tanks were white and 2 × 3 were black. Similarly 12 tanks were kept at a high light intensity (3600-4500lux illumination, surface values); i.e., 2×3 tanks were white and 2×3 were black.

Approximately 1878 newly hatched larvae were stocked in each of 24 cylindri-conical 46-l tanks equalling a density of ~40 larvae.l^{-1}. The setup was in triplicate testing 2 diets, 2 light intensities and 2 background colours in a multifactorial design. Three-day-old first feeding sole larvae were fed enriched IH *Artemia* strain, 2.ml^{-1} until 11 days after hatching (dah). *Artemia* were either enriched by an emulsion of fish oil not supplemented with ARA oil, diet 1 or enriched by an emulsion of fish oil supplemented with 24% ARA oil (Vevodar oil from DSM Food Specialities BV, Holland (Table I). From 12dah until end of the trial all larvae in all tanks were fed EG *Artemia* enriched by commercial Super Selco emulsion. The average water temperature throughout the study was 18.2-18.6 ± 0.6 and flow was 8-10 $l.h^{-1}$. Ammonium, nitrite, and nitrate levels were below detection

Larval dry weight (d.w.) was measured at day 0, 11, and 20. Larvae were sampled on Whatman Ø 25 0.7-µm GF/F glass fibre filters. Samples were dried in an oven at 105°C for 24h and reweighed. At the same days replicate samples of larvae from each tank were collected for measurement of fatty content (10 pcs.$sample^{-1}$). Sampled larvae on filters were covered with nitrogen and frozen at -80°C in sterile 2-ml cryovials until extraction

The fatty acid composition was determined by extraction of lipids by a chloroform / methanol mixture. The fatty acid methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (Lund et al., 2008). Larvae were sampled for histological examinations of the skin pigment cells (i.e., development of melanophores) as well as melanin content. Larval samples for histology and pigment cell development (10 pcs.$tank^{-1}$) were fixed in Bouins fixative. Melanophores just dorsal to the lateral line at the level of the posterior edge of the visceral mass on the (later) dorsal side in an area of $1mm^2$ were counted and extrapolated to the number of melanophores on the whole larvae. Pigmentation was evaluated at 35dah and registered as either hypopigmented, hyperpigmented (i.e. blind side pigmentation) or normal pigmented. The results were analysed by a multiple factorial design in Sigma Stat.

Results and discussion

Larval tissue fatty acid composition of ARA was related to dietary treatment and therefore significantly highest ($P<0.001$) for larvae fed *Artemia* treated with

ARA oil (emulsion 2). When larvae were fed *Artemia* enriched with ARA oil (emulsion 2), it resulted in a highly significant higher level of hypopigmentation ($P<0.001$) than when larvae were fed *Artemia* enriched by a fish oil-based emulsion (emulsion 1) (Fig. 1). The degree of hypopigmentation at 35 days post hatch was related to dietary ARA level and not to light intensity or tank background colour. The proportion of hyperpigmented larvae were significantly ($P<0.001$) more prevalent for larvae not treated with ARA.

Larval survival was significantly different between treatments but was not related to light intensity or tank colour. The size and growth of larvae until 11 days after hatching was not related to dietary treatment, but significantly higher ($P=0.003$ and $P=0.04$) for larvae exposed to 4000lux illumination compared to larvae reared in 100lux illumination regardless of background (Table I).

Table I. Size of larvae (d.w.mg ind^{-1}) 12 days after hatching (dah) as well as growth calculated as specific growth rate (SGR) given as a mean ± standard deviation for all larval groups. In addition size of larvae and survival (%) at settling 21 days after hatching. All in relation to dietary treatment, light intensity, and background colour.

	1[1]-4000[2]-bl[3]	**1-4000-wh**	**2-4000-bl**	**2-4000-wh**	**1-100-bl**	**1-100-wh**	**2-100-bl**	**2-100-wh**
d.w.	0.31±	0.32±	0.28±	0.27±	0.23±	0.19±	0.24±	0.19±
(dah 11)	0.08^{b}	0.03^{b}	0.02^{ab}	0.05^{ab}	0.07^{ab}	0.02^{a}	0.01^{ab}	0.07^{a}
SGR	17.7±	18.3±	17.0±	16.6±	14.5±	13.1±	15.5±	12.5±
(dah 11)	3.2	1.0	0.7	2.1	3.4	0.9	0.5	3.7
d.w.	2.02±	2.28±	1.61±	1.89±	1.31±	1.83±	1.32±	1.75±
(dah 21)	0.33^{bc}	0.46^{c}	0.15^{ab}	0.24^{b}	0.20^{a}	0.31^{b}	0.19^{a}	0.20^{ab}
Survival	43.4±	30.6±	42.9±	37.3±	52.8±	28.6±	54.5±	34.2±
(3-21 dah)	7.4^{abc}	10.6^{a}	3.7^{abc}	7.7^{abc}	4.1^{b}	1.4^{a}	5.7^{bc}	6.8^{ab}

A different superscript in a horizontal row denotes a significant difference $P < 0.05$. [1]1, 2 (i.e., diet 1 or 2, 1 =89% fish oil, 0% ARA oil, 7% lecithin, 4% E vitamin. 2= 65% fish oil, 24% ARA oil, 7% lecithin, 4% E vitamin); [2]100, 4000 (i.e., surface light intensity, lux); [3]bl., wh. (i.e., tank background, black or transparent/white

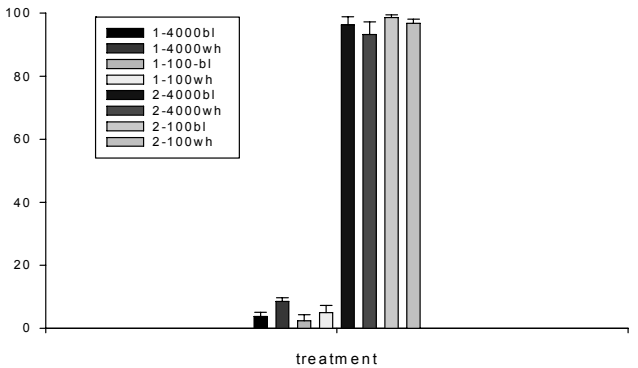

Fig. 1. Hypopigmentation as a result of treatment with the 2 diets in combination with light intensity and background colour.

The development and differentiation of adult melanophores on the dorsal side differed for larvae treated with dietary ARA than without ARA (Fig. 2). Further these cells underwent cytolysis as compared to larvae not treated with ARA.

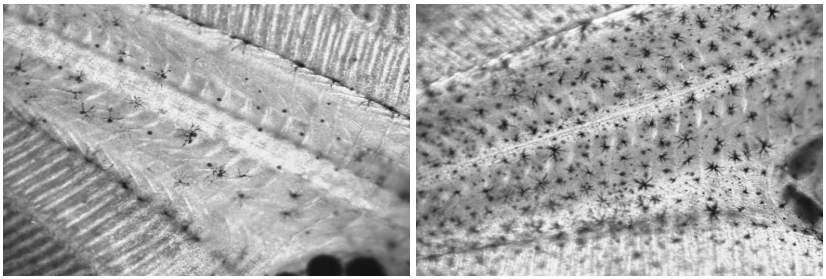

Fig. 2. Example of development of pigment cells (left) in larvae fed *Artemia* enriched with 24% ARA and (right) in larvae without supplementary ARA.

Minor effects on abundance of pigment cells were related to light intensity and background colour.

Conclusion

The study confirmed previous studies that common sole larvae fed dietary ARA during premetamorphosis (i.e., 7 days only) induces hypopigmentation as more than 90 percent of larvae were malpigmented. Visual light at different intensities as well as tank background colour did not influence on pigmentation.

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EFFECT OF FEEDING, SALINITY, AND STOCKING DENSITY ON *LOPHIOSILURUS ALEXANDRI* LARVICULTURE USING BIOLOGICAL FILTERS

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Introduction

In neotropical freshwater larviculture, research has been done looking for best growth and survival rates, in laboratory conditions, using different live food, salinities, and stocking densities (Luz and Zaniboni Filho, 2001; Luz and Santos, 2008; Santos and Luz, 2009). However, few studies have looked at the use of biological filters (Pedreira, 2003). The aim of this work was to investigate the effect of different live food, salinity, and stocking density on *Lophiosilurus alexandri* "pacamã" (Siluriforme: Pseudopimelodidae) larviculture using biological filters.

Materials and methods

In the first experiment, *L. alexandri* larvae at eight days post-hatching (length = 12.7±0.5mm; weight = 17.9±2.5mg) were stocked at a density of 15 larvae.l^{-1} in 16 5-l aquaria. The photoperiod was 10L:14D. The water temperature was 25.9±0.4ºC and dissolved oxygen (DO) was 5.4±0.6mg.l^{-1}. Two different salinities (S0 = freshwater and S2 = 2‰ salinity) and two types of food (A = *Artemia* nauplii and Z = wild zooplankton) were used in a 2×2 factorial design with four replicates each.

The daily prey concentrations (*Artemia* nauplii or zooplankton) were 700, 1050, and 1400 organism.larvae^{-1} from the 1st to the 5th, from the 6th to the 10th, and from the 11th to the 15th day of feeding, respectively. Larvae were feed at 0900, 1300, and 1700h. The hatched *Artemia* nauplii were concentrated in a 5‰ little volume for quantification and feeding. Zooplankton was collected in fertilized ponds and presented *Keratela tropica*, *Filina* sp., *Conochilus* sp., *Polyarthra* sp., *Brachionus caliciflorus*, *Diaphanosoma* sp., *Moina* sp., *Ceriodaphnia* sp, and copepod nauplii.

Inside each aquarium was a submerged biological filter with a 500-ml capacity. The biological filter had an Air lift tower (2cm diameter) emerging 5cm above water level. A grit biofilter media (1.3cm average diameter) was previously prepared for biofilm growth during 30 days.

In the second experiment, *L. alexandri* larvae at eight days post-hatching (length = 12.9±0.4mm; weight = 19.2±0.7mg) were stocked in slightly salt water (2‰) in 12 5-l aquaria with three different stocking densities (D15 = 15; D30 = 30; and D45 = 45 larvae.l^{-1}) with four replicates each. The photoperiod was 10L:14D. The water temperature was 26.4±0.7ºC and the DO was 5.2±0.7mg.l^{-1}.

In this experiment the biofilter media was a mixture of 70% of grit (1.3cm average diameter) and 30% of shell media (1.1cm average diameter). Larvae received *Artemia* as food in the same feeding protocol to the first experiment.

The pH values were 7.52 and 7.53 and alkalinity values were 30.6 and 30.3mg $CaCO_3.l^{-1}$, to freshwater and slightly salt water (commercial salt was used), respectively. During the experiments, at 0800h samples of water to determine ammonium ion were done in the last day (experiment 1) and each three days (experiment 2). Alkalinity and pH (experiment 1 and 2), turbidity and salinity (only experiment 2) were verified at the end of the experiment. After the water samples, the aquaria were siphoned, when 10% total volume was renewed. After 15 days of feeding, survival and growth were assessed.

Survival, growth, alkalinity, pH, and ammonium level were compared by two-way ANOVA in the first experiment. In the second experiment data were compared by one-way ANOVA, and ammonium ion by two-way ANOVA. Means were compared using Tukey`s test ($P<0.05$) using the SigmaStat 3.5 program.

Results and discussion

In the first experiment interaction effect between salinity × food was registered ($P<0.05$) to weight with higher values to S2A (152mg). Intermediate values were verified to S0A (137mg) and smaller to S2Z (32mg) and S0Z (33mg), respectively, showed advantage of salinity and *Artemia* use. The length was significantly influenced by food ($P<0.01$) and salinity ($P<0.05$), with the higher values for A and S2 (Table I). Survival, alkalinity, pH, and ammonium ion were influenced only by food ($P<0.01$) (Table I). *Artemia* nauplii showed better survival and worst water quality.

In the second experiment no significant differences were registered on growth and survival between the densities used (Table II). Higher density showed lower alkalinity and pH and higher turbidity. The salinity showed proportional increase to the density. This is due the *Artemia* nauplii concentred volume offered. The density of D45 showed increase in ammonium during the time and the higher

levels at the end of the work (Fig. 1). A better pH and alkalinity values were registered in the second experiment compared to the first ones. Probably, this fact was due the shell media used in the second experiment.

Table I. Means values (±standard deviation) of total length (TL) (mm), survival (S) (%), Alkalinity (Ak) (mg $CaCO_3.l^{-1}$), pH and ammonium ion (AI) (µg.l^{-1}) after 15 days of feeding under different live food and water salinity.

Treatments	TL	S	Ak	pH	IA
			Means for Food		
Artemia (A)	24.8±0.6a	80.1±5.9a	5.8±3.3b	5.4±0.5b	1034±526b
Zooplankton (Z)	16.5±0.4b	67.5±7.4b	21.0±2.8a	6.5±0.1a	326±232a
			Means for Salinity		
Freshwater (S_0)	20.4±4.3b	73.4±10.5	13.7±6.7	6.1±0.4	760±592
2‰ salinity (S_2)	20.9±4.7a	74.2±8.2	12.0±10.4	5.8±0.8	601±502

Means followed by the same letters on the vertical did not differ by Tukey's test.

Table II. Means values (±standard deviation) of weight, total length, survival, alkalinity, pH, turbidity, and salinity after 15 days of rearing in different stocking densities.

	D_{15}	D_{30}	D_{45}
Weight (mg)	190.6±12.2	196.8±4.8	192.5±11.3
Length (mm)	26.5±0.4	26.0±0.2	25.6±0.8
Survival (%)	92.0±3.4	94.5±4.8	91.7±4.3
Alkalinity (mg $CaCO_3.l^{-1}$)	67.5±1.2 a	68.8±2.0 a	58.6±2.8 b
pH	7.7±0.04 a	7.6±0.04 ab	7.5±0.09 b
Turbidity (NTU)	1.7±0.5 b	3.0±0.8 b	8.5±2.0 a
Salinity (‰)	2.3±0.05 a	2.5±0.00 b	2.7±0.05 c

Means followed by the same letters on the horizontal did not differ by Tukey's test.

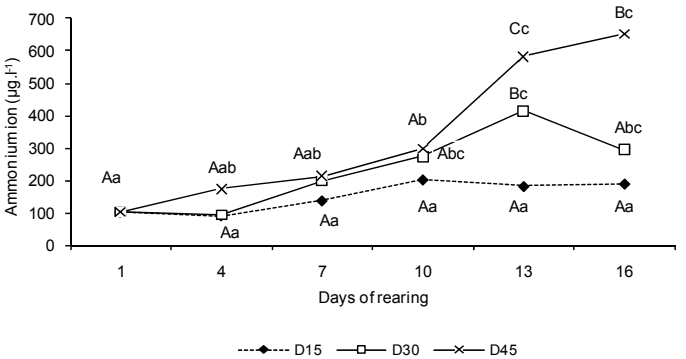

Fig. 1. Means values of ammonium ion levels (µg.l^{-1}) during *L. alexandri* larviculture in different densities. Different capital letters showed significant difference ($P<0.05$) between different densities in the different days of samples. Different small letters showed significant difference ($P<0.05$) within each treatment at a time.

Conclusions

Artemia nauplii and slightly salt water can be used in *L. alexandri* larviculture. The different densities affect the water quality, but without effect on growth and survival of *L. alexandri*. Further studies using different densities and biofilter media quantities are required.

Acknowledgments

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INTERACTIONS OF MICROALGAE AND BACTERIAL COMMUNITIES PRESENT IN THE MICROALGAE CULTURES AND LARVAL REARING SYSTEMS

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Addition of microalgae has been routinely applied in the rearing of marine fish, as it has a positive effect on survival, growth rate, and viability of the larvae. The positive effect of microalgae can be explained by the stabilization of the nutritional state of live feed organisms added to the larval tanks. There are, however, indications that microalgae influence as well the bacterial communities present in a rearing system. The beneficial effect of added microalgae could therefore be explained, in addition to the factors related to the nutritional aspects, with the effect on the microbial balance in the rearing systems.

In marine fish and shellfish hatcheries, microalgae are normally added directly from non-axenic microalgae cultures to the rearing tanks together with the culture medium. Some microalgae strains used in aquaculture show antibacterial activity. It has also been established that microalgae provide a niche for associated bacteria and that some bacterial strains associated to microalgae have antagonistic activity against fish pathogenic or opportunistic bacteria.

Bacteria associated with the microalgae cultures are also added in the larval tanks. The bacterial communities present in the rearing tanks could be influenced by the microalgae cells, or by the associated microbiota, or by both. It has been shown that the microalgae species cultured play an important role on the bacterial load in the microalgae cultures and on the species composition of the bacterial communities present. The method applied for the culture of microalgae may be equally important for establishment of bacterial communities.

The bacterial communities present in microalgae cultures were studied in three different culture systems: (i) small flask cultures, (ii) polyethylene bags and (iii) large bioreactors. The bacterial microbiota was studied at different times with culture-dependent and culture-independent methods. In a study in small flask

cultures, four different strains of microalgae used in aquaculture, showed differences in the number of total bacteria and in the composition of the bacterial communities. In a study with *Chlorella minutissima*, about 550 bacterial strains were isolated and were subjected to basic physiological tests for phenotypic characterization. Amplification and sequencing of 16S rDNA fragments was used to identify 20 representative gram negative bacterial strains, which included bacterial strains reported in the literature to have antagonistic activity. PCR-DGGE was used to obtain the genetic fingerprints, including culturable and non-culturable bacteria, and to compare the influence of the different microalgae culture systems on bacteria microbiota.

In another series of experiments, it was attempted to determine whether there is an antimicrobial activity in microalgae, so experiments were run with axenic cultures of five microalgae species, *Chlorella minutissima*, *Tetraselmis chui*, *Isochrysis* sp., *Arthrospira platensis*, and *Nannochloropsis* sp. The antimicrobial activity was tested against six pathogenic *Vibrio* strains. Incubation with microalgae inhibited the growth of *Vibrio*.

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EFFECTS OF BACTERIUM PROBIOTICS ON GROWTH PARAMETERS AND IMMUNE DEFENCE IN EURASIAN PERCH *PERCA FLUVIATILIS* LARVAE UNDER INTENSIVE CULTURE CONDITIONS

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Introduction

Various probiotics are currently used in animal production not only as effective prophylactics but also to mitigate the emergence of antibiotic resistance as well as the spreading of antibiotics in the aquatic environment. In aquaculture production, it has been shown that some probiotics have beneficial effects on growth parameters and health status (El-Haroun et al., 2006; Aly et al., 2008) but such approach is still limited to few fish species. This study was conducted to examine whether *Bacillus* probiotics have a beneficial impact on growth rate and immune defence status of Eurasian perch larvae.

Materials and methods

A first experiment dealt with the influence of increasing doses of bacterium probiotics on growth related parameters and immune defence of larvae. The bacterium probiotics was the SANOLIFE MIC-F (INVE Aquaculture, Belgium) which consists of a mixture of different species of *Bacillus* (B). Perch larvae were submitted to four different treatments (including a control). Larvae were fed with *Artemia* metanauplii enriched with either 0.5 (B0.5) or 1.0 (B1.0) g of *Bacillus* sp. per litre of incubation medium. In addition to this dietary *Bacillus* supplementation, the bacterium probiotics were mixed with the rearing water of the tanks ($5 g.m^{-3}.d$, BW). The two treatment doses were done within two disconnected recirculating water systems in 40-l tanks of 200 larvae each in triplicate. Control triplicate tanks without probiotics (neither in food nor water) were also set (B0). Larvae (body weight of 2.3 ± 0.8mg at day 14 post-hatching) were fed ad libitum every hour between 9.00 and 19.00 for a 28-day period.

A second experiment was set in mid-May 2009 to evaluate the combining effects of the same doses of the SANOLIFE probiotics as in experiment 1 with stocking

density (10 vs. 20 larvae.l^{-1}). During a 28-day period, Eurasian perch larvae were submitted to the same probiotics protocol feeding as in experiment 1.

For both experiments, growth parameters and immune defence status (lysozyme activity and total immunoglobulins) were evaluated on days 14 and 28 over the bacterium treatment. Results are expressed as the average of the three replicates for each treatment. Data were analyzed by a two-way ANOVA after normalization by log-transformation. Significant differences ($P < 0.05$) were determined by Sheffé-test comparison. The results from the second experiment will be available during the conference session.

Results and discussion

After 14 days of the probiotic treatment, specific growth rate (SGR %/d) was significantly higher for larvae fed the highest dose (B1.0 = 21%) than for the B0 (17%), BW (18%) and B0.5 (18%) groups. The stimulating growth response was sustained until the 28th day of treatment, as indicated by higher values for final body weight and SGR (Table I). Using other types of bacterium probiotics, such promoting growth performance has been reported in other fish species (Makridis et al., 2005; Aly et al., 2008; Bandyopadhyay and Das Mohapatra, 2008), and this was related to a possible positive impact on digestive enzyme activities (Yanbo and Zirong, 2006) or on feed digestibility and utilization (El-Haroun et al., 2006; Watson et al., 2008).

Table I. Effects of increasing doses (0.5 vs. 1.0g.l^{-1}) of SANOLIFE *Bacillus* sp. on growth parameters of Eurasian perch larvae. B0: without *Bacillus*. BW: *Bacillus* in the water tank. B0.5: 0.5g of *Bacillus*.l^{-1}. B1.0: 1.0g of *Bacillus*.l^{-1}

	B0	**BW**	**B0.5**	**B1.0**
Final weight (mg)	140±18^{a}	170±19^{a}	170±20^{a}	200±20^{b}
SGR (%/d)	14.6±0.5^{a}	15.4±0.5^{a}	15.4±0.5^{ab}	16.1±0.4^{b}
Cannibalism II (%)	22±16^{a}	29±12^{a}	15±2^{a}	16±3^{a}
Survival (%)	64±13^{a}	56±12^{a}	64±2^{a}	65±6^{a}

Cannibalism rate was slightly decreased by the probiotic treatment without any marked impact on survival (Table I). As for the survival rate, no marked effect was observed on lysozyme activity and total immunoglobulin levels (Table II) whatever the tested dose, indicating no apparent significant impact of the bacterium treatment on the immune defence status. These results may indicate that the efficiency of *Bacillus* probiotics is species-related because an increased survival and respiratory burst activity as well as disease resistance were reported in Nile tilapia fingerlings after a bacterium treatment based on *Bacillus pumilus* (Aly et al., 2008). These species differences may be related to the interaction between bacterium probiotics and pathogens for which fish species display differential capacity in immune defence. In the present study, stocking density was low and the result from the second experiment will confirm whether such factor account

to the lack of effect on survival and on immune defence because of putative interactions between stress status and populational factors and the immune response to probiotics treatment.

Table II. Effects of increasing doses (0.5 vs. 1.0g.g^{-1}) of SANOLIFE *Bacillus spp* on immune defense status of Eurasian perch larvae. B0: without *Bacillus*. BW: *Bacillus* in the water tank. B0.5: 0.5g of *Bacillus*.l^{-1}. B1.0: 1.0g of *Bacillus*.l^{-1}.

	B0	**BW**	**B0.5**	**B1.0**
Total proteins (mg.g^{-1} tissue)	2.1±0.3^{a}	2.1±0.2^{a}	2.0±0.2^{a}	2.5±0.4^{a}
Total Ig (mg.g^{-1} tissue)	1.4±0.3^{a}	0.93±0.5^{a}	1.4±0.3^{a}	1.4±0.6^{a}
Lysozyme activity (U.ml^{-1})	123±19^{a}	128±14^{a}	127±19^{a}	137±27^{a}

Ig: immunoglobulin

Conclusions

The available results show the interest in using probiotics *Bacillus* sp. to improve growth rate in Eurasian perch larvae but further investigations are needed to demonstrate the benefit for immune defence status.

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A REVIEW OF THE BROODSTOCK MANAGEMENT AND LARVICULTURE OF THE PACIFIC NORTHERN BLUEFIN TUNA IN JAPAN

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Research on broodstock management and larviculture of bluefin tuna *Thunnus orientalis* in Japan has been at the forefront of the world since 1970 thanks to the federal and prefectural governments and universities in a national project intended to optimize productivity of the sea around Japan. In 1979, Kinki Univ. had succeeded in the first spontaneous spawning of the world by broodstock of 5 year-old bluefin tuna in captivity. After spawning in Kinki University in 1980 and 1982, no spawning occurred until 1992, when Maruha Nichiro Holdings, Inc. (MNH) and Nippon Formula Feed Manufacturing Company, Ltd. (NFFMC) became involved in tuna farming and succeeded in the spawning of four-year-old broodstock. Since then, successful spawning of bluefin tuna in captivity has been reported from several sites as well as spawning in Kinki University since 1994. With the successful spawning of bluefin tuna, R&D for tuna larviculture technology has been actively carried out by Kinki University, Fisheries Research Agency (FRA) (formerly, Japan Sea Farming Association), MNH, NFFMC, and Takuyo Ltd. As a result, Japanese scientists and engineers have built upon knowledge on the broodstock management and larviculture; however, there remain technical problems to solve in larviculture.

We have cumulative knowledge on the period of maturation, environmental key factors causing the spawning, the age of onset of spawning, and the pattern of spawning through the approaches of measurement of ambient environment, gonad morphometry, endocrinology, mitochondrial DNA analysis, and daily careful observation of broodstock. There are 9 sites of successful spawning among 4 areas in Japan so far, in which yearly spawning have occurred stably in regions of Amami Island.

Research on larviculture in Kinki University, FRA, MNH, NFFMC, and Takuyo have succeeded in production of tens of thousands of hatchery-raised juveniles based on technologies developed in Japan. As a result, Kinki University succeeded to establish the full life cycle of Pacific bluefin Tuna in captivity, and

also achieved its aquaculture life cycle. Nevertheless, solutions are needed for the remaining technological issues of Pacific bluefin larviculture, such as sinking death on the bottom of tank in the early life stage, the search for appropriate food (kind, size, and nutrition) to tuna larvae between larvae and juvenile stages, cannibalism, bumping against walls, malformation, virus diseases, and so on.

Financial support of the Japan Fisheries Agency and efforts by private companies including the companies above focusing on broodstock management and fingerling production have allowed projects to be conducted toward the goals of the stabilization of tuna farming industry and consequently the conservation of wild tuna resource.

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EFFECT OF VITAMIN A ON THE OSTEOLOGICAL DEVELOPMENT OF EUROPEAN SEA BASS

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Introduction

Skeletal deformities are a frequent problem in European finfish aquaculture. The factors implicated in the development of skeletal deformities are of nutritional, environmental and/or genetic origin. Among nutrients, vitamin A is of great importance since it is involved in differentiation, growth and development of cells and tissues during embryonic and postembryonic development (Haga et al.; 2002, Cahu et al., 2003; Villeneuve et al., 2005). The present study intends to examine the effect of graded levels of dietary vitamin A on the development of deformities in European sea bass (*Dicentrarchus labrax*).

Materials and methods

Sea bass larvae were distributed from 2dph among experimental tanks and fed on microparticulate diets with different levels of retinol acetate (3, 9, 21, 34, 69, 89, and 155×10^3IU.kg^{-1} dry feed), in three replicates. Different dietary regimes were applied during the 9-45 days post-hatching (dph), whereas in the next phase (45-100dph) all population were fed on a common commercial diet (Marin Start-miet AL.0, Le Gouessant, France). During the whole rearing process, temperature, salinity, oxygen concentration, and photoperiod were 20°C, 35‰, >6mg l^{-1}, 24h light, respectively.

From each experimental population, a sample of 47-59 larvae and 47-52 juveniles was randomly taken at 45dph (~15-20mm TL) and at 100dph (~35-45mm TL), respectively. Larval samples were anaesthetised (0.02% phenoxyethanol), fixed in 5% phosphate buffered formalin and stained for bone and cartilage (Park and Kim, 1984). Juvenile samples were anaesthetised, frozen at -20°C and

radiographed. Larval samples were used to examine the most of the deformities, whereas radiographies were used mainly for the study of haemal lordosis (a deformity that develops during the early juvenile stage, Sfakianakis et al., 2006). In total, 1098 larvae and 1053 juveniles were examined. The differences in the deformity incidence among the different nutritional treatments were tested by the G-test (Sokal and Rohlf, 1981).

Results and discussion

Vitamin A significantly affected the development of skeletal deformities in European sea bass. Skull deformities affected the maxillary and premaxillary bones (severe pugheadness) and glossohyal (lateral or ventral transposition). Vertebral deformities involved lordosis or over-mineralization of the haemal vertebrae. Finally, as far as fin deformities are concerned, there were deformations of the dorsal pterygiophores as well as partial to complete lack of the pelvic fins (Fig. 1). The frequency of skull deformities was in general minimised at the lower dietary levels of retinol (Fig.1A-1B). Vertebral deformities presented a different response pattern with their frequency being maximised in the absence of retinol (Fig.1C-1D). Fin deformities exhibited varying response to dietary retinol levels. The frequency of deformed supporting elements of the dorsal fin was reduced at 21×10^{3}IU.kg^{-1}, whereas pelvic deformities were reduced at retinol dietary levels of $>3\times10^{3}$IU.kg^{-1}.

The results of the present study clearly demonstrate that retinol dietary levels for the optimal development of European sea bass larvae greatly depend on the anatomical area under concern. Such a result could be considered as invalid with the theoretically expected unique optimal preferences of a given species. However, it could be easily explained if the ontogenetic scaling of sea bass skeletal elements is taken into account. Indeed, in European sea bass, structures of the cranium such as the jaws and the hyoid arch appear early in development (after hatching and up to 6.5-7.5mm total length, TL) (Gluckmann et al., 1999), largely before the development of vertebrae (9-15mm TL, Marino et al., 1993) and fin supporting elements (9.0-14.0mm standard length, SL, for the dorsal and 11.3mm SL for the pelvic fins) (Koumoundouros et al., 2001; Marino et al., 1993). Under this consideration, our results suggest that vitamin A of maternal origin is enough to support the normal development of European sea bass up to the complete consumption of lipid reserves (5-8 days after the end of yolk-sac larval stage, Koumoundouros et al., 2001), whereas afterwards retinol must be provided by food in a gradually increasing way.

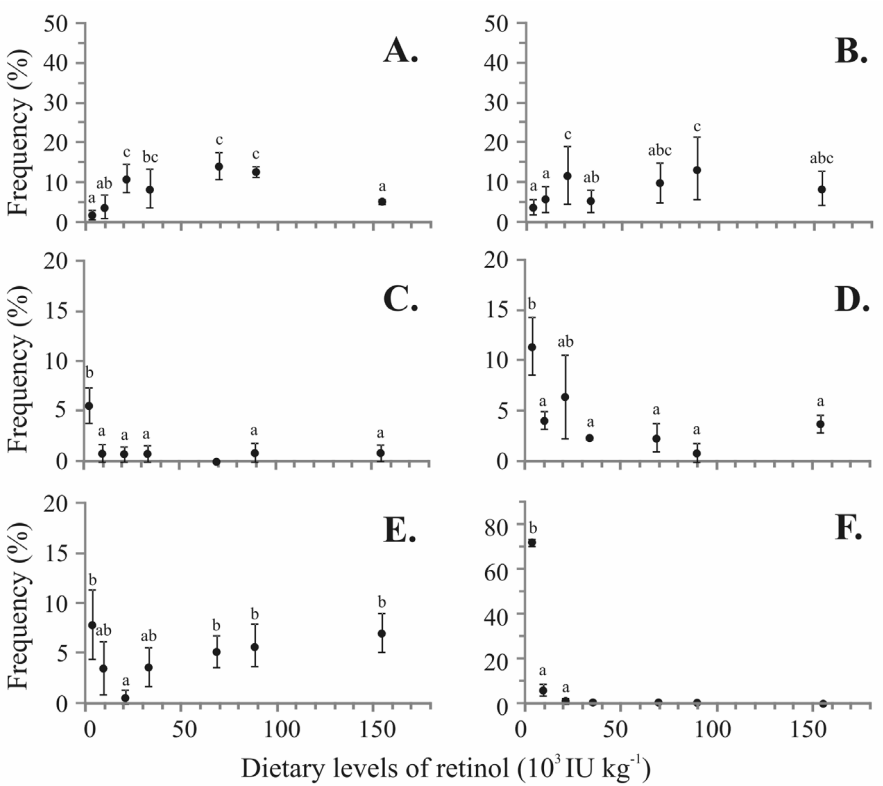

Fig. 1. Effect of dietary levels of retinol on the development of deformities in European sea bass. A. Severe pugheadness. B. Deformed hyoid arch. C. Haemal lordosis. D. Over-mineralization of haemal vertebrae. E. Severe deformation of the dorsal fin. F. Severe deformation of the pelvic fins. Bars equal to ± 1 SE. Different letters indicate significant differences ($p<0.05$).

Conclusions

The dietary retinol requirements of European sea bass change rapidly during larval ontogeny. Future research should focus on the precise estimation of the adequate sequence of retinol levels in both microparticulate and live diets, which are used in the larviculture of European sea bass.

Acknowledgements

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INFLUENCE OF THE PARENTAL DIETS ON THE FATTY ACID DYNAMICS DURING THE ONTOGENIC DEVELOPMENT OF *PALAEMONETES VARIANS* (DECAPODA, CRUSTACEA)

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Introduction

Atlantic ditch shrimp *Palaemonetes varians* (Leach, 1814) is a euryhaline grass shrimp abundant in temperate saltmarshes and estuaries. In the Iberian Peninsula it is used for human consumption or as fish bait, presenting an increase demand, with a good market price. Although there is no official statistic data, high captures are made in saltpans. Therefore, *P. varians* has a high potential as a new candidate species for marine aquaculture.

The culture viability of a species requires the know-how that allows a continuous production in hatchery, and broodstock nutrition is one of the most important issues. Optimization of the broodstock diet is a key factor, due to the influence on sexual maturation and reproduction (Wouters et al., 2001).

Lipids play an essential role on the crustacean ontogenic development. Fatty acid (FA) profile of the ovary has a direct influence on the quality of the eggs, in hatching rate and on the survival and development of the offspring (Ying et al., 2006).

Lipid composition and FA profile change in the broodstock tissues, eggs and embryo during maturation. Hepatopancreas is the lipid storage organ in crustacean, though to be the origin of lipids accumulated in ovaries, however recent studies indicate that the majority of the lipids of the ovary are provided directly from the diet (Wouters et al., 2001).

As in marine fish, shrimp presents a very low biosynthesis of n-3 highly unsaturated fatty acids (HUFA) from their precursors. Therefore EPA (20:5n-3) and

DHA (22:6n-3) are considered essential fatty acids (EFA) and must be provided from the diet.

In this preliminary study, the fatty acids requirements and the culture viability of *P. varians* were investigated. The acceptability and the effect of broodstock diets on the shrimp condition, reproduction and eggs and embryonic fatty acid dynamics were observed.

Materials and methods

Shrimp were collected from the wild and cultured in fibreglass tanks at a density of 320ind.m^{-2}. Experimental trials had a four month duration, and shrimps were fed ad libitum. Five diets with different fatty acids profile were tested. Diets obeyed to nutritional characteristics but also were selected according to their availability and price, that conduced to the following selection: D1 (*Artemia*) – frozen *Artemia* metanauplii enriched with a commercial emulsion (Bernaqua); D2 (Rabbit) – rabbit inert food (Sorgal); D3 (S.One) – trout inert food enrich with SFA (saturated fatty acids) (Sorgal); D4 (Marine worm) – frozen marine polychaeta (*Marphysa sanguinea*) captured in Ria Formosa; D5 (AquaS2) – sea bream inert food (Sorgal). Shrimp eggs were sampled two and three months after the beginning of the trials, respectively ES (early spring sample) and LS (late spring sample), and the embryonic development of the eggs was identified. Fatty acids analyses were conducted for eggs in the first (E1- after spawning) and last embryonic stage (E3- just before hatching).

Wild shrimps were also sampled for treatment D6 as a control group.

Fatty acid methyl esters (FAME) were prepared by acid-catalyzed transesterification (Lepage and Roy, 1986) as modified by Cohen et al. (1988). FAME analyses were performed in a Varian Star 3800 CP gas chromatograph (USA), equipped with an auto-sampler and fitted with a flame ionization detector (FID) at 250ºC. The separation was achieved using a capillary column DB-Wax (30m length × 0.25mm internal diameter and 0.25µm film thicknesses) from Agilent Technologies (USA). After holding at 180ºC for 5min, the temperature was raised to 220ºC at 4ºC.min^{-1}, and maintained at 220ºC for 25min, with the injector at 250ºC.

Results and discussion

P. varians showed a positive acceptability for the five different treatments/diets. Eggs presented a fatty acids profile clearly influenced by the diet, (Fig. 1); similar results were observed by Calado et al. (2005) with a different crustacean species.

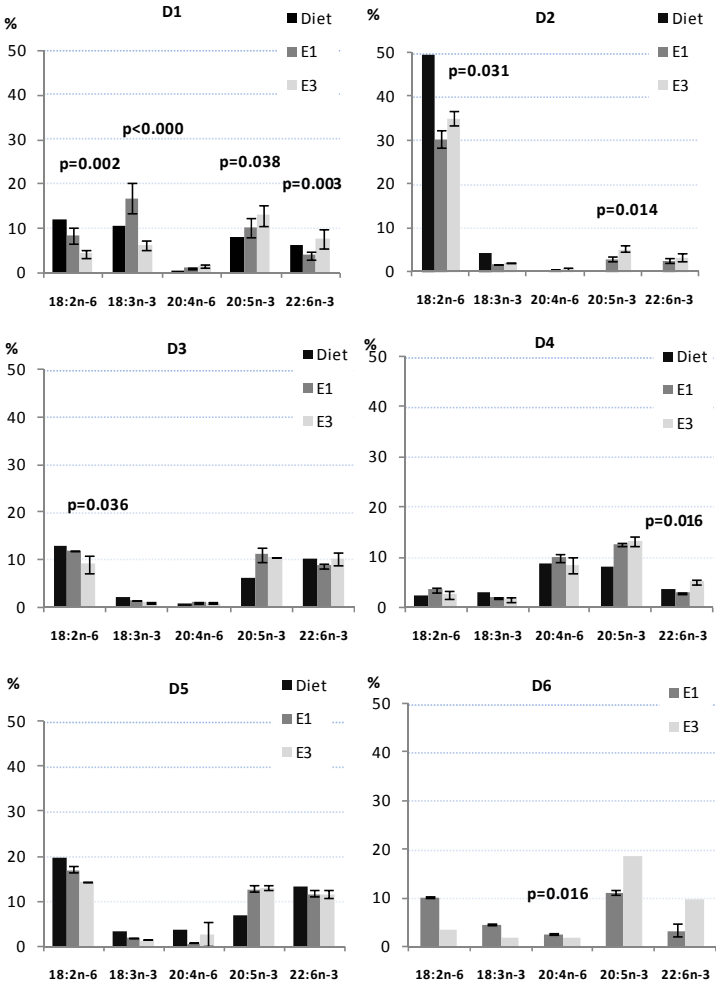

Fig. 1. Fatty acids profile (%) on *P. varians* eggs, during embryogenesis E1- E3, on LS. Significant differences obtained performing an ANOVA, $p<0.05$.

Our study seems to indicate that *P. varians* have not a high demand for DHA, since the eggs content in this FA is generally poorer than diets. Thus, the eggs did not incorporate all the DHA available. Also the content on D6 eggs is lower when compared to other treatments. Yet, during embryogenesis DHA was spared, pointing out its importance during ontogeny and subsequent stages. A less demand for DHA presents an economical and ecological benefit when compared with other cultured species, as marine fish, and allows a more flexible diet formulation, less dependent on other marine resources.

Surprisingly, although diet D2 does not contain HUFA, shrimp eggs fed D2 presents a reasonable content and still an accumulation during embryogenesis. Shrimp fed D4 and D5 presents a HUFA profile similar to D6, although we consider that the DHA content in diet D5 may be excessive for these species.

In all treatments, shrimp eggs presented a high EPA content (higher than the diets) suggesting a conservative strategy for this FA and its importance during embryonic development and early larval stages.

In general, is observed an accumulation of EPA and DHA during embryogenesis and a preferential consumption of 18:3n-3; similar results were reported by Wehrtmann and Graeve (1998) for other caridean shrimps.

Conclusions

This species presented a high efficiency to use fatty acids in a selective way during embryogenesis. They use non-essential or abundant fatty acids as energy source, conserving the essential fatty acids. The n-3 HUFA content of the prehatching stage (E3) provides to the early zoea stages, high independence to the environmental conditions, which is an important ecologic advantage and also suggests that *P. varians* seems to have a higher HUFA biosynthesis than other decapods species. These characteristics, together with their resistance to culture and handling, make this species very interesting for aquaculture production.

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EFFECTS OF LIGHT ON ATLANTIC COD (*GADUS MORHUA*) LARVAE PERFORMANCES: FOCUS ON SPECTRUM

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Introduction

Most farmed marine fish species are carnivorous visual feeders which mainly rely on visual photoreceptors to detect their prey. As such the light environment can directly affect feeding behaviour and consequently larval growth and survival. Light is a complex environmental factor that depends on the lighting systems used (intensity and spectrum), photoperiod, water absorbance properties as well as the specific light sensitivities of the species being reared. While it is well documented that light intensity (Downing and Litvak 1999; Puvanendran & Brown, 2002; Brown et al., 2003) and to a lesser degree photoperiod (Downing and Litvak, 2000) can play a significant role in improving gadoid larvae growth and survival, little is known on the importance of light spectral properties with very few studies on marine larvae reported to date (Downing and Litvak, 2001; Villamizar et al., 2009). This communication will present the findings from a number of studies during which the impact of light spectrum on cod larvae growth and survival to weaning were investigated. The optimised rearing of Atlantic cod is of great commercial interest as juvenile supply is one of the key production bottlenecks that limits industry.

Materials and methods

Three experiments were performed to test the effects of light intensity and spectrum on cod larvae performances using either cold cathode lighting systems (trial 1) or Light Emitting Diodes (LED)(trial 2) as well as test the effects of green water on the light properties of the rearing environment. Eggs were collected from freely spawning cod broodstock (Viking Fish farm, Ardtoe, 2002-2003 year classes) with incubation temperature (9.6 ± 0.5ºC) and salinity (34.3 ± 1ppt) being recorded daily during the trials.

In the first experiment, cod eggs (5000.tank^{-1}) were stocked at stage IV among 16 experimental rearing tanks (80 l) all of which were reared in a clearwater

(without algae) environment and assigned to one of two intensities (0.5W.m^{-2} and 0.25W.m^{-2}) and four spectra (blue-460nm, green-540nm, red-640nm, and white-580nm) in duplicate. The eggs were left in total darkness in static conditions until 100% hatching was observed, at which time continuous light was applied (24L:0D). Novel cold cathode lighting systems (CCL) equipped with dimming systems developed by Intravision Aqua AS (Oslo Norway) were used. The experiment was terminated once the larvae reached 50 days post hatch (DPH). Five replicates of 10 larvae were sampled in each tank every ten days, preserved at -20°C and freeze dried to determine dry weight (DW). In addition, 10 larvae/tank/time point were collected and anaesthetized with MS222 under a dissecting microscope, photographed using a digital camera to measure standard length (SL), myotome height (MH) and eye diameter (ED) using image analysis software. Survival was determined at the end of the trial.

In the second experiment, 4 different light spectra (Blue-455nm, Green-535, Red-640 and White-460/560 light) were tested in triplicate using 12 semi-conical 80 L rearing tanks (same system used in exp. 1). Lighting regimes were set up using a custom designed Light Emitting Diode (LED) lighting system equipped with dimming systems (Intravision Aqua AS, Norway) which was located centrally above each tank. The light intensity was set at 1.39 and 0.37 W.m^{-2} at the water surface and bottom of the tanks, respectively. All tanks were stocked with 5000 stage IV cod eggs with sampling and analyses performed as described for experiment 1.

In the third experiment, rearing tanks were filled with different concentrations (from 50 to 1 200 000 cells.ml^{-1}) of either *Nannochloropsis atomus* or *Pavlova lutheri* algae. The light intensity and spectral profile for each system used in exp. 1 was measured at the tank bottom using a portable spectroradiometer with fiber optic umbilical.

Results and discussion

Results of the first trial confirmed previous findings (Puvanendran and Brown, 2002; Brown et al., 2003) on the effects of light intensity on cod larvae performances. Larvae exposed to higher intensities (0.5W.m^{-2}) showed significantly better growth irrespective of the spectrum (Table I). Furthermore, growth performance (SL, MH, ED, and CI) was best in larvae reared under a blue spectrum (as opposed to red) although weight data did not differ significantly due to high intra-batch variability. These results are in accordance with recently published data on sea bass (Villamizar et al., 2009).

These results were further confirmed in the second trial where significantly improved performances (SL and ED) were observed in larvae exposed to short wavelengths (blue and green) in comparison to a longer wavelength spectrum

red (Table II). Larvae reared under red spectrum were 4-5 times smaller than siblings exposed to white, blue or green spectra (dry weight). However, survival determined at 50 DPH (from number of eggs stocked in the tanks) was low in both trials (2.9-9.3% and 2.5-5.8% in trials 1 and 2, respectively) with no significant spectral or intensity effects.

Table I. Summary of cod larvae performances at 50DPH when exposed to different light intensities and spectrum using CCL lighting (trial 1, n=2, 30 larvae/sampling point/tank). SL: standard length, MH: myotome height, ED: eye diameter, CI: condition index. Swim- bladder inflation was determined at 10DPH. Superscripts indicate significant differences between treatments (ANOVA, $p<0.05$).

	Weight (mg)	SL (mm)	MH (mm)	ED (mm)	CI	Swim-bladder (%)	Survival (%)
White High	3.31±0.46	14.85 ± 0.27^{bd}	2.23 ± 0.07^{bc}	1.32 ± 0.03^{c}	0.15 ± 0.002^{a}	75	9.3
Blue High	3.93±0.58	15.32 ± 0.26^{d}	2.56 ± 0.05^{e}	1.34 ± 0.02^{c}	0.17 ± 0.001^{b}	90	3.8
Green High	3.29±0.36	14.01 ± 0.37^{bcd}	2.13 ± 0.07^{b}	1.26 ± 0.03^{abc}	0.15 ± 0.002^{a}	70	3.2
Red High	3.64±0.65	15.39 ± 0.38^{bcd}	2.39 ± 0.09^{bc}	1.32 ± 0.03^{c}	0.16 ± 0.003^{ab}	55	2.9
White Low	2.15±0.36	12.10 ± 0.32^{ac}	1.81 ± 0.07^{a}	1.13 ± 0.02^{a}	0.15 ± 0.003^{a}	75	5.2
Blue Low	2.84±0.41	13.77 ± 0.29^{bc}	2.09 ± 0.06^{b}	1.24 ± 0.02^{abc}	0.15 ± 0.002^{a}	80	3.6
Green Low	3.25±0.56	13.87 ± 0.29^{c}	2.15 ± 0.11^{b}	1.24 ± 0.04^{b}	0.15 ± 0.003^{a}	70	5.6
Red Low	2.48±0.26	12.05 ± 0.22^{a}	1.82 ± 0.06^{a}	1.12 ± 0.01^{a}	0.15 ± 0.002^{a}	85	6.6

Table II. Summary of cod larvae performances at 50DPH when exposed to different spectrum using LED lighting (trial 2, n=3, 30 larvae/sampling point/tank). SL: standard length, MH: myotome height, ED: eye diameter, CI: condition index. Superscripts indicate significant differences between treatments (ANOVA, $p<0.05$).

	Weight (mg)	SL (mm)	MH (mm)	ED (mm)	CI	Survival (%)
White	2.96 ± 0.47^{b}	12.57 ± 0.54^{b}	4.57 ± 0.10^{b}	1.24 ± 0.04^{b}	12.29 ± 0.32^{b}	3.40±0.81
Blue	3.78 ± 0.92^{b}	13.86 ± 0.44^{c}	1.73 ± 0.09^{b}	1.40 ± 0.04^{c}	12.30 ± 0.33^{b}	3.33±1.18
Green	3.14 ± 0.33^{b}	13.16 ± 0.55^{b}	1.64 ± 0.12^{b}	1.28 ± 0.05^{c}	11.94 ± 0.48^{b}	2.58±0.64
Red	0.77 ± 0.70^{a}	9.41 ± 0.23^{a}	0.75 ± 0.04^{a}	0.91 ± 0.02^{b}	7.91 ± 0.24^{a}	5.81±0.53

Finally, results showed that *Nannochloropsis atomus* and *Pavlova lutheri,* the two main algal species used in cod larviculture, can influence the photic environment within the rearing systems depending on algal species spectral absorbance and densities (data not presented).

Together, these results clearly demonstrate the importance of light characteristics and especially spectrum on marine larviculture. Depending on species specific light sensitivities, particular wavelengths may maximize visual sensitivity, as shown by Bowmaker (1991) in deep-sea fishes or, visual contrast, as for fish living in complex photo-environments, like in shallow or coastal waters. There is therefore, the possibility that larvae visual system could be predisposed to perform best under spectral conditions most frequently encountered in its particular

ecological niche (Moksness et al., 2004). However it must be considered that light sensitivities may change with development.

Conclusions

These trials have implications for current commercial hatchery practices suggesting methods of improving husbandry methodologies. There remains a lack of standardisation of protocols (especially for light) in the industry but clearly the use of new lighting technologies could cut operating costs while also providing specific light environments tailored to the photic affinities of larval cod. More research and development is needed in this area to determine the photosensitivities of larval cod visual pigments throughout development so that the full benefit of enhanced growth and survival can be obtained through the implementation of new hatchery protocols.

Acknowledgements

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RELATIONSHIP AMONG OXYGEN CONSUMPTION, GROWTH, AND SURVIVAL OF LARVAL FISHES

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Introduction

Currently, some finfish larviculture industries still have periods with some mortality which need clarification of the causes of this mortality and improvement of larviculture techniques to eliminate such causes. Increased knowledge of physiological characteristics of finfish larvae permits development of suitable larvicultures technique for improvement of larval healthy and quality (Fushimi, 2001). In order to determine effects of changes in physiology on growth and development of larval fishes, we analyzed the oxygen consumption of larval fishes; i.e., Japanese flounder, ocellate puffer, red sea bream, and devil stinger.

Materials and methods

Larvae of each species were initially stocked at the following densities: Japanese flounder: 9 larvae.l^{-1}; ocellate puffer: 5 larvae.l^{-1}; red sea bream: 20 larvae.l^{-1}; devil stinger: 15 larvae.l^{-1}. In each rearing of ocellate puffer, red sea bream, and devil stinger, rotifers were enriched for 8h with *Nannochloropsis oculata* for 24h, and *Artemia* nauplii were enriched for 24h with Marine-Omega A (Nisshin Marine Tech Co. Ltd., Yokohama, Japan). In the flounder rearing, rotifers were enriched with DHA Protein SELCO and *Artemia* nauplii were enriched for 24h with DC Protein SELCO (INVE Technologies NV, Dendermonde, Belgium). Experiments were performed until larvae developed to juveniles. Every day 3-20 larvae were sampled and their oxygen consumption was measured.

Larval oxygen consumption was measured with the water bottle method (Kurokura et al., 1995). Syringes were used as variable respiration chambers; the seawater in syringe included 3-20larvae. The difference between initial and final oxygen contents during experimental period (30-60min) was evaluated as larval oxygen consumption. The weight of consumed oxygen was calculated from the consumed oxygen content and the difference between initial and final weight of syringe including seawater and larvae. A portion of the water in the syringe was introduced to an oxygen monitor (oxigraph9, Central Kagaku Corp., Tokyo, Ja-

pan). The weight of consumed oxygen per dry weight of larvae was plotted and the change was observed with the growth of larvae.

Results

After hatching out, yolk sac larvae did not increase oxygen consumption. However, they increased consumption from mouth opening to the onset of notochord flexion. After that increase, oxygen consumption decreased from the onset of or during notochord flexion, and then stabilized. However, around that time when it stabilized, the mortality increased.

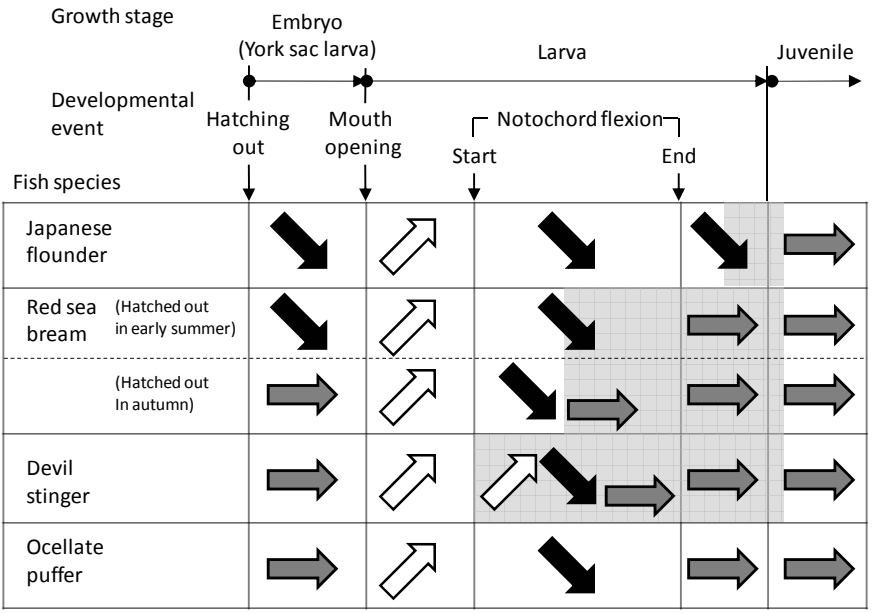

Fig. 1. Schematic explanation of change of oxygen consumption.mg^{-1} dry weight.min with morphological development in 4 fish species larvae. Down arrow indicate decrease of oxygen consumption, level arrow indicate stable consumption and up arrow indicates increase. Grayed area indicates that period when mortality is frequent.

Discussion

Larval oxygen consumption changed during morphological development and those changes occurred before mortality increased (Fig. 1). From these results, it can be concluded that metamorphosis or development from mouth opening to notochord flexion needs much energy and, therefore, metabolism becomes active. On the other hand, larvae have mortality after the increase of oxygen consumption and the timing of beginning of mortality varies among species (Fig. 1).

This may be because the stress by energy consumption from mouth opening to the onset of notochord flexion negatively influenced some larvae and the tolerance for stress varies among species.

The improvement of larviculture techniques by taking into account the change of metabolism will lead to the decrease of mortality and the improvement of larval quality.

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DEVELOPMENT OF DIGESTIVE ENZYMATIC ACTIVITY IN THE PACIFIC RED SNAPPER, *LUTJANUS PERU*

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Introduction

The Pacific red snapper *Lutjanus peru* has a high potential for culture in the Pacific coast of Mexico. In recent years broodstock spawning has been obtained by hormonal induction. However, massive mortality occurred during the first days of development, including first feeding at day 2 after hatching. With the object to evaluate the digestive capacity of the Pacific red snapper larvae, we evaluated the digestive enzymatic activity of trypsin, chymotrypsin, lipase, amylase, and cathepsine-like during the embryonic development, the yolk-sac larvae and in first-feeding larvae.

Materials and methods

Fertilized eggs of 3 spawns were obtained by means of hormonal induction in Pacific red snapper broodstock (Pelcastre-Campos, 2006). The eggs were maintained in a 100-l conical tank at 25ºC. Samples were collected at different developmental stages: newly-fertilized eggs, early cleavage, blastula, gastrula, hatching, 24h and 48h after hatching. All samples were frozen at -70°C until analysis.

The samples of each stage were homogenized and centrifuged (13 000rpm, 10min at 5ºC). The supernatant was stored at -70ºC to be used for enzyme analysis. The concentration of soluble protein in pooled samples was determined by the Bradford (1976) method and the digestive enzyme activity was expressed in units.mg protein^{-1}. The concentration of trypsin (E.C.3.4.21.4) was obtained using BAPNA (Na-Benzoil-DL-Arginine-P-Nitroanilide) as substrate following the method of Erlanger et al. (1961). Chymotrypsin (E.C.3.4.21.1) activity was evaluated using SAAPNA as substrate. The amylase (E.C.3.2.1.1) and lipase (E.C.3.1.1.3) activities were quantified with soluble starch and β-Naphtyl-Caprylate, respectively (Versaw et al., 1989). Cathepsine-like activity was

measured using hemoglobin (modified from Anson, 1938). All assays were performed by triplicate with a spectrophotometric method.

Results and discussion

All the enzymatic activities tested were present in all the stages of the initial ontogeny of the Pacific red snapper larvae, except for the chymotrypsin activity (Fig. 1). The trypsin activity during the embryonic development suggests its role in the activation of other proteases like the cathepsin-like since cathepsins are responsible for yolk utilization during embryogenesis (Carnevali et al., 2001), which is supported by the high cathepsin-like activity during the embryonic development of the Pacific red snapper. All the enzymatic activities increased at hatching. However, only the lipase and cathepsin-like activities augmented after hatching, reaching the highest activity at 48h. This is in accordance with previous reports as these enzymes are involved in the yolk-sac and oil globule utilization during the endogenous nutrition period (Fyhn and Govoni, 1995; Kamler, 2008). On the other hand, the amylase and trypsin activities decreased by the time of first feeding. In the case of trypsin the highest activity is observed at 24 hours after hatching but decrease considerably at 48h probably due to the lack of prey during the firs feeding. The same pattern has been described for the amylase activity (Shan et al., 2008).

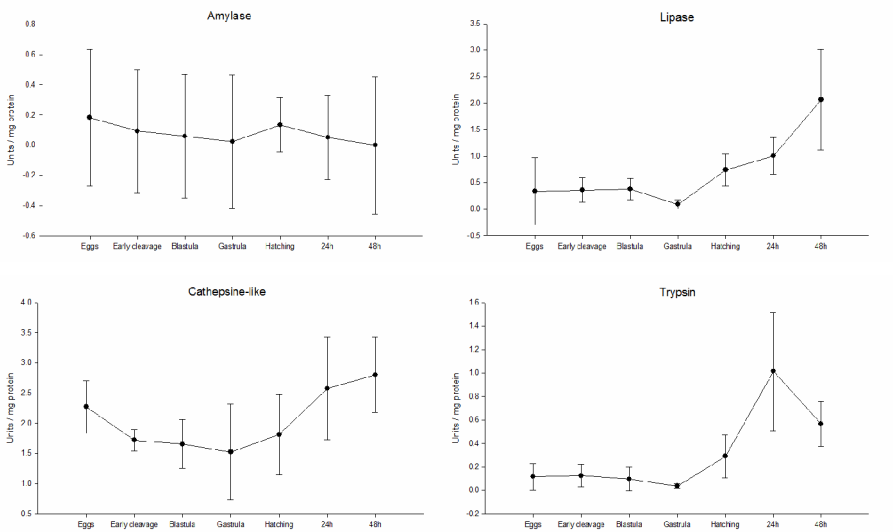

Fig. 1. Digestive enzymatic activity during the embryonic development, in the yolk-sac larvae (24h) and in first-feeding larvae (48h) of the Pacific red snapper *Lutjanus peru*. Each point represents the average activity. Vertical bars denote the standard deviation (n=3).

Conclusions

The presence of the digestive enzymatic activity in first-feeding larvae of the Pacific red snapper suggests that the high mortality rates encountered during culture of this species are not related to a lack of digestive capacity but to other factors namely prey size or culture conditions.

Acknowledgements

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EXPRESSION OF LC-PUFA SYNTHESIS ENZYMES DURING ZEBRAFISH EARLY EMBRYONIC DEVELOPMENT

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Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) are essential in crucial physiological processes, many of which are particularly vital during embryonic development. This study investigated the expression of genes encoding enzymes involved in LC-PUFA biosynthesis, namely fatty acyl desaturase (Fad) and Elovl5- and Elovl2-like elongases, during zebrafish early embryogenesis. Expression of these three enzymes enables zebrafish to synthesise all LC-PUFA from C18 essential fatty acids, and therefore zebrafish are an excellent model to study developmental regulation of LC-PUFA synthesis in vertebrates.

Materials and methods

Zebrafish embryos collected from mating of single broodstock couples were isolated and raised at 28.5ºC. To study the expression of the target genes during zebrafish embryogenesis, total RNA was extracted from pools of 20-30 embryos collected at 0, 3, 6, 9, 12, 14, 24, 48, and 72 hours post-fertilization (hpf). Five µg of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega). Qualitative expression of *fad, elovl5* and *elovl2* transcripts during embryonic development was determined by reverse transcriptase PCR (RT-PCR). Primers used for RT-PCR on embryo cDNA samples are shown in Table I.

To examine the spatial expression of zebrafish *fad*, *elovl5*, and *elovl2*, whole-mount in situ hybridization (WISH) was performed on 24-hpf embryos using digoxygenin (DIG)-labelled antisense riboprobes as previously described (Rotllant et al., 2008). Antisense riboprobes were made from linerarised full length zebrafish *fad*, *elovl5* and *elovl2* cDNAs.

Results and discussion

The presence of transcripts of *fad*, *elovl5*, and *elovl2* during embryogenesis (Fig. 1) suggests that zebrafish embryos are capable of de novo LC-PUFA biosynthesis. All three genes are expressed from the zygote stage (0hpf), indicating maternal transfer of mRNA to the embryo. This highlights that the maternal role in LC-PUFA supply to fish embryos is not only transfer of preformed LC-PUFA, but also transfer of mRNA transcripts that can potentially be translated to active proteins.

Table I. Sequence of the primer pairs used and GenBank accession number of the sequence used as reference for primer design for temporal expression patterns by RT-PCR.

Transcript	Primer	Primer sequence	Accession No.
fad	FadF	5'-AGGAGGTGCAGAAACACACC-3'	AF309556
	FadR	5'-CTCGCCAGATTTCTCCAAAG -3'	
elovl5	Elovl5F	5'-CTCAGGGTCACAGGATGGTT-3'	NM200453
	Elovl5R	5'-CTCCATTAGTGTGGCCGTTT-3'	
elovl2	Elovl2F	5'-AAAGAGATACCCGCGTGAGA-3'	NM001040362
	Elovl2R	5'-TTGGAGTTGGCTCCGTTTAG-3'	
β-actin	βActinF	5'-CTCTTCCAGCCTTCCTTCCT-3'	NM131031
	βActinR	5'-CACCGATCCAGACGGAGTAT-3'	

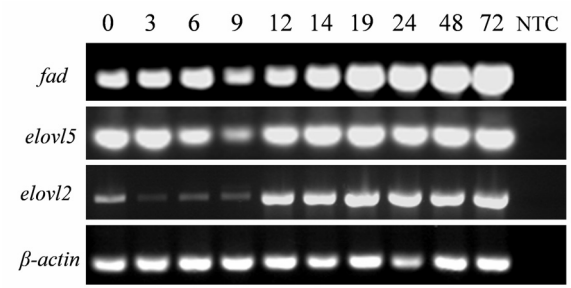

Fig. 1. RT-PCR analysis of the temporal expression patterns of *fad*, *elvol5* and *elovl2* during zebrafish embryogenesis (0 to 72hpf at 28.5ºC). Expression of the housekeeping gene *β-actin* is also shown. NTC, no template control.

The expression patterns of *fad* and *elovl2*, with noticeable increasing expression from 12 hpf onwards (Fig. 1), allow us to speculate that the biosynthetic pathway in the embryo is not fully activated until development of the central nervous system and retina occurs (Kimmel et al., 1995). The spatial expression patterns of *fad* and *elovl2* observed in zebrafish embryos at 24 hpf supports this hypothesis (Fig. 2).

Thus, zebrafish *fad* (Fig. 2A) and *elovl2* transcripts (Fig. 2C) were widely distributed in the head region possibly because high biosynthetic activity is required

to provide LC-PUFA for the formation of neural tissues (Lauritzen et al., 2001). WISH analyses also showed that the target genes are expressed in the yolk syncytial layer (YSL) (Fig. 2 insets), indicating that YSL is likely to be active in remodelling PUFA during zebrafish embryogenesis.

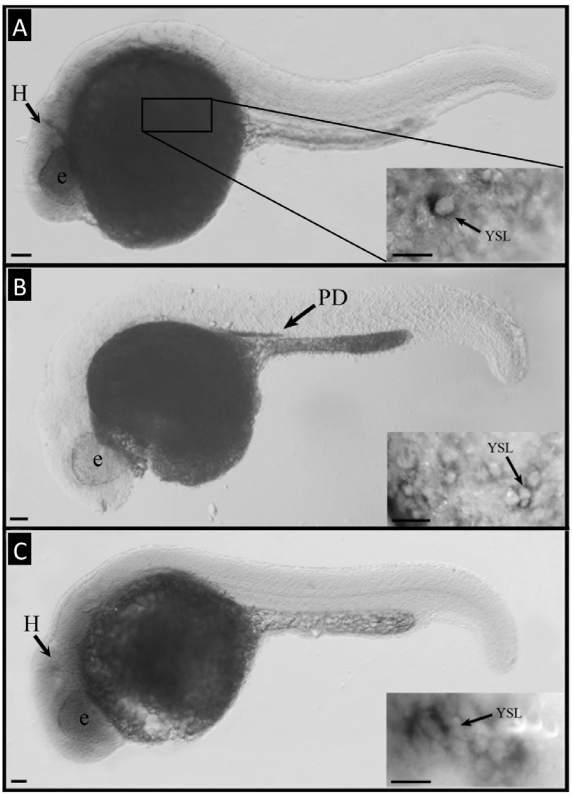

Fig. 2. Whole mount *in situ* hybridization showing the expression patterns of *fad* (A), *elovl5* (B), and *elovl2* (C) in 24-hpf embryos hybridized with antisense probes. YSL, yolk syncytial layer; PD, pronephric ducts. Scale bars: 100μm.

In addition to hydrolysis of the abundant lipids contained in the yolk (Wiegand, 1996), the YSL may also influence the composition of the hydrolysed and absorbed fatty acids in a number of ways including conversion of C18 PUFA and alteration of EPA/DHA ratio prior to transfer to the developing embryonic tissues. Interestingly the Elovl5 elongase was specifically expressed in the pronephric ducts of 24 hpf embryos (Fig. 2B). Although Elovl5 elongase has been reported to be expressed in kidney of adult fish (Zheng et al., 2005), there is no obvious explanation for such a specific expression in the pronephric ducts of the embryonic kidney. In addition to functions including blood filtration, waste extraction, salt recovery, and water balance, our results suggest the em-

bryonic kidney may also play a role in fatty acid elongation but the purpose is unclear. Further investigations are required to elucidate these findings.

Conclusions

The presence of transcripts of enzymes of LC-PUFA biosynthesis supports the hypothesis that LC-PUFA biosynthesis occurs in zebrafish embryos. Moreover, expression of the target genes at zygote stage demonstrates that the maternal role in LC-PUFA supply involves not only transfer of preformed LC-PUFA, but also transfer of mRNA transcripts that can potentially be translated to active proteins. Spatial expression results demonstrate high levels of *fad* and *elovl2* transcripts in the head area of 24-hpf embryos, suggesting biosynthetic activity related to the high requirements for LC-PUFA of developing neuronal tissues. The YSL may be important in remodelling of yolk fatty acids during embryogenesis, as seen from expression patterns of all three genes in 24 hpf embryos. The pronephric ducts showed specific expression of the elongase *elovl5*, suggesting an as yet unknown role in fatty acid metabolism during zebrafish embryogenesis.

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A NEW METHOD FOR THE STUDY OF ESSENTIAL FATTY ACID REQUIREMENTS IN FISH LARVAE

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Introduction

The need to provide adequate dietary levels of essential fatty acids (EFA) for early developing fish with unusually high growth rates and large developmental demands is undoubtedly one of the highest priorities in larval rearing. Dose-response approaches are commonly used in juvenile and adult fish nutrition studies but have been difficult to adapt to marine fish larvae, which are initially fed on live preys with a variable and difficult to control nutritional composition. Nonetheless, the recent development of new products and live food enrichment technologies has enabled the use of dose-response designs to study EFA requirements in marine fish larvae (e.g., Bransden et al., 2005; Villalta et al., 2008). However, key indicators of larval performance commonly used, such as survival, growth, pigmentation and stress resistance, might not be the most sensitive to determine exact EFA requirements. Based on the premise that EFA, and docosahexaenoic acid (DHA) in particular, are mostly retained in body tissues and are a poor substrate for the energy-generating β-oxidation system (Sargent et al., 1999), we envisaged a new method to study EFA requirements in fish larvae. The methodology is based on following the metabolic fate of tube fed ^{14}C-radiolabelled fatty acids (FA), in conjunction with dose-response studies. The underlying hypothesis is that dietary EFA supply above larval requirements will result in its increased oxidation, so the requirement for a particular EFA might be determined as the level from which it becomes significantly more catabolised (Morais and Conceição, 2008). This is the basis of a research project aiming to validate this hypothesis whose first preliminary results will be presented here.

Materials and methods

Two rearing trials were so far conducted with Senegalese sole (*Solea senegalensis*) post-larvae, looking at the dietary supply of graded levels of DHA (Trial 1) and eicosapentaenoic acid (EPA; Trial 2). In trial 1, post-larvae were reared

from 16 days after hatching (DAH) until 28DAH and were fed one of 3 experimental treatments, in triplicate tanks: 1) *Artemia* enriched on a soybean oil emulsion, containing no dietary DHA (NDHA); 2) *Artemia* enriched on a fish oil emulsion, a medium DHA regime (MDHA); and 3) *Artemia* enriched on a mixture of Easy DHA Selco (INVE Aquaculture) and Microfeed (EWOS), containing a high DHA level (HDHA). More details can be found in Morais and Conceição (2008). Trial 2 was performed from 24 to 35DAH. In this case, post-larvae were fed one of 5 treatments (diets A-E), in duplicate, composed of *Artemia* enriched with emulsions containing graded levels of EPA. This was achieved by including Incromega TG7010SR (Croda) at 0, 10, 20, 30, and 40g.100g^{-1} emulsion; DHASCO and ARASCO (Martek) levels were kept constant in all emulsions, at 30 and 15g.100g^{-1} emulsion, respectively. In both trials, enrichments using commercial products followed manufacturer's instructions and those using oil emulsions were conducted at a density of 150 nauplii.ml^{-1}, during 16h, with 0.6g.l^{-1} being added in two doses (0.4g.l^{-1} at 0h and 0.2g.l^{-1} at 8h). A single batch of enriched *Artemia* from each treatment was produced and was kept frozen for the duration of the experiment. The FA composition of the enriched *Artemia* was determined and the levels of EFA are shown in Table I.

Table I. Levels of EFA (% total FA) present in enriched *Artemia*.

Treatment	EFA composition in *Artemia* (% total FA)		
	DHA	EPA	ARA
		Trial 1	
NDHA	Nd	1.5	0.6
MDHA	3.0	6.8	1.0
HDHA	8.1	6.2	1.4
		Trial 2	
A	3.3	1.9	3.1
B	4.2	4.3	3.3
C	4.3	6.8	3.1
D	5.4	11.3	4.0
E	8.6	19.5	5.6

Tube feeding was performed at 28DAH and 34-35DAH in trials 1 and 2, respectively (as described in Morais et al., 2005), to examine the absorption and catabolism of ^{14}C-DHA in trial 1 and ^{14}C-EPA and ^{14}C-arachidonic acid (ARA) in trial 2 (all radiolabeled tracers from ARC), after an incubation period of 24h. Results are presented as percentage of absorbed (total minus evacuated) and catabolised dpm, in relation to total counts.

Results and discussion

The absorption of tube fed ^{14}C-DHA in trial 1 was extremely high (94-95%) and independent of diet (Fig. 1A). However, a significantly higher proportion of the absorbed DHA label was oxidised in larvae fed HDHA, compared to NDHA,

with an intermediate, non-significantly different, catabolism being found in MDHA-fed larvae. This suggests that increasing dietary supply of DHA does result in its increased oxidation for energy purposes and opens up possibilities to determine DHA larval requirements using this methodology.

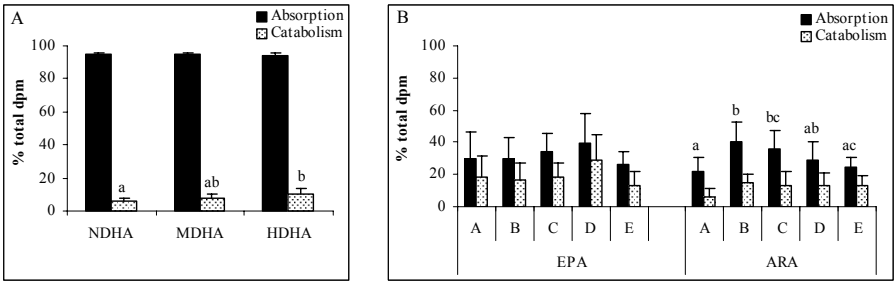

Fig. 1. Absorption and catabolism of radioactive labels (% in relation to total dpm tube fed) of Senegalese sole post-larvae fed differently enriched *Artemia* diets. A- Trial 1, tube feeding of ^{14}C-DHA; B- Trial 2, tube feeding of ^{14}C-EPA and ^{14}C-ARA.

In the second trial, a much lower fraction of the tube fed radiolabel was absorbed by sole post-larvae (26-39% for EPA and 21-41% for ARA; Fig. 1B), in comparison to DHA. Although these results were obtained from different trials and are thus not directly comparable, it could well be that, within PUFA (which are thought to be preferentially absorbed than less saturated FA), specific mechanisms exist for DHA absorption and transport. This should be examined in future experiments. On the other hand, in spite of the clear gradient in dietary EPA supply (Table I, diets A-E), no significant differences were noted in the percentage of ^{14}C-EPA catabolised by the larvae (Fig. 1B). The metabolism of ARA was also analysed, given the known competitive interactions between dietary EPA and ARA and the importance of not only their absolute levels but also of relative EPA:ARA dietary ratios in the diet (Sargent et al., 1999). Treatments B and C (EPA:ARA ratios of 1.3 and 2.2, respectively) showed significantly higher ^{14}C-ARA absorption than A and E (EPA:ARA ratios of 0.6 and 3.5, respectively; treatment E only significantly different from B). However, in terms of ^{14}C-ARA catabolism, no significant differences were noted between dietary treatments.

Conclusions

Results obtained with Senegalese sole post-larvae fed different levels of DHA have shown that the percentage of catabolised DHA increases with dietary supply. Further experiments are planned to test more graded levels of DHA, keeping other EFA levels constant, in order to determine the EFA requirements for this species during the post-larval stage. Testing finer dietary DHA gradients, may

allow more accurate estimations of requirements by determining the inflection point at which the label oxidation significantly increases. This methodology might then be tested in different species and stages of development and is expected to contribute to advance the knowledge on marine fish larvae nutrition. However, the assumption that an increase in dietary supply would result in increased oxidation for energy purposes did not prove to be true for EPA. EPA is a much better substrate for β-oxidation than DHA, as reflected in its much higher catabolism, independently of dietary treatment. It is therefore likely that the methodology suggested here might not be appropriate for the estimation of larval requirements for EPA (and possibly also for ARA). Nonetheless, given that *Artemia* nauplii always have measurable EPA levels, these results should be confirmed by a microdiet-based experiment using lower levels of EPA, in combination with lower amounts of both DHA and ARA, than those tested so far.

Acknowledgements

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MINERALIZATION PATTERNS IN THE OPERCULUM OF GILTHEAD SEA BREAM, *SPARUS AURATA*

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Skeletal deformities are a major cutback in fish production worldwide. During the rearing process of gilthead sea bream (*Sparus aurata*), an inward folding of the opercular bone is particularly common. Affected individuals show significantly diminished growth rates and a consequent downgrading of the marketing value of the product. In order to alleviate its occurrence, hatcheries need applicable screening criteria; e.g., body shape. The abnormality develops at an early larval stage: 17 days post hatching (dph) and most probably earlier. In this study, (1) nano-CT scanning is used for quantifying deviations in opercular mineralization co-occurring with abnormal opercular phenotypes; and (2) Masson's trichrome and toluidin blue histological staining are tested for their applicability to characterize irregular osteoid deposition and mineralization in early ontogenetic stages of larval development. The results of the histological analyses are presented here; the advantages and the problems of different histological staining are discussed.

We selected a range of phenotypically normal to severely deformed *S. aurata* larvi (0-65dph, up to 2mm in length, from Maricoltura di Rosignano Solvay, Italy). Three larval heads were embedded in paraffin, frontally and serially sectioned at a thickness of 5-20µm, and stained with alternating (a) Masson's trichrome and (b) toluidin blue. Another four larval heads were embedded in (c) epon, cut with a diamond knife at 1.5-5µm, and stained with toluidin blue.

(a) Toluidin on paraffin did not reveal the different tissue components of interest (osteoblasts, osteoid, and mineralized tissue were indistinguishable). The stain penetrated well, but was overall too blue. It was not possible to cut thinner sec-

tions because of severe tissue damage and deformation. (b) Masson's trichrome on paraffin stained rather faint, but the different tissue components were distinguishable. The colours differed however from what is described in literature, which makes interpretations difficult. Thicker sections (10-14µm) resulted in stronger staining and more regular colours, but small entities (e.g., osteoblasts) became unrecognizable and blurry spots emerged. (c) The epon slices curled up immediately after cutting with the diamond knife, and the toluidin did not stain properly. However, the regions that were more densely stained showed very detailed results, enabling us to distinguish the non-mineralized from the mineralized tissue.

In conclusion, toluidin staining on epon and Masson's trichrome staining on paraffin sections are promising, but protocols need to be adjusted. In this study, different cutting techniques and thicknesses have been tested. Furthermore, preliminary results with respect to the usefulness of transmission electron microscopy for studying mineralization are presented. Once the earliest signs of deviation from normal skeletal development are pinpointed, the obtained histological characters can allow us to correlate body shape characters with opercular deformations at the earliest stages.

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ONTOGENY OF CRANIOFACIAL STRUCTURES IN ATLANTIC COD (*GADUS MORHUA*), PRESENCE OR ABSENCE OF OSTERIX, A PROTEIN REQUIRED FOR OSTEOBLAST MATURATION WHICH IS REGULATED BY PGE_2

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Introduction

Experiences from both experimental and commercial production of cod larvae and juveniles reveal relative high occurrence of deformed craniofacial structures.

These deformities are less common in cod fed their natural diet, marine zooplankton, compared to rotifers which are normally used in cod hatcheries. We know from the production of halibut that the ossification process is slower when larvae are fed commercial diets (Sæle et al., 2003), which indicates that commercial diets are suboptimal for skeletal development. In the project "Investigation of mechanisms of bone development in craniofacial structures of Atlantic cod through pathways regulated by prostaglandins and retinoic acid" we aim to increase our understanding on how certain nutrients (vitamin A and arachidonic acid) may affect bone development.

A major step prior to and during the production of bone matrix is the maturation or differentiation of mesenchymal cells into active osteoblasts. This process requires up-regulation of the genes *Runx2* and *Osterix*. Zhang et al (2002) showed that PGE_2 (prostaglandin derived from arachidonic acid) could rescue the defect osteogenesis in osteoblast cultures lacking the expression of *COX-2*.

We are therefore interested in the onset of *Osterix* expression in relation to ossification processes as well as understanding how the different diets may affect the ontogeny of craniofacial bones.

Material and methods

Evaluation of craniofacial structures

Larvae were collected at 2 hatcheries, one using marine zooplankton (Lofilab AS, Norway) until 27 days after hatch (DAH), the other using rotifers (Marine Harvest Cod, Norway) until 23DAH where formulated diet (Gemma Micro) and rotifers were co-fed for a period of 7 days followed by the use of only formulated diet. Collected larvae were fixed on PBS buffered 4% paraformaldehyde for 24h and then kept on 70% ethanol at 4°C until processed. Larvae from different developmental stages were bleached and stained with either Alizarin Red S or Alcian Blue according to (Alex M. Schreiber, personal comm.). Calcein was used to observe calcification processes in vivo. Calcein (1% w/v) was dissolved in fresh salt water and filtered through a 45-µm filter. Larvae was then immersed in this water and allowed to drink for about 30 minutes. To eliminate unspecific staining, the larvae was then transferred into a larger amount of fresh salt water and left for a minimum of 40 minutes prior to documentation, using a digital camera (Olympus DP 50) connected to a stereoscope (Olympus SZX12) with a fluorescent filter (set for G excitation of SZX-RFA, exciter filter BP460-560, dichroic mirror DM580, barrier filter OG590). Pictures from all staining techniques will be shown on the poster.

q-PCR

Bio-Rad cDNA-synthesis kit (Bio-Rad) was used to synthesize first-strand cDNA from 500ng of total RNA. A semi-quantitative two step real-time RT-PCR protocol was used according to Olsvik et al. (2007). RT reaction was run in duplicates together with serial dilution series (31, 63, 125, 250, 500, and 1000ng total RNA) used to determine the PCR efficiency. cDNA amplification was monitored using real-time PCR, performed with a Light-Cycler 480 (Roche).The RT-PCR program included an enzyme activation step at 95°C (10min) and 45 cycles of 95°C (10s), 60°C (20s), and 72°C (30s). Relative expression levels were calculated using the comparative Ct method. GeNorm (Vandelsopmele et al., 2002) was used to calculate a normalization factor from the two examined reference genes (*Ubiquitin* and *RPL27*, Sæle et al. 2008). Primers (5'-3') were designed based on our cloned sequences using Primer-Blast. For *Periostin*; forward AGGAGGTGATGGAGCTGTTG, reverse TCTTCAG-GACGTGGTTCTCC, poduct size: 195bp. For *Osterix*; forward ACGTCTTGCCGTACACCTTC, reverse CTGGAATAAGCAGCTCCTCG, product size: 171bp. The *Osterix* (transcription factor Sp7) sequence used to design these primers is not fully evaluated. When BLASTing our 600-bp-long sequence, Sp8 and Sp9, which are known to have strongly homologue sequences, also comes up with equally high resemblance as *Osterix*.

Results and discussion

The poster will show that at the onset of exogenous feeding, only the cleithrum and part of the upper jaws are calcified and further that the jaws are calcified cartilage. In vivo Calcein staining was more sensitive than Alizarin Red S for the detection of early bones but one must be aware that Calcein also stains calcified cartilage. The ossified craniofacial structures develop later in cod larvae fed rotifers compared to larvae fed marine zooplankton (pictures not included in this mini paper). Further, the onset of ossification in larvae fed rotifers coincides with an increase in the expression of *Osterix* and *Periostin*, occurring around 20DAH (Fig. 1). *Osterix* expression is required for the maturation of pre-osteoblasts and is most likely regulated by PGE_2 either directly or via BMP2 (Zhang et al., 2002). *Periostin* is suggested to play a role in recruitment and attachment of osteoblasts (Horiuchi et al., 1999). As expected, these two genes have very similar expression pattern through the development, increasing with increasing level of ossification. Both genes have a relatively low expression level, even at the end of the developmental series. Results from ongoing work on the evaluation of our *Osterix* sequence will be presented on the poster.

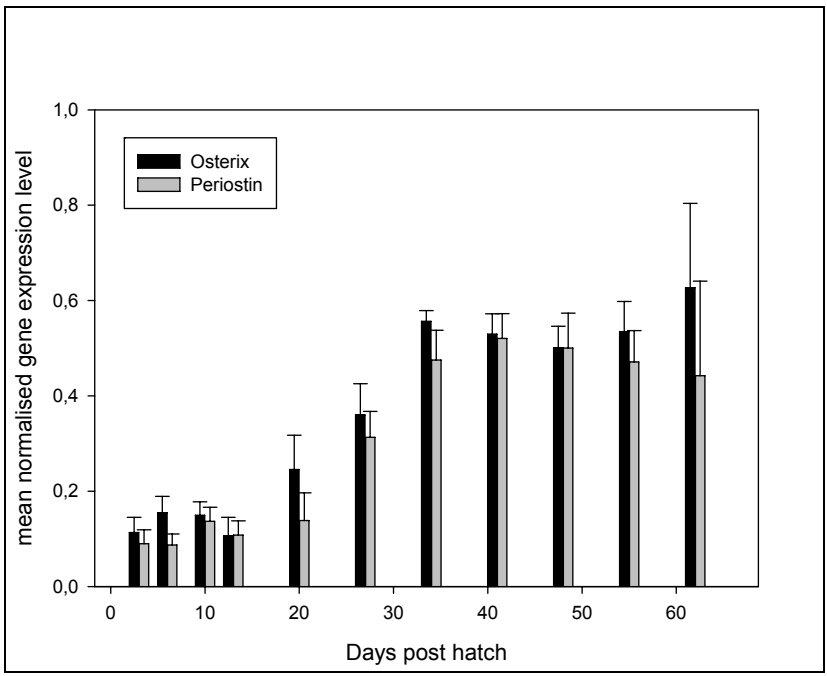

Fig. 1. Normalized expression of *Osterix* and *Periostin* in cod larvae fed rotifers until 23 days after hatch (DAH), then co-fed with roifers and formulated diet (Gemma Micro) for 7 days and finally fed formulated diet only.

Conclusion

Diets affect growth and skeletal development. Cod larvae fed marine zooplankton grow faster compared to rotifer-fed larvae. Rotifer-fed larvae of same size as zooplankton-fed larvae have fewer ossified craniofacial structures. This is in accordance to what has been shown for Atlantic halibut (Sæle et al., 2003). Both cartilage and bone structures are calcified. Preliminary results from Osterix expression show a correlation between this and the ossification of major craniofacial parts as well as a correlation with the expression of *Periostin*¸ an osteoblast marker.

Acknowledgements

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MYOSIN LIGHT CHAIN 2 IN GILTHEAD SEA BREAM (*SPARUS AURATA*): A MOLECULAR MARKER OF MUSCLE DEVELOPMENT AND GROWTH

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Introduction

White muscle is the final product of aquaculture and counts for up to 70% of the fish body mass. The mechanisms of its development and growth are of great physiological significance for the optimization of swimming performance, feeding capacity and overall larval quality and growth potential. Myosin light chain 2 (MLC2) is a component of the myosin molecule. In gilthead sea bream (*Sparus aurata* L.), an important species for Mediterranean aquaculture, two isoforms of MLC2, A and B, have been isolated and characterized. Their expression differentiates throughout development and MLC2A expression marks the phases in white muscle development of at the molecular level (Moutou et al., 2001; Sarropoulou et al., 2006). The purpose of the present study was to further investigate the potential of MLC2 as a molecular marker of muscle development and growth in sea bream. To that direction, we investigated by the means of real-time RT-PCR a) the levels of expression of MLC2 transcripts throughout the crucial stages of development, before and after hatch and up to completion of metamorphosis; b) the regulation of expression of MLC2 transcripts during myoblast proliferation and differentiation in primary muscle cell cultures of sea bream; c) the effect of growth hormone at variable doses in vivo on the expression of MLC2 transcripts in white muscle.

Materials and methods

Sea bream eggs and larvae were collected at 24, 27, 36, and 43hpf and on 1, 4, 15, 20, 24, 46, and 64dph.

Sea bream primary muscle cell cultures were established according to protocol described by Montserrat et al. (2007). Cells were harvested at 24, 36, and 48h and on 3, 4, 8, and 13 days after cultures were set up.

Four groups of sea bream of 57.3g mean weight were administered ovineGH (NIADDK-oGH-15) at 0, 0.1, 1.0, and 10.0μg.g^{-1} BW, respectively as a single i.p injection. White muscle was sampled on days 1, 2, 4, and 7 after oGH administration.

The expression levels of MLC2 transcripts in all the abovementioned experimentations were determined by real time RT-PCR method. Total RNA was isolated from cell samples using the NucleoSpin RNA II Kit (Macherey-Nagel). Total RNA from each sample was reverse-transcribed using the AffinityScript multiple temperature reverse transcriptase (Stratagene). Reverse transcription reactions were performed in duplicate and reaction products were pooled to a common sample. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis. Reactions were performed in a 20-μl volume containing cDNA generated from total RNA template, 300nM each of specific forward (F) and reverse (R) primers and Brilliant SYBR Green QPCR Master Mix (Stratagene). For normalization of cDNA loading, all samples were run in parallel with Elogation factor 1α (EF1α) and relative expression levels were determined. To estimate efficiencies, a standard curve was generated for each primer pair based on serial dilutions of cDNA pool created by mixing a) several developmental stages of sea bream, b) several stages of myocyte differentiation or c) several samples of white muscle, transcripted by total RNA.

Results and discussion

MLC2A expression increased gradually from 24hpf up to metamorphosis, when it peaked to decline afterwards. MLC2B expression remained at steadily low levels throughout development and increased slightly only after metamorphosis (Fig. 1). This expression pattern is in agreement with the previous results obtained with Northern analysis and hybridization in situ. MLC2A appears earlier in development, at the onset of somitogenesis. Its transcription remains high during the hyperplastic phase of muscle development and decreases significantly post-metamorphosis. Hybridization in situ showed that MLC2A expression marks the newly formed white fibers and post-metamorphosis it is limited to small cells located in the inter-fiber space, most probably myogenic proliferating cells. MLC2B exhibits a broader tissue expression pattern, appears post-hatch and its expression is not significantly affected by metamorphosis.

In vitro, cumulative MLC2A expression followed myocyte differentiation pattern. In order to distinguish the different development phases, myogenin, expressed at myocyte differentiation, was included in the analysis From day 4 onwards that they entered the differentiation phase marked by a gradual increase in myogenin expression, MLC2 expression increased significantly and peaked on day 8. MLC2B expression also increased during differentiation, yet the expres-

sion levels were very low. Being part of the contractile apparatus, its expression increases to provide sufficient amount of protein to support the increasing volume and function of the growing muscle cell. The interesting correlation with myogenin expression is in agreement with the presence of six E-boxes for myogenic factor binding in the promoter region of the gene (Funkenstein et al., 2007).

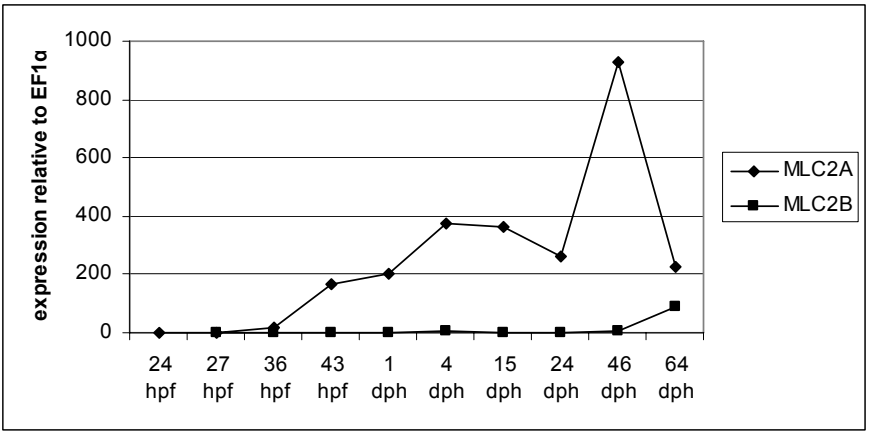

Fig. 1. The expression of the two isoforms of MLC2 during the development of *Sparus aurata*.

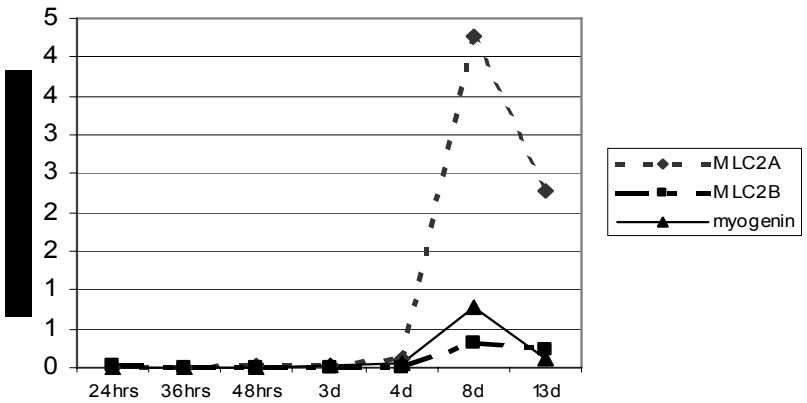

Fig. 2. The expression of the two isoforms of MLC2 and myogenin in primary muscle cell culture of *Sparus aurata*.

Expression levels of MLC2A were significantly elevated in the white muscle of sea bream on day 1 following GH admnistration. The effect was not dose dependent. On the contrary, GH did not elicit a significant effect on MLC2B expression.

Conclusions

Overall, the results of the present study are supportive of the validity of MLC2A as a marker of newly-formed muscle fibers and its potential use for the study of the effect of different physiological factors on muscle development in gilthead sea bream.

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FLOW FIELD CONTROL AT NIGHT TIME ENHANCES SURVIVAL OF *THUNNUS ORIENTALIS* LARVAE

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Introduction

The mortality of Pacific bluefin tuna (PBT), *Thunnus orientalis*, until 10 days after hatching is very high and mainly occurs during night time (Miyashita 2002). As the downward speed of larvae caused from gravitational force and infrequent swimming behavior during nighttime is higher than the upward speed of water generated from aerators in the tank, PBT larvae sink to the tank bottom. Takashi et al. (2006) suggested that the high mortality of PBT during this period could be caused by the direct contact with the tank bottom. In the present study, we measured physical characteristics of flow field in the larval rearing tank and discussed the relationship between flow field at nighttime and PBT larval survival.

Materials and methods

PBT larvae ware reared up to 10 days after hatching at three trials using between 9-15 500 l tanks on each. *Nannochloropsis oculata* and *Brachionus plicatilis* sp. complex were fed to larvae from 2 days after hatching until the end of the experiment. Light condition was provided by fluorescent lamp from 07:00 to 19:00h. An air stone was set on center of tank bottom. Aeration rate at daytime was 300ml.min^{-1} in all tanks, and at nighttime ranged from 0 to 1500ml.min^{-1}. At 10 days after hatching, survival rate at each tank and total length of 20 larvae were measured.

Flow fields in the rearing tanks at nighttime were measured by an acoustic Doppler velocimeter at each tank and average flows and turbulence's energy dissipation rate were calculated. Larval downward speeds at nighttime were also measured. Twenty larvae were collected from the rearing tank at nighttime and were anesthetized. The larvae were introduced into a 2-l cylinder. The sinking time of larvae for 10cm depth in the cylinder was measured. When larval downward speed was higher than water upward speed, this means that the larvae will potentially sink to the tank bottom. Thus, area in the rearing tank in this situation was defined as potentially sinking area.

Results and discussion

The survival rates of PBT larvae at 10 days after hatching were higher in the highest air supply treatment. Larval body density of PBT increases during the larval rearing (Takashi et al., 2006). In the present study, larval downward speed increased until the final day of experiment, and the downward speed at 9 days after hatching was highest. Thus, potentially sinking areas were estimated from the 9 day after hatching larvae downward speed. When air supply at nighttime was increased, water circulation flows became faster and bigger, and the potentially sinking areas on the bottom of the rearing tank became smaller. These means that the risk of larval sinking became lower according to air supply at nighttime. Moreover, turbulence's energy dissipation rates showed that strong turbulences in 1500ml.min^{-1} aeration rate were located over potentially sinking areas. The strong turbulences may inhibit larval intrusion into the potentially sinking areas. In conclusion, increased air supply at nighttime may prevent larval sinking and, therefore, bottom related PBT larvae death.

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ONTOGENY AND CHARACTERIZATION OF SOME INTESTINAL ENZYMES IN COBIA *RACHYCENTRON CANADUM* LARVAE

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Cobia (*Rachycentron canadum*) has been largely recognized as an excellent species for marine aquaculture due to its high market value and fast growth rate. Cobia aquaculture has become increasingly popular in a number of Asian countries, and development of commercial farming of this species is expanding to other parts of the world such as the Americas and the Caribbean regions. However, as an emerging cultured species, a reliable production of high-quality juveniles still remains a bottle neck for cobia farming production. The stomach of cobia larvae is known to become functional during the period 12-20 days post hatch (dph), simultaneous with an increase in pancreatic enzymes during this period. This suggests that cobia larvae could be weaned this early onto microdiets, but previous attempts to wean cobia larvae onto microdiet from 16dph greatly reduced survival up to 25dph, compared to those fed only live feed. So far the development of intestinal enzymes of cobia larvae has not been reported, and thus the intestinal maturation process related to larval growth and condition is not known. Such knowledge is important for a better understanding of their nutritional requirement and their digestive capacity for utilization of formulated diets. In this study, we investigate the development of the three intestinal enzymes alkaline phosphatase (AP), leucine aminopeptidase (LAP) and leucine-alanine peptidase (leu-ala). In addition, development of the enzyme activity of the pancreatic enzyme trypsin was described to evaluate larval condition. Our aim was also to study whether early weaning cobia larvae onto a microdiet from 17dph affected the larval gut maturation process.

The larvae were reared in two 500-l cylindrical composite tanks with initial stocking density of 45 larvae.l^{-1}. In one tank (LF treatment) the larvae were fed cultivated enriched preys: rotifers (*Brachionus plicatilis*) from mouth opening (2dph) to 12dph (3-8 prey.ml^{-1}, 2 times.day^{-1}), and *Artemia* nauplii from 7dph to the end of the experiment at 33dph (1-3 prey.ml^{-1}, 1-4 times.day^{-1}). In the other tank (L-MD treatment), the larvae were reared with the same feeding regime until 17dph when they were co-fed with the Otohime microdiet (Marubeni Nisshin

Feed Co. Ltd, Indonesia) for 10 days, followed by the microdiet alone to 33dph. During the course of the experiment, the larvae from each treatment were sampled for length measurement and assay of the digestive enzymes. Enzymatic activities were analysed from 2dph onward for the larvae in the LF treatment while only from 23dph onward for those in the L-MD treatment.

The larvae grew exponentially from an initial standard length (SL) 3.1±0.02mm at hatch to a finial size 20.6±0.6mm in the LF and 21.3±0.6mm in the L-MD treatment at 33dph. Weaning cobia larvae onto the microdiet from 17dph did not affect larval growth but reduced survival almost 50% compared to those fed only live feed up to 30dph. All the studied enzymes in cobia larvae were detected before onset of first feeding and they increased exponentially with larval SL. The specific activity of brush border membrane (bbm) AP in the LF treatment abruptly increased approximately twofold from 23 to 26dph ($p<0.05$), which corresponded to the SL of 17 ± 0.6 mm and 699 degree days at 26dph. A similar increase of about 2.5-fold for this enzyme activity was observed latter, from 26 to 30dph, in the L-MD treatment ($p<0.05$), which corresponded to the SL of 20.0±0.7mm and 814 degree days at 30dph. The changes in bbm LAP followed a similar trend as observed for bbm AP. However, no significant difference in the specific activity of bbm LAP was found within or between treatments. The specific activity of leu-ala in the LF treatment gradually declined from 7 to 23dph ($p<0.05$) and remained statistically unchanged to the end of the experiment ($p>0.05$). Similarly, there was no significant difference of the activity in the L-MD treatment during 23-33dph.

The gut maturation index, based on the relation between individual activity of bbm AP and a cytosolic enzyme leu-ala, strongly increased concurrently with the abrupt elevation of bbm AP activity in each treatment. The results demonstrated that the maturation of larval intestine was attained at 26dph in the LF and at 30dph in the L-MD treatment. Significantly higher ($p<0.05$) activities of intestinal and bbm AP, LAP, and gut maturation index from 30dph, and the pancreatic enzyme trypsin at 33dph in the L-MD than in the LF treatment demonstrated that the L-MD larvae had a better digestive capacity than the LF larvae from 30dph onward.

In conclusion, weaning cobia larvae to this microdiet from 17dph delayed their intestinal maturation and was also associated with lower survival compared to those fed live feed. However, prolonged feeding cobia larvae with *Artemia* after 28dph reduced their digestive capacity compared to those fed the microdiet.

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COBIA (*RACHYCENTRON CANADUM*) AQUACULTURE IN VIETNAM: RECENT DEVELOPMENTS AND PROSPECTS

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Cobia is an attractive candidate for mariculture due to its rapid growth and succulent flesh. The global aquaculture production of cobia has been increasing rapidly, up to 3000-fold over a 10-year period (1997-2007) while captures have remained stable around 10 000 tons annually. In Vietnam, research on cobia reproduction commenced in 1998 and the first commercial batches were obtained in 2002. Since then, techniques covering various aspects of broodstock management, larval rearing and fry transportation as well as grow-out in sea cages have been improved. Estimated 2008 production in Vietnam was 1500MT, meaning Vietnam is the third largest producer of farmed cobia in the world, after China and Taiwan.

Cobia fingerlings are produced from broodstock kept in sea cages. Cobia eggs can be obtained from natural or hormonal induced spawning of broodstock. Effects of dietary essential fatty acid levels on broodstock spawning performance and egg fatty acid composition of cobia were investigated recently. Cobia broodstock fed experimental formulated diets matured, spawned, and had comparable fecundity, eggs sizes, fertilization rate, hatching rate, and larval survival as compared to those fed trash fish (1.86% n-3 highly unsaturated fatty acid (HUFA) of dry weight), although a tendency of better spawning performance was observed in the group fed trash fish, which had higher level of n-3 HUFA than all formulated diets used. Fatty acid composition of the eggs was significantly different between dietary groups, and reflected that of the experimental diets. The main spawning season of cobia in Northern Vietnam takes place during April-July, and some spawnings occurred during winter (October-November), opening the possibility to expand fingerling production over a longer season.

Cobia larviculture used green water technique and production of enriched live food for the first feeding stage, followed by weaning to formulated diets in recir-

culation systems. As cobia is a fast growing species, further research in nutritional requirements is regarded as a priority to improve larval growth, survival and quality. Using umbrella *Artemia franciscana* instead of enriched rotifers did not affect growth, survival as well as vitality of cobia larvae. This success together with earlier weaning with formulated diets allows shortening the live prey feeding period, simplifying the rearing protocol and improving production as well as quality of cobia seed. In addition, research on nutrition for early juveniles also indicated that high dietary docosahexaenoic acid (DHA) and DHA/EPA ratio resulted in better growth, survival and vitality during transportation. In order to satisfy the demand for cobia seed, mass production of fingerlings in outdoor ponds has been conducted. The combination of cultured live food and natural zooplankton, mainly copepods, resulted in considerable fingerling production. However, sooner or later, the intensive production should be replaced to avoid potential pathogens (i.e., parasites).

The grow-out production of cobia in Vietnam has been conducted in both small-scale wooden raft cages and in high density poly-ethylene circular floating net cages. Cobia can grow more than 5kg per year although their growth ceases when water temperature is below 22°C. Low-cost moist pellets, made of 50% ensilaged trash fish, can be temporarily replaced by trash fish, but not for more than 2 months. Grow-out trials using extruded feed (daily feeding basis) or traditional used raw fish (feeding based on the supply from a fishing harbor) showed that cobia fed pellet feed gained double growth compared to group fed raw fish. At the moment, extruded pellets are used as sole feed in industrial-scale production of cobia in Vietnam.

The major challenges with cobia culture in Vietnam are related to the short breeding season, low temperature, and monsoon winter in the North. High mortality is caused by parasites during the juvenile phase. The viral disease known as VNN can occur during the hatchery phase. Bacterial infections have also been recorded in grow-out facilities when seawater was below 20°C in combination with starvation due to harsh weather conditions.

Vietnam has great potential for cobia aquaculture. However, further research is needed in order to improve intensive fingerling production, extend the breeding season, develop a locally formulated feed for grow-out, and diversify the culture systems in semi-open, open areas, and land-based practices. Related aspects such as post-harvest and/or processing technology and marketing are also essential to accelerate the development of this cobia farming species.

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QUANTITATIVE SHAPE ANALYSIS OF FIXATIVE INDUCED DEFORMATIONS IN EARLY LARVAL SEA BASS (*DICENTRARCHUS LABRAX* L.)

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Introduction

Aquaculture increasingly focuses on upgrading larval fish quality, but the commercial production of *Dicentrarchus labrax* (sea bass) larvae nowadays is plagued by morpho-anatomical abnormalities. To meet the priority of reducing their prevalence, the study of early manifestation of deformities in hatchery conditions can provide vital information for quality assessment. In this context, this study aims to establish a protocol for mounting of seabass larvae and quantitative body shape analysis, focusing on the potential effect of fixatives on body shape from hatching (hereby referred to as day after hatching 0 - DAH0) until 15 days post hatching (DAH14).

This assessment was done not only through the use of traditional biometric analysis, but also of geometric morphometrics that allow a more complete description of shape variation in growing fish (Adams et al., 2004). In the case of early larval stages of seabass, this can be done with the aid of Elliptic Fourier descriptors, a technique that can delineate any type of shape with a closed two-dimensional contour (Iwata and Ukai, 2002).

Materials and methods

Seabass eggs obtained by the Ecloserie Marine de Gravelines hatchery in France were transported to the Laboratory of Aquaculture & Artemia Reference Center, University of Ghent, Belgium in an isolated room with constant temperature of 16±1°C and continuous purple light with an intensity of 50lux. They were split in three groups, with hatched larvae reared until DAH14. At DAH2 and DAH14 a total of 30 specimens per group were collected, anesthetized with MS222 and photographed in a completely flat position on glass slides under a Olympus SZX9 stereoscopic microscope, while still alive. Images were captured using a

Colourview 8 digital camera, and analysed with Image J and Corel Draw 12. After a few minutes of exposure to air, the larvae quickly become dehydrated and unsuitable for further analysis. To avoid this situation, extra care was taken to use as little seawater around the specimens as possible in order to eliminate any optical distortions that could interfere with the image analysis.

Group A larvae were fixed in 8% formalin, group B in 70% ethanol, and group C in 8% formalin for 48h and then transferred to 70% ethanol. Formalin has the ability to form cross-linking between proteins in the tissues. Ethanol does not do this properly, but is good for storing specimens. Pictures were taken again after four months of storage. In order to facilitate observations and to evaluate different staining protocols for usefulness in studying structural ontogeny, specimens were then stained with histological stainings such as toluidine blue, alizarin red, haematoxylin-eosin and alcian blue as suggested by Gurr (1962), and mounted on glass slides with "Mounting Medium" by Richard-Allan Scientific.

To evaluate the effect of the different treatments, an outline-based morphometric analysis was performed – i.e., Elliptic Fourier Analysis – on the body outline of the larvae. Variation in shape during the three treatments was studied through a principal component analysis (PCA) on the Elliptic Fourier coefficients. These Fourier analyses were performed using the SHAPE freeware software by Hiroyoshi Iwata (http://life.bio.sunysb.edu/morph/index.html).

Results and discussion

Fixed group B larvae were unsuitable for shape analysis due to a large degree of fin folding and body curving, which was clearly smaller in anesthetised larvae of all groups. There was a shrinkage effect after fixation (Fig. 1). The largest shrinkage occurred in larvae of DAH2 fixed in 8% formalin for 48h and then transferred to 70% ethanol (Fig. 2).

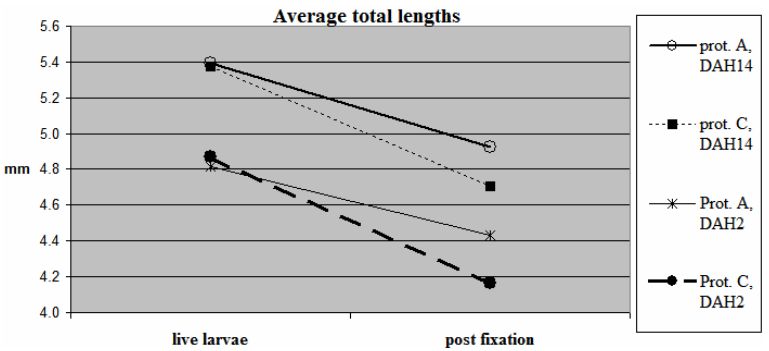

Fig.1. Shrinkage effect of fixatives in larvae of groups A & C. Differences in groups C-DAH14, A-DAH2, and C-DAH1 are statistically significant with $P<0.01$, and Prot. A-DAH14 with $P<0.05$.

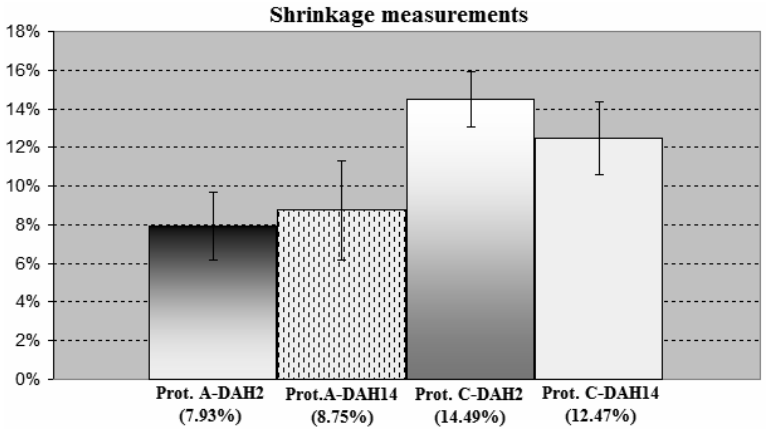

Fig.2. Degree of shrinkage in larvae of groups A & C, based on total length.

Elliptic Fourier analysis showed that shape changes due to fixation were bigger in all groups at DAH14 (Fig. 3), and that mounting introduced further shape variation by the induction of artefacts, making the mounted larvae unfit for morphometric analysis.

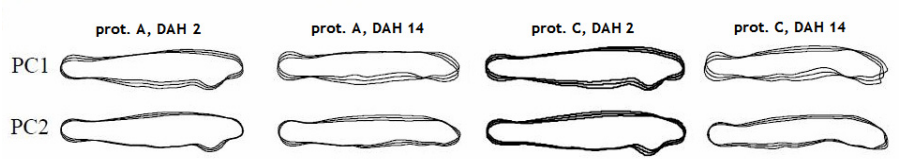

Fig.3. Contours of the two principal components of larvae shape variation due to fixation. These figures do not include shape variation induced after mounting, which was clearly noticable in the specimens due to their fragility and to the dehydration induced by the mounting medium.

In the Elliptic Fourier analysis represented in Fig.4, 61.27% of the variation is explained by PC1 and 13.19% by PC2. The graph demonstrates the differences in this shape variation between groups. Each one included its total number of larvae outlines both before and after fixation, in order to demonstrate the ability of the protocols to reflect the shape changes over time. The larvae outlines of twice the standard deviation of PC1 and PC2 are placed in the edges of the axes (-2SD and +2SD). As the larvae grow older, there is an evident shape change that reflects the depletion of their yolk sac, as well as the elongation in their form and the decrease in their body height. The shape difference between the two treatments becomes bigger as larvae reach DAH14, as can be seen from the two clusters of groups A-DAH14 and C-DAH14 spreading further apart from each other.

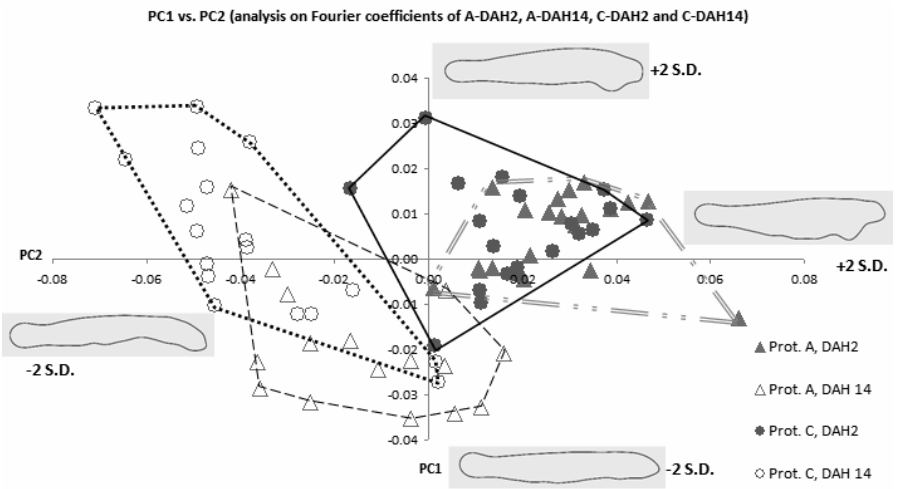

Fig.4. Plot of results of principal component analysis on Elliptic Fourier coefficients of larval outlines.

The use of different staining protocols revealed the presence of neuromasts at DAH2 (haematoxylin-eosin), epithelium epidermal cells at DAH2 (toluidine blue), and the cleithrum (pectoral girdle) at DAH14 (alizarin red).

Conclusions

Due to the fact that all protocols induced varying degrees of specimen shrinkage, the testing of different fixation techniques may be advisable for specimen storage and mounting. Geometric morphometrics demonstrated the ability to reflect larval shape changes in our experimental setup. The artefacts induced by our mounting procedure rendered it suitable only for purposes of qualitative analysis. However, this outline-based technique can be applied in all cases of early larval sea bass until DAH14, where a quantitative analysis of shape changes may assist in applied hatchery research for larval quality assessment.

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CHANGES IN LIPID CLASS AND FATTY ACID COMPOSITION IN STRIPED JACK (*PSEUDOCARANX DENTEX*) EGGS

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Introduction

The production of high quality fertile eggs with high larval survival and growth rates is the key to successful aquaculture. Chemical composition of fish eggs is species-specific and reflects the embryonic demands both for nutrition and growth (Yanes-Roca et al., 2009). Striped jack (*Pseudocaranx dentex*) is one of the economically most important fish among the Carangidae family in Japan reared from the 70s decade. Still, little information is available on their spawning behavior and chemical components of the produced eggs. The aim of the present study was to examine the lipid content, lipid classes, and fatty acid composition of striped jack eggs and related to their quality from the early, middle and late spawning season.

Materials and methods

The striped jack broodstock, collected from the wild in 2001, were kept in a $10m^3$ tank until 2008. Fish were fed near satiation once a day, six days a week with a moist pelleted diet (Lansy Breed Maturation, INVE) and twice a week with a mixture of low commercial value. Spontaneously spawned eggs were collected daily in a net basket and stocked in 20-l cylindroconical incubation tanks with filtered water and air supply. To estimate the total number of produced eggs, ten samples of 5ml each were analysed under the stereoscope, using the method of Fernandez-Palacios et al. (1994). In each sample two fractions were distinguishable: the percentage of viable buoyant eggs and the second fraction corresponding to the sinking eggs (non-viable). Every week 30 viable eggs were collected for biometric measurements under the microscope, using a program image analysis (Axion Vision Release 4.3 Zeiss). Samples were taken at the beginning, middle and end of the spawning season and analysed for total lipid content and fatty acid composition. Lipids were extracted with a chloroform-methanol mixture (1:2 v/v) according to Bligh and Dyer (1959). Lipid classes were separated using silica column and solvent elution sequence proposed by

Guckert et al. (1985) and Smith et al. (1986). In order to determine the fatty acid profiles, methyl esters (FAMEs) were prepared according to the Lepage and Roy method (1986) modified by Cohen et al. (1988). Chemical analyses were carried out, at least, in duplicate, and the present results are the average of the values obtained with standard deviation. Excel and SPSS 14th edition were used to perform statistical analysis. Data were subjected to univariate ANOVA followed by Tukey's multiple comparison test. A Spearman correlation factor was performed due to the data normality.

Results and discussion

In our culture conditions, the spawning season of wild striped jack kept in captivity lasted two months (May 5 to June 29), corresponding to temperatures ranging from 19.5ºC to 21.9ºC. During the spawning season (20 spawning days), the total number of eggs collected was estimated at 10.8 millions (10 789 780), of which 42.90% were sinking eggs and 56.63% were buoyant eggs. The number of eggs collected at each release varied between 15 600 and 1 430 400 with an average number of eggs per batch of 674 361. The egg releases and viability rate are shown in Fig. 1. The striped jack eggs were spherical, non-adhesive and pelagic with a mean diameter of 0.969±0.027mm and an oil globule of 0.279±0.017mm, similarly to the results found by several authors (Watanabe et al., 1998; Vassallo-Agius et al., 2001). The mean daily egg diameter fluctuated, but towards the end of the season the mean size declined when water temperature rose above 21ºC. There was a negative correlation between egg diameter and temperature (r-value= -.263; p-value=.000).

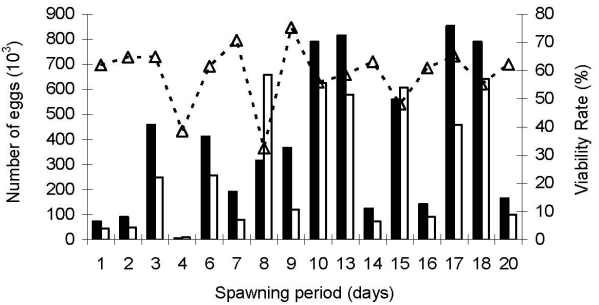

Fig. 1. Spawning frequency, number of eggs per release (black bars: viable eggs; white bars: non viable eggs) and viability rate (%) of striped jack during the spawning season.

It is known that lipids affect spawning and the egg quality of many fish species due to their role as an energy source and as structural components in membrane biogenesis. Total lipid (TL) and the lipid class were studied. The fatty acid com-

position of TL, neutral lipid (NL), glycolipids (GL), and polar lipid (PL) were determined in striped jack eggs during spawning season.

TL not differ significantly throughout the spawning season, with a mean value of 16.15±1.59% (on dry basis). Fatty acid profile showed a typical pattern of marine fish eggs. The most abundant polyunsaturated (PUFA), monounsaturated (MUFA) and saturated (SFA) fatty acids observed were docosahexaenoic acid (22:6n-3), oleic acid (18:1n-9) and palmitic acid (16:0), respectively (Table I). In TL the (n-3) series were much more abundant than those of the (n-6) with an overall ratio (n-3)/(n-6) around 5%. During the spawning season, and in general terms, it appeared that the levels of EPA, DHA, (n-3) HUFA and (n-3)/(n-6) tended to decrease near the end ($p<0.05$). On the other hand, no significant differences in SFA and PUFA values were found between the beginning and the end of the spawning period.

The lipid class data show that the major portion of the lipid is the NL (46.9-83.5% of TL) with a maximum value in the middle of the season where the viability rate is higher. The percentage of monounsaturated fatty acids (MUFA), particularly oleic acid, was higher in NL (43.67-45.41). Also, in this fraction predominate the n-3 fatty acids (21.31-30.65%), namely the C18:4, C20:5, and C22:6, with a significant decrease during the spawning season. In addition, PUFAs are more accumulated in the middle of the season

Table I. Main fatty acid composition of total lipid (TL) and the neutral fraction (NL) in striped jack eggs along the spawning season*.

	TL			NL		
	Initial	**Middle**	**Final**	**Initial**	**Middle**	**Final**
Total Lipid+	17.33±0.75	16.94±0.08	15.75±0.96	8.13±0.35	11.71±0.06	10.30±0.63
14:0	1.83±0.00	1.76±0.01	2.06±0.09	2.09±0.17	1.88±0.02	2.02±0.05
16:0	15.03±0.03	14.80±0.01	14.34±0.01	12.19±0.55	11.51±0.06	12.76±0.11
18:0	3.58±0.02	3.52±0.01	4.18±0.24	1.70±0.13	0.81±0.12	1.17±0.04
Total SFA	28.91±0.07	29.58±0.12	26.75±0.31	16.37±0.56	14.78±0.24	16.60±0.22
16:1	5.85±0.03	5.37±0.05	5.65±0.17	7.52±0.25	6.32±0.12	6.75±0.08
18:1	18.75±0.04	18.46±0.23	19.59±0.26	26.07±0.04	24.92±0.14	25.89±0.13
20:1	0.76±0.01	1.02±0.01	1.11±0.00	0.72±0.05	1.57±0.01	1.58±0.00
Total MUFA	25.48±0.00	24.97±0.28	27.01±0.43	45.41±0.64	43.67±0.07	44.23±0.10
18:3n-3	0.69±0.00	0.79±0.00	0.71±0.02	0.86±0.03	1.85±0.02	1.85±0.00
18:4n-3	2.85±0.02	3.00±0.03	2.63±0.16	4.72±0.05	3.89±0.02	3.48±0.09
20:4n-6	0.08±0.00	0.08±0.00	0.09±0.00	0.00±0.00	0.00±0.00	0.00±0.00
20:5n-3	5.96±0.00	5.21±0.08	5.64±0.12	5.26±0.32	4.26±0.05	3.72±0.02
22:6n-3	21.14±0.03	20.00±0.42	19.90±0.89	16.37±0.77	11.34±0.26	9.42±0.10
Total PUFA	39.91±0.25	39.86±0.44	40.45±0.91	38.22±1.20	41.55±0.31	39.18±0.31
n-3	32.86±0.28	31.09±0.51	32.61±1.01	30.65±1.18	24.46±0.22	21.31±0.19
n-6	5.98±0.02	7.64±0.05	6.55±0.10	0.00±0.00	0.14±0.01	0.15±0.00
(n-3)/(n-6)	5.50±0.06	4.07±0.09	4.98±0.23	204.21±1.56	169.59±8.41	144.51±0.28
(n-3)HUFA	28.97±0.02	26.95±0.54	29.22±1.17	23.96±1.05	17.66±0.26	14.88±0.16
DHA/EPA	3.55±0.00	3.84±0.03	3.52±0.08	3.11±0.04	2.66±0.03	2.53±0.01

* Results expressed in % of total fatty acid area. Values are mean ±SD, n=4; + % on dry basis.

Conclusions

To establish the most productive months in terms of eggs viability, striped jack eggs were analysed for total lipid, lipid class and fatty acid composition. The spawning season of striped jack in captivity lasted two months, showing variations in the floating and hatching rate. Total lipid content did not differ significantly throughout spawning season but the lipids fractionation indicates that the neutral lipid (the main fraction) has a maximum value in the middle of the season where the viability rate is higher. The results of this study indicate that striped jack eggs are rich in PUFAs which are essential in terms of fertilization success and larval development.

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EFFECT OF ENRICHED *HUSO HUSO* AND *ACIPENSER PERSICUS* LARVAE WITH HUFA AND VITAMIN C ON THEIR NUTRITIONAL VALUE

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Introduction

Economically, sturgeons are very important in Caspian Sea fisheries. *Acipenser persicus* and *Huso huso* are two important species of sturgeons in the Caspian Sea: *A. persicus* is endemic to Iranian waters and *H. huso* is the largest fish in the Caspian Sea producing the most valuable roe. Although the importance of *Artemia* in Iran sturgeon larviculture was first reported by Takami (1987), and is widely practiced in commercial hatcheries, no studies have been reported on effects of feeding of *Acipenser persicus* and *H. huso* larvae with various forms of brine shrimp *Artemia* on vitamin C and fatty acid profile.

Materials and methods

Fish larvae of both species after yolk sac absorption were randomly distributed in 8 different treatments and 4 replicates. All tanks were connected to a flow-through system with a flow rate of 1 l.min^{-1}.tank.

In total, eight feeding treatments were tested. The first seven feeding regimes were newly-hatched *Artemia* nauplii (N), *Artemia* nauplii enriched with saturated lipid (SL) supplemented with 0, 10, or 20% ascorbyl palmitate (treatments 2-4), and *Artemia* nauplii enriched with highly unsaturated fatty acids emulsion (HUFA) supplemented with 0, 10, or 20% ascorbyl palmitate (treatments 5-7). *Artemia* nauplii in all 6 batches were enriched using the standard enrichment protocol of Léger et al. (1987). The fish larvae in treatment 8 were fed decapsulated *Artemia urmiana* cysts (DC). The larvae were fed at 25% body weight per day (BW.day^{-1}), with a feeding frequency of 6 times per day. The pH (7.3-7.5),

temperature (19-20±1°C) and dissolved oxygen level (7.00-7.65mg.l^{-1}) of each tank were monitored twice a day.

Fatty acids and vitamin C analyses
Batches of *Artemia* nauplii samples (enriched and non-enriched), decapsulated cysts, and fish larvae from each treatment were collected regularly and immediately stored at -80°C until later analysis. The samples were analyzed for ascorbic acid by reverse-phase ion-pair high performance liquid chromatography (HPLC) procedure coupled with electrochemical detection and internal standard quantification based on iso-ascorbic acid (IAA) according to Nelis et al. (1997).

Fatty acid composition of nauplii and fish larvae samples was determined by gas chromatography. Fatty acid methyl esters (FAME) were prepared via a modified procedure of Lepage and Roy (1984). This method implicates a direct acid catalyzed transesterification without prior extraction of total fat. To each sample 10% of an internal standard (20:2n-6) was added prior to the reaction. FAME was extracted with hexane. After evaporation of the solvent the FAME was prepared for injection by dissolving them in iso-octane (2mg.ml^{-1}). Quantitative determination of fatty acids was done by a Chrompack CP9001 gas chromatograph, equipped with an auto sampler and a temperature programmable on-column injector (TPOCI) (Lepage et al., 1984).

Statistical analysis
Results were analyzed by analysis of variance, ANOVA. The homogeniety of variances and the normal distribution test was done according to Levene and Shapiro-Wilk test and comparison among treatment means were made by Duncan's multiple range test (DMRT) (Sokal and Rohlf, 1969). All statistical analyses were conducted using SPSS (version 13) and tested at $p < 0.05$.

Results and discussion

The results obtained in this study showed a significant increase in HUFA and vitamin C level in the *Artemia* nauplii after 24 hours enrichment with self emulsifying lipid emulsion rich in DHA and EPA and AP (Fig. 1). The amount of EPA and DHA in *Artemia* nauplii increased 5- and 7-fold, respectively. The quantity of these two fatty acids further increased significantly when AP was incorporated into the lipid emulsion ($P < 0.05$), which may be due to the antioxidative nature of ascorbic acid.

Fatty acid analysis of the fish larvae revealed that the highest amount of HUFA was incorporated into body tissue of the fish fed HUFA-enriched Artemia (Fig. 2). However, it was observed that the fish larvae fed on non-enriched Artemia and decapsulated cysts of *Artemia* also had considerably high EPA and DHA. Based on this finding it can be assumed that *A. persicus* and *H. huso* larvae are

able to synthesize these highly unsaturated fatty acids immediately from the first day of active feeding.

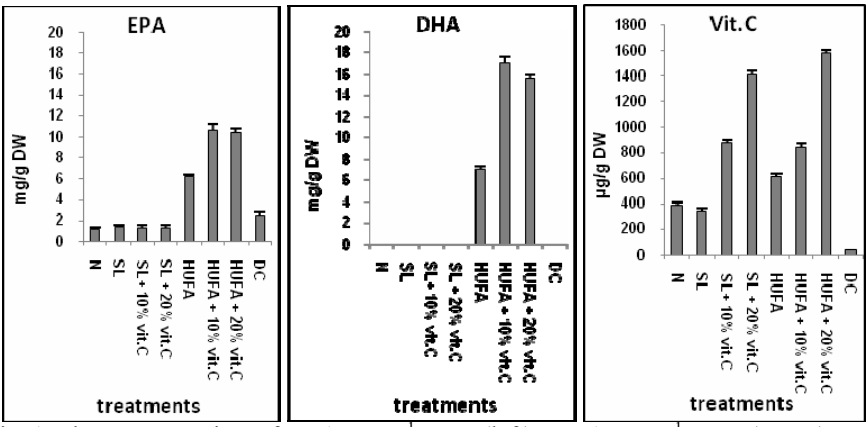
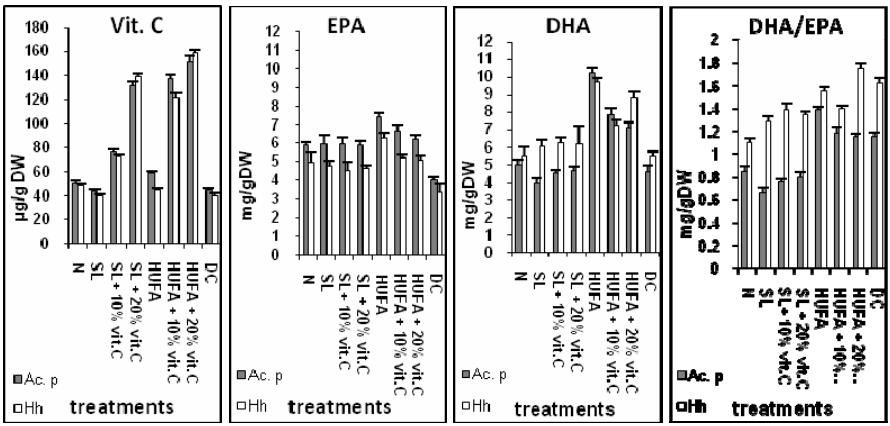


Fig. 1. The concentration of EPA mg.g^{-1} DW (left), DHA mg.g^{-1} DW (center), and ascorbic acid µg.g^{-1} DW (right) measured in decapsulated cysts and *Artemia urmiana* nauplii with/without different enrichments.



A B C D

Fig. 2. Contents of Ascorbic Acid µg.g^{-1} DW (A), EPA mg.g^{-1} DW (B), DHA mg.g^{-1} DW (C), and DHA/EPA (D) in *A. persicus* and *H. huso* larvae fed on different feeds.

The amount of vitamin C increased significantly in *Artemia* nauplii supplemented with AP and also in fish larvae fed on these enriched nauplii compared to the other feeds without AP supplementation ($P < 0.05$) (Fig. 2). This study confirms earlier findings that a diet of HUFA and vitamin C-enriched *Artemia* nauplii results in bio-accomulation in fish larvae (Merchie et al., 1995; Evjimo et al., 1997; Coutteau and Mourent, 1997; Gapasin et al., 1998; Han et al., 2000).

These research findings prove that the use of *Artemia* nauplii as live food enriched with DHA and EPA, supplemented with vitamin C is reflected in the whole body tissue of sturgeon fish larvae (Fig . 2).

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EFFECT OF GRADED LEVELS OF ARACHIDONIC ACID ON REPRODUCTIVE PHYSIOLOGY, SPAWNING PERFORMANCE, AND EGG QUALITY OF ATLANTIC COD

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The levels of arachidonic acid (ARA) are higher in eggs of wild fish than in farmed fish and in fish fed Mediterranean trash fish than in those fed formulated feeds with northern hemisphere oil. Further, supplemental ARA gives better spawning performance and egg quality in both Japanese flounder and Atlantic halibut. The present experiment was designed to investigate whether ARA has similar effects on the spawning performance of Atlantic cod. The hypothesis was that increasing levels of ARA, would induce increasing levels of eicosanoids in the gonad of female cod, which then would stimulate estradiol production and vitellogenisis and improve fecundity and egg quality.

Cod broodstock held in net pens were fed 0.5, 1.0, 2.0 or 4% ARA (of total fatty acids) for 9 months prior to spawning (May-January). Samples were taken every month to analyse fatty acids, eicosanoids, hormone profiles and vitellogenin. Just before spawning, the fish were moved to spawning tanks with collection of floating and sinking eggs to assess realised fecundity. Floating eggs were incubated both in the hatchery and in nunc plates and fertilisation and survivals were determined.

There was a linear increase in ARA concentration in the female gonads, from 1.8 to 7.1 % of total fatty acid in response to the increase in dietary ARA ($p<10^{-6}$). Prostaglandin E_2 and F_2 (PGE_2 and PGF_2) concentrations showed large within group variation, however, regression analyses revealed a significant increase in PGE_2 concentration in November and January ($p<0.001$) and in PGF_2 concentration in January ($p<0.004$), in response to dietary ARA. In February, after the fish had started spawning, there was no such relationship. Plasma estradiol peaked in January in females fed 1 and 2% ARA and in February in fish fed 0.5 and 4% ARA, while plasma vitellogenin concentrations peaked in December in fish fed 0.5% ARA and in November in fish fed the other three diets. Relative fecundity was higher in fish fed 1% than in fish fed 4% ARA, while the other groups had intermediate fecundity. Egg fertilisation rate and survival was not affected by the

diets. The spawning period was extended with 2-3 weeks in fish fed the diet with 4% ARA, compared to the other groups.

The experiment shows that ARA affects the reproductive physiology of cod and that the optimum level of dietary ARA indicated by the results is 1-2% of fatty acids.

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DELIVERY OF WATER SOLUBLE MICRONUTRIENTS TO ROTIFERS (*BRACHIONUS PLICATILIS*)

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Several species of marine fish larvae fed rotifers have reduced growth, lower survival and higher rates of malformation compared to larvae fed wild zooplankton such as copepods. For this reason copepod nutritional content has been used as a benchmark when manipulating the nutritional content of rotifers. The effect of modifying the fatty acid profile of rotifers has been carefully studied and has shown to significantly improve larval quality. Meanwhile, the modification of rotifer water soluble vitamin and mineral concentration has not been thoroughly investigated. Especially for the minerals iodine, manganese, copper, zinc, and selenium there is large difference in concentration between rotifers and copepods. The concentrations of these minerals in rotifers are from twofold lower for Mn and up to 30-fold lower for Se compared to copepods and they may therefore be below the requirement level for marine fish larvae.

Manipulation of the nutritional content in rotifers is mainly done during short-term enrichment (1-3h) prior to feeding the fish larvae. However, it is important to remember that in comparison to *Artemia*, rotifers can also have the nutritional content modified during the culture stage (barring the added nutrient doesn't having a negative effect on the rotifer culture). Rotifers supplied with Cu, Zn, and Se during the culture phase reached controllable and high levels of the respective minerals. As shown for *Acartia* and *Artemia*, long-term accumulation of micronutrients during the culture phase may lead to different tissue distribution than short-term exposure, which may have an impact on bioavailability. Manipulation of the nutrient level during the culture phase also leaves room for focusing on manipulation of other nutrients during the short-term enrichment phase.

The most common method for the delivery of water soluble micronutrients to rotifers is by dissolving the nutrients directly in the aqueous phase. Delivery through the aqueous phase is easy and there is no need for sophisticated delivery vehicles. However, the uptake efficiency has in many cases shown to be very low and therefore this method can be both expensive and not environmental sound. The rotifers uptake efficiency of Mn, Zn, and Se delivered as chelated

and organically bound minerals in particles was from 2 to 50 times as efficient as minerals dissolved in the aqueous phase.

An aqueous delivery will lead to an unknown route of uptake, and it will not be known to which extent the nutrient is taken up over body surface, breathing organ or by drinking and thereby through the digestive system. The route of uptake will most likely vary significantly from large complex vitamins, where the only route of uptake may be through drinking, to minerals in its ionic form which may have a high diffusion rate over the body surface and breathing organ. Several results have shown a relative low uptake efficiency of aqueous dissolved vitamins, while relative better uptake efficiency has been shown for minerals in ionic form. The detoxification/storage product may be different for the different routes of uptake. This may play an important role for determining the bioavailability of the minerals in feeding trials. Cod larvae fed Se enriched rotifers had a 4.5-fold higher Se concentration than control larvae by 29dph (3.99±0.15 versus 0.88±0.02mg $Se.kg^{-1}$ DW, respectively). However, cod larvae fed rotifers enriched with Se enriched yeast retained 8% more Se than those fed Na-Se enriched rotifers. It is not known if this difference is due to route of uptake or difference in source of Se. These results show that care must be taken when comparing nutritional trials were rotifers are enriched with minerals through different vectors and in different chemical forms.

A protocol has now been developed for enrichment of rotifers with controlled levels of Mn, Cu, Zn, and Se over large concentration ranges. The concentrations of minerals were reproducible at all concentration levels and the respective minerals can be delivered within a narrow window. This is a good tool for running dose response studies. To reach copepod levels of Cu, Mn, Se, and Zn within the same batch of rotifers, less than a 10% replacement of the commercial enrichment product was necessary. This is important considering the large quantities of other nutrients such as lipid and proteins that needs to be delivered.

In most hatcheries rotifers are enriched once or twice a day. Depending on feeding regime, fish larvae will therefore eat rotifers that have been stored from 2-20h. The capability of the rotifers to retain the nutrients for a prolonged period is therefore important. The retention period may vary largely depending on the delivery method (particles vs. aqueous), length of the enrichment period and the enriched nutrient. Se-enriched rotifers had a minimum of 86% retention after 10h storage, while iodine-enriched rotifers lost, depending on length of enrichment, 80-100% within 2.5h storage. These results show the importance to know the retention period for all nutrients that are to be investigated in nutritional studies. The recommended requirement of different nutrients may be based on feeding trials with an unknown retention period. This is important to consider when changing the enrichment protocol or the feeding regime, especially for nutrients with a narrow optimal window between requirement and toxicity.

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STATUS AND CHALLENGES IN COD LARVAL PRODUCTION

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The seafood industry represents Norway's largest export industry after oil and gas. Both governmental and industrial interests have focused on Atlantic cod in order to bring the species into commercial cultivation. Cod is now Norway's third largest aquaculture species in terms of production. One main challenge in cod farming is production of high quality juveniles at a reasonable price. The production of cod juveniles was earlier undertaken in closed fjords (basins) using extensive methods. The first successful intensive production of cod juveniles in Norway was done at SINTEF in 1987. Several hatcheries for cod have been established, and today nearly all are based on the intensive production method. Two hatcheries are producing cod larvae by extensive methods, using harvested copepods as live feed, and these larvae, serving as a reference for the intensive producers, have a higher growth rate and less deformities than intensive produced cod larvae. The industry has expressed a need for easy methods to estimate the larval quality, and development of a larval quality standard has high priority.

A challenge in juvenile production is the unexpected variation in egg quality. Egg batches with low hatching success or asynchrony hatching results in variable larval survival. Another problem in the current juvenile production is that cod farms, by some unknown reason, suffer unacceptably high losses after transfer of juveniles to the sea. This problem has called for major attention in research and developments in order to find the optimal nutritional and feeding protocols for cod larvae and juveniles. The problem is suggested to originate in specific challenges in the biology of cod larval development or insufficient live feed quality. Several hatcheries and nurseries apparently apply comparable protocols in terms of water treatment, nutrition and handling. Despite this, considerable variations in growth, survival, and quality of the fish are often reported. These variations in success of production indicate other factors affecting the vitality of the larvae, such as e.g, environmental, broodstock and human factors.

An economically sustainable cod farming industry depends on a stable supply of large quantities of high quality cod juveniles at low costs. However, the production process of marine juveniles involves a range of rather complex operations, including production of live feed organisms, first feeding of larvae, multiple gradings, vaccinations, and juvenile ongrowth. These factors impose strict requirements on the production process in terms of knowledge, rearing protocols, equipment and logistics. Even if cod juvenile production involves complex biological issues, the production processes could benefit technologically by adopting principles known from other process oriented industries. General technology trends in industrial development suggest that incorporation of specialized instrumentation, automation and control systems may improve operational stability, product quality and efficiency, at the same time as they will increase profit margins by reducing labour intensive operations in the production process. Considering Norwegian conditions where labour costs are exceedingly high, this observation is even more important. These features correspond well to the requirements of large quantities, high quality and low unit prices of cod juvenile production. The key differences between process oriented industry (metallurgical and petrochemical industry, power plants, paper mills, textile industry, automotive industry etc.) and aquaculture production is that the latter involves living organisms interacting in a complex biological processes. Proper understanding and quantitative descriptions of the mechanisms governing growth, functional development, survival and quality of larval and juvenile fish are important topics of active research. Because design of efficient process control systems depends on intimate knowledge of the underlying processes, it is essential that researchers from the areas of aquaculture biology and control engineering cooperate in developing modern production systems for juvenile fish. NTNU and SINTEF recently completed the research program “CODTECH – a process oriented approach to intensive production of cod juveniles”. In this presentation results from CODTECH and some other projects will be reviewed and ideas for new marine larval production technologies will be launched.

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CAN ATLANTIC HALIBUT (*HIPPOGLOSSUS HIPPOGLOSSUS*) BE VACCINATED DURING LIVE FEED PHASE?

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Introduction

Atlantic halibut larvae are primitive at hatching, and have a prolonged yolk-sac stage. Marine larvae hatch in an environment where they are exposed to numerous pathogens, and high rates of mortalities have been observed during first feeding stages in halibut aquaculture (Grøntvedt and Espelid, 2003; Austin, 2006). This emphasises the need to establish adequate prophylactic counter measures like vaccination and use of probiotics. Development of successful vaccines depends mainly on the ability to activate the specific immune system, and synthesise antibodies and memory cells. However, immunisation before the fish is able to mount an effective immune response can induce tolerance resulting in the lack of response to later stimulation (Patrie-Hanson and Ainsworth, 1999; Rombout et al., 2005). Thus, ontogeny of the kidney, thymus, and spleen in addition to detection of B- and T- cell markers during larval development in Atlantic halibut was studied.

Materials and methods

Eggs from one female from the halibut broodstock maintained at Austevoll Aquaculture Research Station were stripped, and fertilised with sperm from a single male. Eggs and larval stages up to 45dph were maintained at 6°C, and larval stages from 45dph and onwards at 12ºC as described (Mangor-Jensen et al., 1998). The larvae were fed with enriched *Artemia* until weaning to dry feed at approximately 120dph. All sampling at 49, 52, 59, 66, 73, 80, 87, 94, 102, 108, 115, 122, 129, 144, and 159dph (318, 354, 438, 522, 606, 690, 774, 858, 954, 1026, 1110, 1194, 1278, 1458, and 1638ddph, respectively) was carried out from tanks.

To avoid disturbing larvae during the critical yolk sac phase and to ease sampling during the rearing period in silo, some fertilised eggs were also transferred to 6-well plates at 10dpf with 10ml of sterile sea water (SSW) with a salinity of 25ppt as described earlier. Approximately 8 eggs were transferred to each well, and the eggs were inspected daily. The day after hatching, 2 larvae from each well were transferred to new plates to reduce the risk of bacterial contamination from the empty egg shells. Larvae from multi-well plates were sampled at the following dph: 1, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, and 45 (6, 18, 42, 60, 84, 102, 126, 144, 168, 186, 210, 228 and 270ddph, respectively).

The larvae were fixed in 4% phosphate-buffered paraformaldehyde, dehydrated in ethanol, cleared in xylene, infiltrated in paraffin, and embedded in histowax. Serial sectioning (3µm) of larvae was performed for morphological analysis, IHC, or in situ hybridisation using a Leica RM 225 microtome (Leica Microsystems). Histological sections were dewaxed and stained with Haematoxyline-Erythrosin-Safran (HES). Immunohistochemistry was performed using mouse anti-halibut IgM monoclonal antibody (Aquatic diagnostics) as described previously (Patel et al., 2009a). For in situ hybridisation, probes against halibut IgM (Patel et al. 2009a), and CD4 (Patel et al., 2009b) were prepared using the DIG-AP RNA labelling kit (Roche Molecular Biochemicals) following the manufacturer´s protocol.

For real time RT-PCR, Taqman probes were designed such that they spanned exon-exon boundaries in order to avoid amplification of genomic DNA. The assays for B- and T-cell markers such as IgM, CD8α, CD8β and CD4 have been described earlier (Patel et al., 2008; 2009a; 2009b).

Results and discussion

All three lymphoid organs, spleen anterior kidney and thymus were morphologically well developed during late metamorphic stages. Real time RT-PCR analysis revealed that IgM mRNA expression at 66dph and later, while a clear increase in expression of CD4, CD8α and CD8β mRNA could be seen at 80dph and onwards. The immuno-histochemical detection of IgM showed the presence of IgM protein in both kidney and spleen at 94dph, while in thymus it could not be detected until 108dph. Since antibodies against the T-cell markers for Atlantic halibut are not yet available, it was not possible to analyse when the functional proteins are developed.

In conclusion, Atlantic halibut seems to develop its immune system during the metamorphic stages. Thus vaccination of juvenile before 94dph may result in tolerance. The right time for vaccination after 94dph, depends on the detection of functional proteins of other immune markers, and thus such studies are warranted.

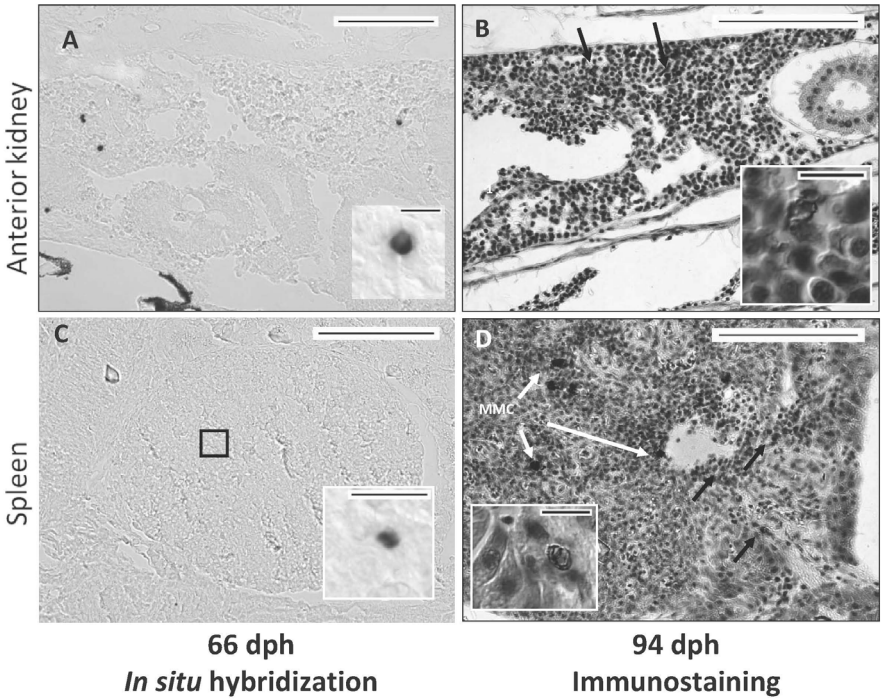

Fig. 1. Expression of IgM in kidney and spleen from Atlantic halibut during ontogenesis. I*n situ* hybridisation showing IgM mRNA expression in the (A) kidney and (C) spleen. Immunostaining with an anti-halibut IgM monoclonal antibody (red staining) in the (B) kidney and (D) spleen. The scale bar in A, and C is 100µm; while in B and D is 50µm. The scale bars in the insets in all panels represents 10µm. Red staining in panels B and D indicates IgM positive staining. Abbreviation: Melanomacrophage (MMC). Figure modified from (Patel et al., 2009a).

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EFFECT OF DIFFERENT BIOFILTER MEDIA QUANTITIES ON *LOPHIOSILURUS ALEXANDRI* LARVICULTURE

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Introduction

Freshwater fish larviculture in Brazil is often carried in outdoor fertilized ponds. Recently, larviculture also has been done under laboratory conditions with continuous water flow and high flushing rate. Research using closed systems is not plentiful on neotropical freshwater larviculture (Pedreira, 2003). The aim of this study was to investigate the effect of different biofilter media quantities on *Lophiosilurus alexandri* (Siluriforme: Pseudopimelodidae) larviculture.

Materials and methods

L. alexandri larvae at eight days post-hatching (length of 12.9±0.4mm and weight of 19.2±0.7mg) were stocked at a density of 15 larvae.l^{-1}, in 16 5-l aquaria. The photoperiod was 10L:14D. The water temperature was 25.1±1.2ºC and dissolved oxygen was 4.9±0.7mg.l^{-1}. Four different biofilter media quantities were chosen: Q_0 – without biofilter media; Q_{40} – 40g.l^{-1} of biofilter media; Q_{80} – 80g.l^{-1} of biofilter media; and Q_{160} – 160g.l^{-1} of biofilter media, with four replicates each. Larvae were fed at 0900, 1300, and 1700h. The daily prey concentrations were 700, 1050, and 1400 *Artemia* nauplii.larvae^{-1} from the 1st to the 5th, from the 6th to the 10th, and from the 11th to the 15th day of feeding, respectively.

Inside of each aquarium a submerged biological filter was installed that had an Air lift tower (2cm diameter) emerging 5cm above the water level. A grit biofilter media (1.3cm average diameter) was used to complete the different biofilter media quantities, previously prepared for biofilm growth during 30 days.

At the beginning of the experiments pH, turbidity, and alkalinity was 7.09, 0 NTU, and 30mg $CaCO_3$.l^{-1}, respectively. Samples of water to determine ammonium ion and nitrate levels (Koroleff, 1976) were done every five days. The

aquaria were siphoned daily to remove waste, after which 10% total volume was renewed. After 15 days of feeding, survival, total length, wet weight, and water parameters were measured.

Data of survival, growth, and water were compared by one-way ANOVA. Ammonium ion and nitrate levels were compared by two-way ANOVA. Means were compared using Tukey's test at 5% probability level using SigmaStat 3.5.

Results and discussion

The different treatments did not affect survival and growth of *L. alexandri* after 15 days of rearing (Table I).

After 15 days of feeding, pH and alkalinity were lower in the without biofilter media treatment (Table I) and decreased over time, showing increased acidification of water. In the other treatments, pH and alkalinity were similar between them, and higher compared to the initial levels. The turbidity was seen only in the without biofilter media treatment (Table I), suggesting that the biofilter media also provided some mechanic filtration.

Table 1. Means values (± standard deviation) of survival, wet weight, and total length of *L. alexandri*, and pH, turbidity, and alkalinity after 15 days of rearing with four different biofilter media quantities.

	Q_0	Q_{40}	Q_{80}	Q_{160}
Survival (%)	75.4±1.2	75.9±4.5	80.4±11.3	72.7±7.9
Weight (mg)	183.7±39.5	206.6±20.9	185.8±17.3	222.0±24.7
Length (mm)	26.4±1.8	26.9±0.9	26.0±0.6	27.9±0.9
pH	6.6±0.32b	7.4±0.04a	7.4±0.10a	7.4±0.10a
Turbidity (NTU)	7.5±0.7a	0.0b	0.0b	0.0b
Alkalinity (mg $CaCO_3.l^{-1}$)	4.8±0.5b	39.7±0.4a	40.4±1.8a	51.1±3.6a

Different letters on horizontal are statistically different by Tukey's test ($P<0.05$).

The ammonium ion and nitrate levels were significantly influenced by biofilter media quantities ($P<0.001$), days of rearing ($P<0.001$), their interaction ($P<0.001$). During the experiments, ammonium ion levels (Fig. 1) were similar among the different biofilter media quantities and higher for the without biofilter media treatment, suggesting that the different biofilter media quantities were sufficient to maintain lower values of this compound. In the without biofilter media treatment, there was an increase in the five first days of feeding, and afterwards, these levels were constant. This suggests the nitrification process occurs in the water column in aquarium without biofilter media, as this requires a longer time.

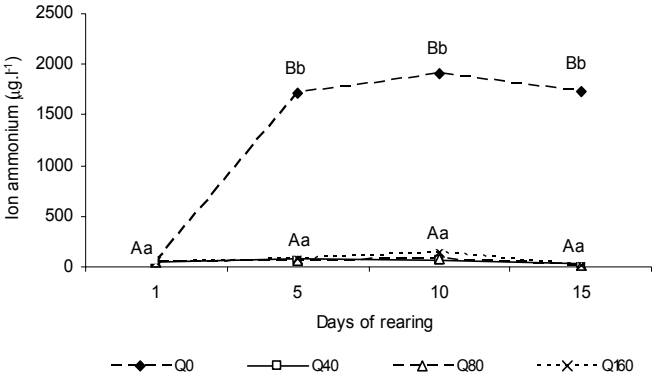

Fig. 1. Means values of ammonium ion levels ($\mu g.l^{-1}$) during the *L. alexandri* rearing with four different biofilter media quantities (0, 40, 80, and $160 g.l^{-1}$). Different capital letters showed significantly difference ($P<0.05$) between different biofilter media quantities in the different days of samples. Different small letters showed significantly difference ($P<0.05$) inside of each treatment during the time.

The nitrate levels (Fig. 2) were seen to increase in different biofilter media quantities, indicating the efficiency of the used system. In the treatment without biofilter, this increase occured after five days of feeding, reaching similar values of the others treatments. This fact confirms the nitrification process in the water column as described by Ozório et al. (2004).

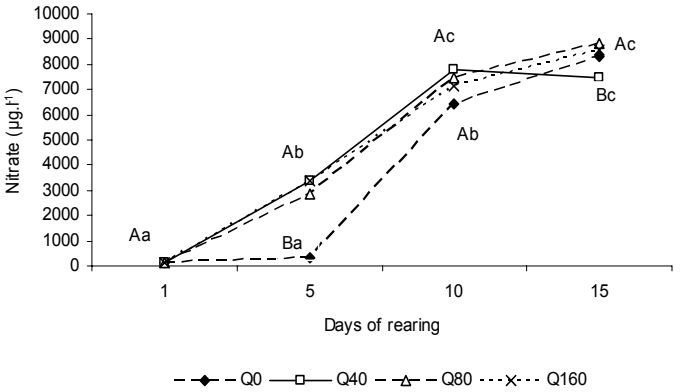

Fig. 2. Means values of nitrate levels ($\mu g.l^{-1}$) during the *L. alexandri* rearing with four different biofilter media quantities (0, 40, 80, and $160 g.l^{-1}$). Different capital letters showed significantly difference ($P<0.05$) between different biofilter media quantities in the different days of samples. Different small letters showed significantly difference ($P<0.05$) inside of each treatment during the time.

Conclusion

The different biofilter media quantities improve water quality during *Lophiosilurus alexandri* larviculture; however, the differences seen among the use or omission biofilter media did not affect survival and growth.

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RECENT DEVELOPMENTS ON BROODSTOCK MATURATION AND REPRODUCTION OF INDIGENOUS PENAEID SPECIES IN BRAZIL

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The interest in culturing indigenous penaeid species has increased lately due to *Litopenaeus vannamei* disease outbreaks, especially in southern Brazil. Although sustainable production of the indigenous *Farfantepenaeus paulensis*, *F. brasiliensis*, and *F. subtilis* is greatly desired in Brazil, it has been constrained by the current dependency on wild-caught broodstock. To overcome this problem, research efforts have focussed on the development of domesticated stocks and optimization of their reproductive performance under laboratory conditions.

As in most penaeids, *Farfantepenaeus* spp. present two different phases in its life cycle: an oceanic one, marked by reproduction and larval development, and another represented by growth in estuarine areas. Wild broodstock are usually found in depths from 40-60m and seem to prefer soft substrates to burrow during the day and emerge at night for feeding. Since *Farfantepenaeus* females have closed-thelycum, mating takes place when males are in the intermolt period and females have recently molted (soft cuticle). After the hardening of the exoskeleton, mature females may fertilize several spawns with the sperm obtained in a single mating event.

Sexual maturity of *Farfantepenaeus* males has been estimated on the basis of joined petasma, presence of spematophores, and sperm counts, while visual observation of females, specifically the shape and color of the ovary, has been routinely used to evaluate the stage of maturation under laboratory. Histological analyses have been used to describe ovarian maturation and its relationship with visual changes in maturing *F. paulensis* and *F. brasiliensis*. Unilateral eyestalk ablation is the most effective way to induce ovarian maturation and spawning of *Farfantepenaeus* species in captivity. Furthermore, the positive effects of eyestalk ablation in ovarian development were also observed through histological analysis of spent ovaries from unablated and ablated *F. paulensis*.

Lack of mating is currently the major limitation in captive maturation of *F. paulensis*. To circumvent the lack of mating, artificial insemination has proven a useful notwithstanding labor-intensive option. The efficiency of artificial in-

semination was compared in conventional (mixed) versus unisex maturation systems of *F. paulensis*. Results demonstrated that the presence of males had no apparent effect on the reproductive performance of artificially inseminated females and therefore it might be possible to make better use of the maturation facilities by holding females separately from males.

Despite that wild-caught females generally have a comparatively superior performance than their captive counterparts, the high costs and unpredictability associated to the capture in the wild poses a difficulty for the production of nauplii. Therefore, some studies were carried out to assess the feasibility of producing domesticated broodstock. When similarly-sized *F. paulensis* broodstock from different sources were compared, a larger number of eggs/spawn and higher total egg production were recorded for the wild females (32g), but domesticated females (33g) produced more nauplii as a result of a higher percentage of fertilized spawns.

The future success of culturing indigenous *Farfantepenaeus* species would depend upon increasing supplies of high quality seed. Current efforts to close the life cycle and improve reproductive performance of these species in captivity would hopefully be able to design an efficient breeding program through the selection of desirable characteristics reflected in culture performance. New challenges, associated especially with the nutritional requirements during grow-out and the development of genetically improved captive broodstock, must be achieved through research efforts in the private and public sectors.

LARVI '09 – FISH & SHELLFISH LARVICULTURE SYMPOSIUM
C.I. Hendry, G. Van Stappen, M. Wille and P. Sorgeloos (Eds)
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INCREASING THE LEVEL OF SELENIUM IN ROTIFERS (*BRACHIONUS PLICATILIS 'CAYMAN'*) IMPROVES THE HEALTH OF COD LARVAE (*GADUS MORHUA*)

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Intensive production of Atlantic cod (*Gadus morhua*) larvae frequently results in large variations in survival, high rates of skeletal deformity, and low growth rates. One of the main reasons for this appears to be the inadequacy of the first feeding diet. Intensively reared cod larvae are fed on cultured rotifers, but it has been shown that rearing larvae on their natural diet, copepods, results in much higher cod larvae quality. For example, rotifer fed cod larvae rarely have specific growth rates (SGR) exceeding 10%.day^{-1} dry body mass (DW), while those fed copepods can have SGR exceeding 25%.day^{-1} DW.

Accumulating evidence suggests that the superior nutritional composition of copepods is an important reason for the subsequent high cod larvae quality. While copepods have ample concentrations of essential minerals, often much higher than the national research council's recommendations for juvenile fish, rotifers appear to contain many minerals at low or possible deficient concentrations. The mineral found with the largest difference and thus potentially the most deficient in rotifers is selenium (Se). Selenium levels of rotifers (0.08-0.09mg.kg^{-1} dry weight (DW)) are over 30-fold lower than copepod levels (3-5mg.kg^{-1} DW) and 3 to 8-fold lower than the Se requirements for juvenile fish.

Selenium is an essential micronutrient for vertebrates due to its function in Se dependent enzymes, which includes the glutathione peroxidases (GPx) and the deiodinases (ORD). The GPx family are part of the body's antioxidant system, while ORD control the ratios of thyroid hormones which are important in regulating growth, metabolism, and in metamorphosis. Therefore, increasing the level of Se in rotifers to copepod level may increase cod larvae quality by helping to maintain the correct thyroid hormone and antioxidant status of the animal.

A protocol was developed that enabled rotifers to be enriched with Se under hatchery conditions with minimal replacement of other enrichment ingredients.

Rotifers were co-enriched with a standard enrichment product plus a Se containing yeast to 3mg.Se.kg^{-1} DW (Se+rotifers), to replicate the level found in copepods. These rotifers were then fed to cod larvae (Se+larvae) from start feeding at 3 days post hatch (dph) until 29dph. Larvae samples were taken at 2, 7, 17, 26 and 29dph and analysed for length, weight, mineral composition and Se dependent enzyme levels, while survival was measured at 29dph. Larvae were then reared until 120dph, and the weight, length and skeletal deformities were analysed to determine the effect of increased pre metamorphic Se on later development.

Se+larvae had a continual uptake and retention of Se during the larval period, and at 29dph contained 4.5-fold higher concentrations of Se than controls. At 17 dph, Se+larvae had significantly higher ($p<0.01$) GPx activity compared to controls, but no effect of the treatment was seen on ORD activity. Both Se+ and control larvae had significant changes in GPx and ORD activity between days during the larval period. Survival of the fish (≈100%, 29dph) was not affected by Se+rotifers. Se+larvae were significantly shorter ($p<0.05$) than controls at 7 and 17dph, but by 26dph and onwards there was no difference. Dry weight and SGR were similar between groups throughout the trial. At 120dph there was no effect of Se+rotifers on growth or skeletal deformities.

The increase in whole body Se concentration in Se+larvae demonstrates that Se+rotifers successfully delivered Se to cod larvae. Higher GPx activity at 17dph in Se+larvae demonstrates that Se+rotifers also increased the concentration of bioavailable Se in larvae. In addition, it shows that control rotifers delivered inadequate Se concentrations to support optimum antioxidant status in larvae. The results suggest that cod larvae, like other vertebrates, place priority on retaining ORD activity at the expense of GPx activity under Se deficiency. Decreased lengthwise growth during the early larval stage and higher GPx activity in the mid larval stage in Se+larvae, along with the significant changes in Se-dependent enzyme activity with age in both larvae groups, supports the idea that Se requirements change during cod larvae development. The data proves that increased Se concentrations can be successfully delivered to larvae via enriched rotifers, and that rotifers enriched to 3mg.Se.kg^{-1}DW improved health parameters of cod larvae.

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THE IMPORTANCE OF AROMATIC AMINO ACIDS DURING FISH ONTOGENY

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Introduction

Aromatic amino acids (AA; phenylalanine and tyrosine) are the precursors of thyroid hormones, which regulate fish metamorphosis. This is a critical stage for fish larvae, especially in flatfish, where major changes occur in every organ system. Therefore, to achieve a successful transition to juvenile stage, aromatic AA requirements may increase during flatfish larval metamorphosis and dietary supplementation may be beneficial.

This work aimed to evaluate changes in aromatic AA metabolism throughout metamorphosis of Senegalese sole (*Solea senegalensis*), an important flatfish species for South-Eastern European aquaculture. In addition, this study also aimed to assess the effect of dietary tyrosine supplementation on aromatic AA metabolism during the same ontogenic stages of Senegalese sole development.

Materials and methods

This study comprised two experiments in which the "in vivo: method of controlled tube feeding described by Rust et al. (1993) and modified by Rønnestad et al. (2001) was applied in Senegalese sole larvae at pre-metamorphic (11-12 days after hatching – DAH), metamorphic (16-18 DAH) and post-metamorphic stages (23-25DAH). In experiment I, larvae were tube-fed L-[U-^{14}C] phenylalanine (Phe; Amersham Biosciences) and tyrosine (Tyr; ARC, USA) at the different metamorphic stages, in order to evaluate aromatic AA metabolism throughout metamorphosis. Before being tube-fed, Senegalese sole larvae (n=20) were anaesthetised with MS-222 (33µM). After tube-feeding, larvae were transferred to incubation chambers filled with seawater (7.5ml), which contained all labelled ^{14}C resultant from fish evacuation (evacuated fraction). An airflow connection was provided between each chamber and a KOH trap (5ml, 0.5M) to collect $^{14}CO_2$ produced by labelled AA oxidation (catabolised fraction). Fish were sampled at the end of incubation period (6h), while $^{14}CO_2$ remaining in water was collected as described by Rønnestad et al. (2001). Trichloroacetic acid (TCA; 500µl, 6% w/v) was added to larval samples for 24h at 4°C, in order

to extract ^{14}C-labelled free AA (FAA fraction). Larval bodies were subsequently solubilised with Solvable (500µl, PerkinElmer, USA) for 48h at 50ºC for analysis of ^{14}C-body fraction.

In experiment II, the effect of dietary tyrosine supplementation on sole aromatic AA metabolism was assessed throughout metamorphosis (same stages as in Experiment I). Two types of solutions were prepared: a control saline solution (seawater:distilled water 1:3) and a Tyr-supplemented solution (Tyr-Ala dipeptide; Sigma-Aldrich, Germany; 3% of protein from a normal *Artemia* meal). To each of these solutions was added L-[U-^{14}C] Phe or Tyr. Therefore, four different solutions were obtained and used for tube-feeding: Tyr supplement with ^{14}C-Tyr, Tyr supplement with ^{14}C-Phe, saline solution with ^{14}C-Tyr and saline solution with ^{14}C-Phe. Sole larvae fed upon *Artemia* metanauplii enriched with commercial products for a half hour before being tube-fed with one of the above solutions (n=15). Larvae were anaesthetised before being tube-fed. Sole larvae were then transferred to seawater filled incubation chambers individually connected to KOH traps, following the procedures described in experiment I. At the end of the incubation period (24h), larvae were sampled and the $^{14}CO_2$ in incubation water was collected. Each fraction (body, FAA, catabolised and evacuated) was determined for all samples as described in experiment I.

Sample disintegrations per minute (DPM) were determined by adding Ultima Gold XR (PerkinElmer, USA) scintillation cocktail and counting in a Beckman LS 6000IC liquid scintillation counter (Fullerton, CA, USA). Metabolic budgets were calculated after subtracting blanks of each fraction. Results for each fraction were expressed as a percentage of total tracer fed. In experiment I, data were tested by one-way ANOVA followed by Tukey's multiple comparison tests. In experiment II, data were tested by Student's t-test. The significance level was $p \leq 0.05$. All results expressed as a percentage were previously arcsine transformed (Zar, 1999).

Results

In experiment I, no significant differences were found for body and evacuated fractions of Senegalese sole larvae tube-fed ^{14}C-phenylalanine during the pre-metamorphic, metamorphic and post-metamorphic stages. However, phenylalanine catabolised fraction significantly decreased throughout larval development, while its retention in FAA fraction significantly increased at metamorphosis. In addition, larvae retained more ^{14}C-tyrosine in body fraction during pre-metamorphic than during metamorphic or post-metamorphic stages (Fig. 1). Tyrosine was also significantly more retained in FAA fraction during metamorphic than during pre-metamorphic stage. Conversely, tyrosine was less catabolised during metamorphic than during pre and post-metamorphic stages. Evacuated

fraction for tyrosine was significantly lower in larvae during pre-metamorphosis than during metamorphosis and post-metamorphosis.

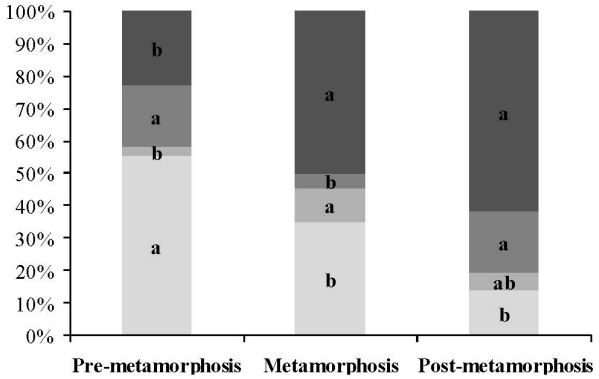

Fig. 1. Percentage of tube-fed ^{14}C-tyrosine present in body (□), free amino acid (▒), catabolised (■) or evacuated (■) fractions of *S. senegalensis* larvae at pre-metamorphic, metamorphic and post-metamorphic stages. n = 20. Different letters within fractions represent significant differences among ages.

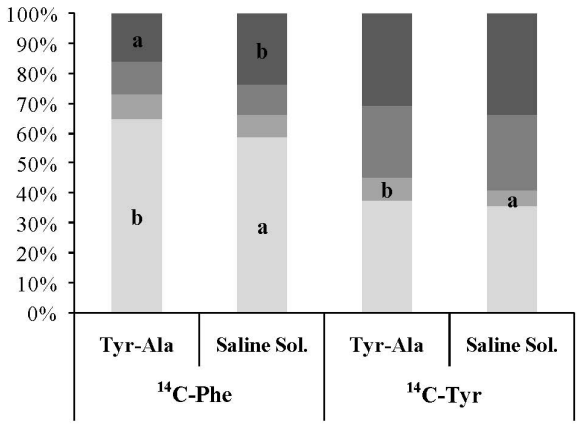

Fig. 2. Percentage of tube-fed ^{14}C-phenylalanine (Phe) and ^{14}C-tyrosine (Tyr) present in body (□), free amino acid (▒), catabolised (■) and evacuated (■) fractions of *S. senegalensis* larvae fed (Tyr-Ala) or not (Saline Sol.) a tyrosine supplement at metamorphosis. n = 15. Different letters within fractions represent significant differences for each radiolabelled solution in presence or absence of dietary tyrosine supplementation.

In experiment II, no significant differences were found between treatments during Senegalese sole pre-metamorphic or post-metamorphic stages. However, tyrosine supplementation significantly increased phenylalanine retention in body

fraction during metamorphosis (Fig. 2). Conversely, tyrosine supplement significantly decreased phenylalanine evacuated fraction at this stage. At this point, tyrosine supplement also increased tyrosine retention in larval FAA fraction.

Discussion

This work showed that aromatic AA (phenylalanine an tyrosine) were more retained in Senegalese sole larvae during pre-metamorphic and metamorphic stages. This suggests high physiological requirements for aromatic AA during these stages, probably for production of thyroid hormones, which are important for larval metamorphosis completion (Delgado et al., 2006). In addition, dietary tyrosine supplementation increased aromatic AA retention during Senegalese sole metamorphosis. At this stage, tyrosine supplementation increased phenylalanine retention in larval bodies, possibly by decreasing tyrosine biosynthesis from phenylalanine. Moreover, dietary tyrosine supplementation increased tyrosine retention in FAA fraction, suggesting higher tyrosine availability for coping with metamorphosis-related processes. This study showed that aromatic AA are especially important during Senegalese sole metamorphosis and dietary aromatic AA supplementation during this critical stage of Senegalese sole development may be important.

Acknowledgments

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DETERMINING HYDROBIOLOGICAL LARVAL REARING PARAMETERS FOR *CRASSOSTREA GIGAS* IN SMALL VOLUME FLOW-THROUGH CONTAINERS

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Introduction

The development of procedures for rearing *C. gigas* larvae in flow-through systems has allowed the development of efficient tools that can help us to understand the ecophysiological needs of this species (Rico Villa et al., 2008). Analogous approaches are being considered for the European flat oyster *Ostrea edulis* and European clam *Ruditapes decussatus*, two species with lower fecundity (1-2 million oocytes vs. 20-30 million in *C. gigas*). This constraint, combined with the need to rear a large number of families of any species for genetic reasons, have favoured the development of through-flow larval rearing in small volumes. The specific form of larval containers and their low volume (in our case 5-l translucent methacrylate cylinders) led us to verify the impact of the hydrodynamics of this culture environment on *C. gigas* survival, growth, and competence to settle, the Pacific oyster thus serving as a biological model.

Materials and methods

Three consecutive trials, run at a density of 50 individuals.ml^{-1}, were performed to identify and determine the importance of zootechnical parameters influencing *C. gigas* larval rearing. The effects of seawater flow direction (downwards or upwards) were first tested, combined with sieve position in the tank. The effects of seawater renewal rate (SWR: 35, 100, 180, and 300%.h^{-1}) and airflow levels (0, 10, 20, and 30ml.min^{-1}) were then estimated. These trials led to the definition of the best hydrodynamic configuration, which was then applied and tested at four larval densities (50, 100, 200, and 400 larvae.ml^{-1}).

Two-day-old D larvae were put in the tanks at the desired densities, in 1µm-filtered UV-irradiated natural seawater maintained at 25°C and 34ppt salinity. Experiments were conducted over two weeks, during which larvae were fed a mixed diet of *Isochrysis affinis galbana* (T-ISO: strain CCAP 927/14) and *Chaetoceros gracilis* (strain UTEX LB2658) at a 1:1 ratio of cell biovolume. Feeding

rate was adjusted according to larval biomass increase, to ensure a constant level of 1400µm^3.µl^{-1} around the larvae at all times. Daily food ration at the entry to the rearing system was therefore progressively increased, as detailed in Rico-Villa et al. (2009), and regularly verified using a Multisizer 3 equipped with a 100-µm aperture tube. Experiments were continued until first settlement was observed on the translucent tank walls, and were also based on larval growth and competence records made at intermediate check points. Larval mortality was estimated regularly on samples by counting the translucent larval shells under the microscope. Larval length was assessed by measuring the shell length of 200 individuals by image analysis (WinImager 2.0 and Imaq Vision Builder 6.0 software for image capture and analysis, respectively). From day 12, the number of pediveligers ready to settle (competent larvae characterized by the presence of an eyespot) was counted. Lastly, in the fourth experiment, examining high larval density, total counts of culturable heterotrophic bacteria (marine agar) and vibrios (TCBS) in the culture tanks and larvae were estimated using the plate count method; results were expressed in colony-forming units (CFU.ml^{-1} or CFU.larvae^{-1}).

Differences ($p<0.05$) in growth, survival, competence, and settlement were analysed by one-way ANOVA, using XLSTAT 2008, after data transformation (arcsin[square (.100^{-1})]) where necessary.

Results and discussion

When seawater inflow was top to bottom, rather than bottom to top, larval survival was lower at the end of the experiment on day 13 (41% ± 18 vs. 77% ± 2, respectively). Larval length was also lower, showing a 50µm difference. When exposed to high seawater renewal rate, larval survival was affected from day 7. Thus, on day 15, mortality concerned ≈50% of the population at 300% SWR vs. 20-25% at ≤180%. Larval length was depressed at high SWR, with a 15-30µm difference in size. A lack of aeration was detrimental for larval survival on day 10, whatever the seawater renewal rate (30%.h^{-1} SWR: 41 ± 32 vs. 86% ± 9; 100%.h^{-1} SWR: 54 ± 7 vs. 94 ± 7%). In contrast, a difference in air inflow between 10 and 30ml.min^{-1} had no influence on larval survival (77-93%), larval length (272-285µm) or competence (73-81%) on day 15. Subsequent larvae were then reared following the best conditions: bottom to top seawater inflow, 100%.h^{-1} renewal, and the air supply at 20ml.mn^{-1}.

In this situation, an increase in larval density from 50 to 400 larvae.ml^{-1} did not show any effects on larval survival prior to metamorphosis, which remained particularly high (95-98%). In contrast, on day 14, larval growth was depressed at high density (212 vs. 242µm). However, the main difference was shown for metamorphosis, as there was a clear delay in its onset between these density levels (from day 16 to 25). The quantitative analysis of the seawater microbial

population revealed a noticeable increase in total bacteria with larval density prior to metamorphosis (from 40×10^3CFU.ml^{-1} at 50 larvae.ml^{-1} to 150×10^3CFU.ml^{-1} at 400 larvae.ml^{-1} on day 15: Fig. 1a). Vibrios were mainly present at 400 larvae.ml^{-1} density from day 12, showing a peak concentration of 20×10^3CFU.ml^{-1} (Fig. 1b). However, on day 16, the level of vibrios remained relatively low in crushed larvae, with values varying from 15CFU.larvae^{-1} at 50 larvae.ml^{-1} to 164CFU.larvae^{-1} at 400 larvae.ml^{-1}.

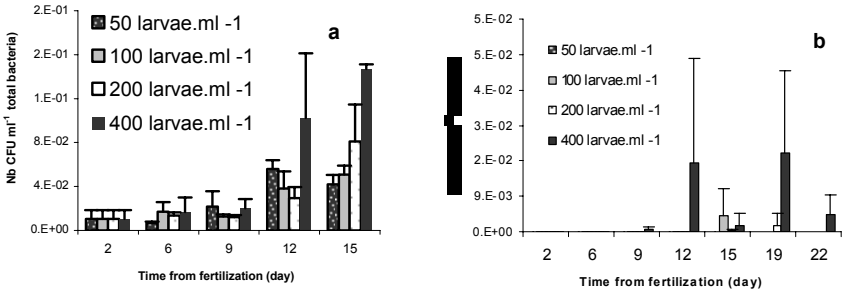

Fig. 1. Evolution of total counts of culturable heterotrophic bacteria (a) and vibrios (b) in rearing seawater, observed at different larval densities from 50 to 400 larvae.ml^{-1} from day 2 to settlement.

Conclusions

The interactions between seawater inflow direction, seawater renewal rates and aeration are specific to each type of structure (shape and volume) and to the biological stage of development of the species considered. In 5-l cylindrical vessels, seawater renewal rate is the most important factor for *C. gigas* larval development with 100%.h^{-1} as the most efficient. In optimized hydrobiological conditions, larval density >200 individual.ml^{-1} represents a limit resulting in a delay in oyster settlement, while a simultaneous increase in vibrio concentration in larvae and rearing seawater probably indicates the beginning of physiological disorders at this level.

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COULD ANTIOXIDANT STATUS AND OXIDATIVE STRESS BIO-INDICATORS BE USED TO ASSESS SHRIMP OFFSPRING QUALITY?

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Introduction

More than 40 generations of *Litopenaeus stylirostris* captive broodstock have been reared, to date, in New Caledonia. However, commercial hatcheries have been subjected to mortality outbreaks in the last four years. The sustainability of shrimp farming is closely linked to the larvae capacity to withstand stressful events. In this context, is it possible to correlate larval quality to the antioxidant status of the animal?

Materials and methods

From day 0 (D0) to day 9 (D9), survival rate and mean stage index (MSI) were recorded daily in July and October 2007, on two and three tanks respectively. To determine the antioxidant status of the animals in each tank, larvae were sampled at three different stages (nauplius at D0, zoea at D4, mysis at D8). They were flash frozen in liquid nitrogen and then kept in freezer (-80°C) until use. Four endogenous antioxidants and two secondary oxidation products from lipids and proteins were analysed according to the previously described methods adapted to microplate reader (Bioteck® Synergy HT) (Castex et al., 2009):

- Superoxide Dismutase SOD (Marklund and Marklund, 1974), Catalase CAT (Clairbone, 1985)
- Glutathiones GHS (Akerboom and Sies, 1981)
- Glutathione peroxydase GPx (adapted from Gunzler et al., 1974)
- Malondialdehyde MDA (Richard et al., 1992), Protein Carbonyl (Lenz, 1989; Rodney et al., 1990).

Results and discussion

Final survival rates were higher in October (64%) than in July (10%) (Fig. 1A). The differential mortalities occurred after D3. The low survival (LS) came with lower MSI from D5 compared to the high survival (HS) MSI (Fig. 1B).

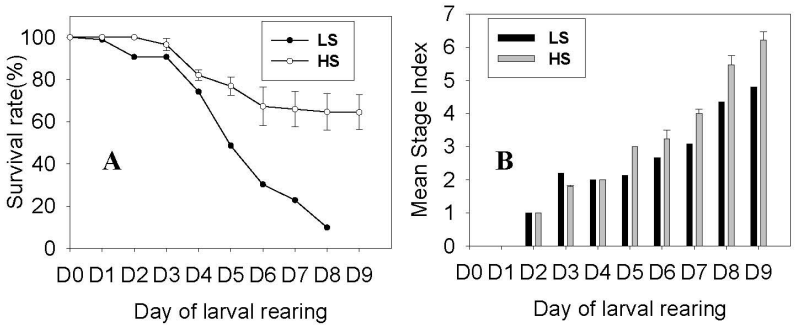

Fig.1. Evolution of the survival rate (A) and Mean Stage Index (B) according to the rearing day for the low survival batch (LS) and high survival batch (HS).

For most of the antioxidant parameters, zoea stage was characterized with higher levels compared to nauplius and mysis stage. Very low or no SOD was detected at the nauplius stage while it was relatively high at zoea (Fig. 2A).When comparing the evolution between LS and HS, higher levels in GSH (Fig. 2B) were obtained with HS whereas CAT was more active for LS (Fig. 2C).

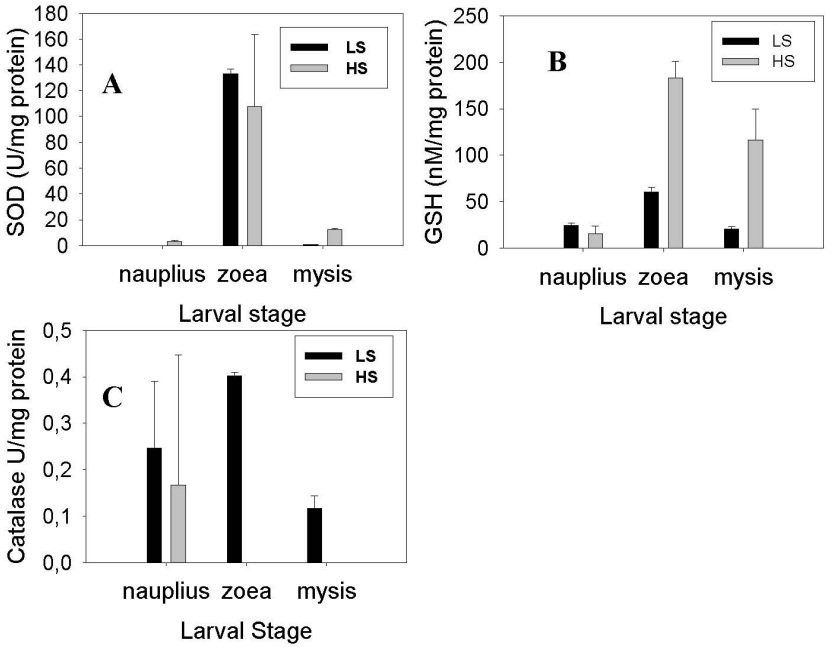

Fig. 2. Evolutions of SOD (A), GSH (B) and CAT (C) according the larval stages for LS and HS batches.

Regarding oxydative stress damages, lipids peroxydation (MDA) in nauplius is much higher than in later stages (Fig. 3A). MDA in LS larvae were higher than in HS ones. Contrarily, carbonyl concentration was stronger for HS larvae and two to six times as high in zoea compared to other larval stages (Fig. 3B).

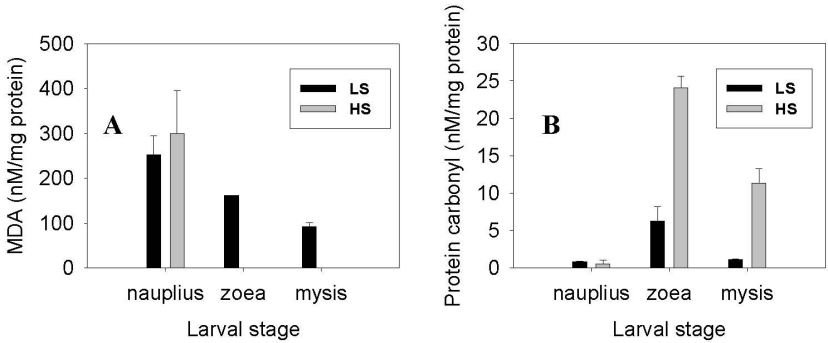

Fig. 3. Evolutions of MDA (A) and protein carbonyl (B) according the larval stages for LS and HS batches.

Zootechnical differences between the two batches could be due to the breeders' quality and/or different environmental conditions from a period to the other. In this experiment, the differences in biotechnical parameters have resulted in differences in antioxidant status.

Lipid peroxidation is favoured in the nauplii which obtain most of their energy from the catabolism of lipoprotein yolk reserves (Agard, 1999). With a higher metabolism compared to the other stages (Lemos and Phan, 2001), nauplius could be more sensitive to the free radicals produced by the respiratory chain. When the nauplius turns into zoea, the feeding regime changes with the supply of artificial food, involving a modification of oxidative activity (Lemos and Phan, 2001). The higher survival and better MSI from the HS batch could be the result of a better protein conversion into energy. However, the high levels of protein oxidation in the two batches suggest a higher oxidative stress level in zoea compared to the other stages.

The toxic reactive oxygen species (ROS) produced by the aerobic metabolism can be eliminated in different defence processes which imply either free-radical scavengers (glutathiones) or specific enzymes (SOD, CAT, GPX). The high SOD activity in LS batch probably leads to an H_2O_2 accumulation which increases catalase activity. The absence of catalase activity in HS batch suggests that GSH is the preferential pathway to remove the ROS. This hypothesis is confirmed by the high GPx activity (result not shown) and this antioxidant defence system probably contributes to the good zootechnical results in HS batch.

Conclusions

These preliminary results show that the shrimp response to an "oxidative stress" depends on the larval stage but also on the larval rearing quality. More experiments are required to confirm these first observations.

This research is aimed at identifying the risk factors and to prevent the oxidative stress in larval commercial production. From a practical angle, some zootechnical or nutritional recommendations could be suggested to improve the antioxidant status of the shrimp during its larval ontogenesis. .

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METAMORPHOSIS OR THE LARVA-JUVENILE TRANSITION: STEERING THE FUTURE OF THE FOODFISH INDUSTRY

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The ability to consistently produce high quality juveniles is essential for aquaculture. Current initiatives are hampered by the post-metamorphic appearance of developmental anomalies such as deformities, poor growth or aberrant sex differentiation. Available evidence suggests that the influence of environment, early diet or pollutants during the embryonic or exogenous feeding phase of teleost larvae steers such anomalies. The dynamic metabolic programming taking place during these critical periods appears to permanently impinge on the physiology, organ system development as well as gender and intersex. Some of the endocrine mechanisms that are especially active during metamorphosis are challenging to study due to the multiplicity of the genes that code for the signalling pathways. Here we review recent findings and discuss potential avenues for steering the post-metamorphic development of foodfish.

The larval-juvenile transition is an endocrine-driven series of overlapping events involving neurology, endocrinology, morphology and physiology. Recent comparative studies indicate that the Fish Specific Genome Duplication, which occurred about 320mya, permitted not only the evolution of an exceptionally large pigmentation-gene repertoire among vertebrates, but also indicate that fundamental regulatory mechanisms may be unexpectedly variable, such as the non-muscle expression of myoglobin in carp and the differences in endogenous regulation of melatonin release from the pineal of sole and tench. Even if we consider that metamorphosis is a Thyroid Hormone mediated transition (Fig. 1), differential regulation of its many receptor forms allows development with particular responses to stress, environment and physiological demands.

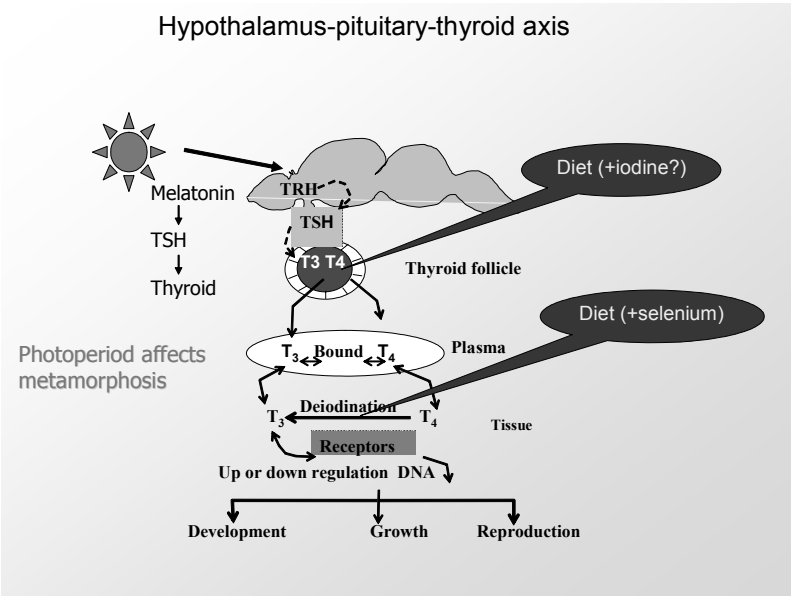

Fig. 1. Brief overview of the Hypothalamus-Pituitary-Thyroid axis. TRH=Thyroid releasing hormone, TSH=thyroid stimulating hormone, T_3= triiodothyronine, T_4=tetraiodothyronine.

The plasticity of response in the early stages has long-term consequences for scope of growth and phenotype, not only for fish but also for many phyla. This implies that improved understanding of the underlying regulatory mechanisms and their response spectra in early teleost stages should improve overall production at later stages. However, genomic approaches are generally large scale, requiring homogenization of small larvae for screening, and thus obfuscating individual response elements. A critical point, then, is selection of sample material, as larvae of the same age but very different developmental stages can be present in the same tank, and therefore already represent a variety of spatio-temporal patterns of cell activation which are therefore difficult to interpret as data points. The highly plastic embryonic cells of teleosts respond according to their derivation, feed type, density and growth factors by exhibiting different phenotypes, patterns of differentiation, molecular signals, epigenetic stability and growth potential, all over a broader time scale than previously thought. Thus disciplines with direct application to the larval-juvenile transition include the emergent fields of metabolic and epigenetic programming, nutrigenomics, neural plasticity, networks of interacting developmental tissues and genes, as well as endocrine priming. Further investigation of the effects of larval environment on the final foodfish quality and quantity will require new skills and new questions of the investigators, as well as focussing on more levels of biological organization.

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FATTY ACID COMPOSITION OF EARLY STAGES OF *DIPLODUS SARGUS* (L., 1758) AND DIETS

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Introduction

Diplodus sargus is a marine species with potential interest to fish farming in the Mediterranean due to its high market price and demand. *D. sargus* larval rearing has promising survival rates (Saavedra et al., 2006), but has some severe constraints such as high incidence of vertebral deformities (Dores et al., 2006; Saavedra et al., 2008) and a decrease on the growth rate when reaching the juvenile stage (Cejas et al., 2003). These problems might be related to an inadequate feeding protocol as there is a lack of information regarding *D. sargus* nutrition requirements. Larvae of several marine fish species have shown to require n-3 highly unsaturated fatty acids (HUFA), particularly docosahexaenoic (DHA) and eicosapentaenoic acid (EPA) in order to have a normal development and growth (Izquierdo et al., 1992). The aim of this study was to determine the fatty acid profile of *D. sargus* larvae through ontogeny and compare it to *D. sargus* diet.

Materials and methods

This study was carried out at the Aquaculture Research Station of IPIMAR, in Olhão, South of Portugal. The larvae were reared in a conical cylindrical fibreglass tanks at a density of 100 larvae.l^{-1}. Feeding protocol consisted on *Brachionus plicatilis* enriched with Protein Selco (INVE Aquaculture, Belgium) from 3 to 20 days after hatched (DAH). At day 12, larvae started having *Artemia* nauplii (BE 480, INVE Aquaculture, Belgium) and *Artemia* metanauplii, enriched with Super Selco (INVE, Aquaculture, Belgium) from 17DAH until 39DAH. Dry feed (Nippai, Japan) was given from day 25 until the end of the experiment (45DAH). Three larvae samples were taken from three tanks at 0, 2, 5, 8, 12, 17, 25, 35, and 45DAH for chemical analysis and compared with the live and dry feed. Fatty acids composition was determined using the transesterification method by basic catalysis (Park et al., 2001).

Results and discussion

Diplodus sargus diets had quite different fatty acid profiles and are shown in Table I.

Table I. Fatty acid composition (% total FA) of white sea bream (*D. sargus)* diet. Values are averages of three separated analyses. Different superscript letters in the same row represent significant differences ($p<0.05$).

	Rotifers	***Artemia* nauplii**	***Artemia* metanauplii**	**Dry feed**
18:2n6 (LA)	6.98^{a}	5.85^{a}	6.36^{a}	3.33^{b}
18:3n3 (LNA)	1.06^{a}	20.15^{b}	16.11^{c}	0.71^{d}
20:4n6 (ARA)	1.23^{ab}	0.86^{a}	1.93^{b}	0.62^{a}
20:5n3 (EPA)	5.71^{a}	2.72^{b}	6.94^{c}	11.31^{d}
22:6n3 (DHA)	5.26^{a}	1.06^{b}	5.69^{a}	10.73^{c}
Σ SFA	23.52^{a}	21.57^{a}	17.17^{b}	41.83^{c}
Σ MUFA	40.54^{a}	32.56^{b}	33.30^{b}	25.27^{c}
Σ PUFA	26.54^{a}	36.09^{b}	43.36^{c}	31.04^{d}
n-3 HUFA	13.31^{a}	3.86^{b}	13.25^{a}	22.83^{c}

Table II. Fatty acid composition (% Total FA) of white sea bream *(D. sargus)* larvae. Values are averages of three separated analyses. Different superscript letters in the same row represent significant differences ($p<0.05$).

% Total FA	**0DAH**	**2DAH**	**5DAH**	**8DAH**	**12DAH**	**17DAH**	**25DAH**	**35DAH**	**45DAH**
14:0	2.98^{a}	1.75^{ab}	0.93^{b}	0.56^{b}	0.71^{b}	1.60^{ab}	1.53^{b}	1.75^{ab}	1.45^{b}
16:0	28.22^{a}	25.49^{ac}	23.54^{ab}	18.23^{b}	16.77^{b}	20.46^{bc}	16.80^{b}	19.12^{bc}	22.49^{ab}
18:0	6.11^{a}	6.88^{ac}	9.43^{d}	11.16^{e}	10.78^{e}	9.00^{bd}	8.20^{bf}	8.02^{bcg}	7.84^{cfg}
ΣSFA	38.85^{a}	35.51^{ab}	35.56^{ab}	31.46^{ab}	29.76^{b}	32.99^{ab}	28.20^{b}	30.75^{ab}	34.52^{ab}
16:1	5.43^{a}	4.21^{abc}	2.75^{bc}	2.27^{b}	2.69^{bc}	5.02^{ac}	4.00^{abc}	3.33^{abc}	2.82^{bc}
18:1	14.47^{ac}	13.83^{a}	13.85^{a}	13.47^{a}	14.70^{ac}	23.48^{b}	21.44^{b}	20.60^{bd}	17.70^{cd}
ΣMUFA	21.30^{a}	19.36^{a}	18.01^{a}	17.48^{a}	19.93^{a}	31.30^{b}	29.27^{b}	26.85^{bc}	22.33^{ac}
18:2n6	4.64^{ade}	4.72^{ade}	1.82^{b}	5.66^{ce}	7.37^{c}	6.25^{ac}	5.10^{ade}	3.92^{bde}	3.20^{bd}
18:3n3	0.48^{a}	0.41^{a}	0.34^{a}	1.62^{b}	1.28^{ab}	4.53^{cd}	5.51^{c}	4.32^{d}	1.70^{b}
20:4n6	1.31^{a}	1.64^{ac}	1.89^{bcd}	2.15^{be}	2.04^{bcd}	1.59^{ad}	2.08^{bc}	1.71^{ace}	1.58^{ad}
20:5n3	5.01^{a}	5.31^{a}	5.05^{a}	5.55^{a}	6.31^{a}	5.27^{a}	10.82^{b}	11.69^{b}	12.42^{b}
22:5n3	0.92^{a}	1.06^{a}	0.97^{a}	1.57^{ab}	2.06^{b}	1.91^{ab}	1.98^{ab}	1.48^{ab}	1.40^{ab}
22:6n3	20.51^{ad}	25.59^{ac}	30.50^{c}	27.22^{ac}	23.58^{acd}	9.84^{b}	11.46^{be}	11.48^{be}	17.97^{de}
ΣPUFA	36.76^{ad}	43.76^{abc}	44.09^{ab}	47.52^{b}	46.49^{ab}	33.16^{cd}	40.37^{abc}	39.15^{abc}	41.39^{abc}
n-3 HUFA	26.44^{be}	31.96^{bce}	36.52^{c}	34.34^{bc}	31.94^{bcd}	17.01^{a}	24.69^{ade}	24.65^{ade}	31.79^{bce}
DHA/ EPA	4.10^{bc}	4.82^{b}	6.04^{a}	4.91^{b}	3.74^{c}	1.85^{d}	0.99^{d}	0.98^{d}	1.45^{d}
EPA/ ARA	3.82^{d}	3.23^{de}	2.67^{ef}	2.58^{f}	3.09^{eg}	3.33^{dg}	5.03^{a}	6.85^{be}	7.84^{c}

Larvae fatty acids profiles showed significant differences between almost all larval ages (Table II). These changes were often associated with the change of the type of the diet. During the endogenous phase (0 to 8DAH), the changes in the fatty acids profiles probably reflect their utilization by the larvae (Cejas et al., 2004). Thus, SFA especially 14:0 and 16:0 and MUFA, especially 16:1, de-

creased their contents during this period in the larvae. This accentuated decrease of lipid reserves during the first days of feeding was also reported by other authors (Rodríguez et al., 1998, Koven et al., 1989). These fatty acids, mainly those with shortest chains, are probably used as the dominant substrate for energy production from hatching to first days of larval feeding (Rønnestad et al., 1994).

The fatty acids profile obtained in this study show that the content of EPA and DHA during this endogenous phase doesn't show any significant differences, indicating a clear tendency to be spared. DHA is specifically incorporated into neural tissue phospholipids, playing an important role in synaptogenesis and rectinogenesis during early larval stages (Bell et al., 1995; Navarro et al, 1995). On the other hand, the content of ARA increased significantly. Along with EPA, ARA is considered an essential fatty acid (EFA) since possesses a vital function as the main precursor of a wide variety of biologically active compounds known as the eicosanoids (Bell and Sargent, 2003).

Once the exogenous feeding has started larvae fatty acid composition continued to change. The fatty acids profiles show that although supplemented, the EPA and DHA contents found in the live feed diets are less than half of the dry feed. That was reflected in EPA content of the larval carcass which increased when dry feed was introduced but not in the DHA content that decreased after 17DAH.

Conclusions

In conclusion, larval fatty acid profiles generally seemed to reflect diet fatty acid composition as well as preferential use of certain fatty acid on different larval stages. Results pointing out that DHA, EPA and ARA are spared during the depletion of the yolk sac for biological function. Further studies are needed to optimize fatty acid balance of feeds for *D. sargus* rearing.

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STUDIES ON LARVAL NUTRITION OF LARGE YELLOW CROAKER *PSEUDOSCIAENA CROCEA*: A REVIEW

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Large yellow croaker is one of several traditionally important fishes in China. It has been widely cultured in China after success of hatchery. In the recent years, a series of studies have been conducted to investigate nutritional physiology and to develop cost-effective micro-diet for large yellow croaker larvae. These studies focused on feeding behavior, digestive physiology, nutrient requirements, and micro-feed processing technology.

Feeding experiments showed that large yellow croaker at all stages demonstrated an obvious feeding rhythm with a feeding peak around 18:00. Besides this peak, the postlarvae and juveniles of this fish had another feeding peak around 10:00. Large yellow croaker larvae seldom fed during the night, indicating that light intensity determined their feeding activity and they have a crepuscular feeding rhythm. Feeding frequency of 2, 4, 8 and 12 meals per day were also designed to examine the appropriate feeding frequency of croaker larvae. Results showed that 8 meals d^{-1} had significantly higher survival, growth, and digestive enzymes activities ($P<0.05$) compared with other groups.

Large yellow croaker larvae like most of other marine fish undergo major morphological and functional changes during the first weeks of life. The ontogenetic development of the gut and accessory organs in this croaker was investigated using light microscopy from hatching up to the juvenile stage (40 days post hatch, dph). The analysis in digestive tract structure and function suggest that this fish could be divided into pro-larval stage (0 to 5dph), post-larval stage (6 to 20dph) and juvenile stage (21dph). Enzymatic assays were also conducted from 1dph to 40dph in larvae fed the following live prey sequence: rotifers, *Artemia nauplii*, copepods, and then frozen copepods. Assays were done on whole larvae until 19dph, then on dissected larvae and on purified brush border membranes of intestine. Digestive enzymes such as pancreatic enzymes, amylase, and trypsin showed high activities from early stages, even before mouth opening. Between 23 and 25dph, alkaline phosphatase and aminopeptidase abruptly increased, suggesting maturation of the intestinal digestive process.

Dose response method was used to determine the nutrient requirement of croaker larvae. Following 28-30d feeding experiments, the broken line model or the second order polynomial regression was used to estimate the requirement of protein, methionine, lysine, lipid, phospholipid, arachidonic acid and vitamin C. Compared with juvenile and adult, large yellow croaker larvae have a relatively higher dietary requirement for protein (57.1%), methionine (2.58%), lysine (3.38%), lipid (17.7%), phospholipids (5.72%), arachidonic acid (0.93%), and vitamin C (89.6mg.kg^{-1}).

Five approximately isonitrogenous and isoenergetic microbound diets were formulated with carrageenan, sodium carboxymethyl cellulose, sodium alginate, gelatin as binders, respectively, and a diet with no special binder as a control. A 30-day feeding experiment showed that the survival and growth of this croaker larvae fed sodium alginate and carrageenan diet was significantly higher than carboxymethyl cellulose, gelatin and the control diets (P<0.05). These results showed that carrageenan and sodium alginate are suitable binders in production of microbound diet of large yellow croaker larvae compared with other types. Four approximately isonitrogenous and isoenergetic microbound diets were also formulated with different stuff particle size (Diet 1-100μm, Diet 2-71μm, Diet 3-48μm and Diet 4<25μm). The water stability increased with decreasing the stuff particle size reduction. Fish fed Diet 3 with 48μm particle size had significantly higher survival, growth and specific activity of brush border membranes enzymes, alkaline phosphatase and aminopeptidase. These results showed that appropriate size for feed ingredient was 48μm in micro-diet of large yellow croaker larvae.

On the basis of the above data, artificial micro-diet has been formulated and used to replace live prey in production of large yellow croaker larvae. However, little is known on the molecular and hormonal mechanisms controlling feeding, digestive physiology and nutrient requirements, which need to be further studied. Also, the high quality micro-diet should be formulated to replace live prey in order to decrease feed cost, increase growth and survival, improve larvae quality, and maintain the sustainable development of marine aquaculture.

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PRESENT STATUS OF *MACROBRACHIUM ROSENBERGII* HATCHERIES IN BANGLADESH

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Introduction

In 2007, *Macrobrachium rosenbergii* production reached 24 000 metric tons in Bangladesh (FAO, 2009). The reasons for the rapid expansion of culture and increase in production include less susceptibility to viral diseases than penaeid shrimp (such as white spot syndrome virus), suitability to culture in different production systems (e.g., integration with rice culture, polyculture with carps in ponds), rapid growth, and higher market price.

Insufficient production and poor quality of hatchery-produced postlarvae (PL) are major constraints to further improve this production. The objectives of this study were to gather current information on different aspects of *M. rosenbergii* hatchery operations in Bangladesh including broodstock, larval rearing, cost benefit, and constraints.

Materials and methods

A survey questionnaire was prepared and field-tested. Data was collected through filling the questionnaire by visiting hatcheries, interviewing and or telephone conversation with hatchery owners, managers, government and non-government officials and berried female suppliers. The study was conducted from January and May 2009. Data were analyzed using Microsoft Excel.

Results

In 2008, sixty four *M. rosenbergii* hatcheries produced 175 million PL in Bangladesh (Table I). These were operated by private entrepreneurs (65.6%), non-governmental organizations (NGOs) (18.8%) and Department of Fisheries (DoF) of Government of Bangladesh (15.6%). Hatcheries can be divided into large (more than 5 million PL per year), medium (1-5 million PL per year), and small (less than 1 million PL per year). Only 23% of the hatcheries were large in op-

eration owned by private entrepreneurs (except a NGO hatchery). Hatcheries located in south east (Chittagong division) and southwest of Bangladesh (Khulna and Barisal division) contributed 85% of the total production. Hatchery operation period was March - August and produced PL mainly 2 cycles per year.

Table I. Distribution of *M. rosenbergii* hatcheries and PL production in millions in the year 2008.

Division	Total number of hatcheries	Number of hatcheries were in operation	PL production (millions)
Barisal	10	7	22.4
Chittagong	21	13	31.4
Dhaka	23	11	18.7
Khulna	43	26	94.9
Rajshai	24	6	7.3
Sylhet	3	1	0.3

Concentrated seawater (brine) (100-200g.l^{-1}) were collected either from Chokoria in Coxsbazar district or Munshigonj in Satkhira district. Several hatcheries located in Chokoria, Patenga, Kuakata, Satkhira pumped seawater directly from ocean or brackish water from adjacent rivers.

Eighty seven percent hatcheries used flow-through system, except 8 hatcheries of a NGO used clear water recirculation system. Water exchange in flow-through system hatcheries ranged 20-40% per day and alternatively 80-90% every third day. In the recirculation system, water was recycled @ 100-200% of the tank volume per day through bio-filter. All hatcheries were equipped with sand filter. The methodology to prepare sand filter and bio-filter was described earlier (BOBP, 1994).

Wild berried females (50-200g) were collected from rivers and farms of Khulna and Barisal division or local farms. Price of berried female were 3.5-8.5 US$/piece and 1.2-3.5 US$ /piece collected from river and farm, respectively.

Newly hatched larvae were stocked @ 50-120 larvae.l^{-1}. Larvae started feeding newly hatched *Artemia* nauplii 2-3 times per day and from 7-10 days onwards combination of *Artemia* nauplii with homemade egg custard (FAO, 2002) 4-7 times per day. The metamorphosis rate was 20-30%, with a maximum 40%. First metamorphosis (PL1) was reported at day 18, PL 50% (d) at 25-30 days and PL 90% (d) between 35-45 days.

The chemicals used in the hatcheries were bleaching powder, sodium thiosulfate, formalin, sodium bicarbonate, hydrochloric acid, povidone iodine, EDTA, and lime (CaO). The antibiotics include oxytetracycline, ciprofloxacin, erythromy-

cin, malachite green and methylene blue. Only three hatcheries reportedly used probiotics either Sanolife MIC of INVE Aquaculture or Epicin.

The average operating cost of production per million PL was $US7654 (Table II). The major cost include *Artemia* cyst 30%, staff salary 19%, brine 11%, energy (electricity and fuel) 10%, and berried female 8%. The selling price of one million PL ranged $US10 218 - 21 980.

Table II. Average operating cost per million PL of *M. rosenbergii* hatcheries producing more than 5 million PL per year.

Item	Unit	Unit cost (US$)	Quantity	Total cost (US$)
Brine	metric tons	36.5	22.3	814.0
Berried female	kg	21.9	26.7	587.7
Staff salary	man months	52.0	27.7	1440.0
Electricity	KWH	0.05	5640.0	282.0
Gasoline				
Diesel	litre	0.7	570.0	399.0
Lubricants	litre	1.6	60.0	96.0
Chemicals				
Bleaching powder	kg	0.6	68.0	40.8
Formalin	litre	1.7	36.0	61.2
Antibiotics	kg	80.3	0.6	48.2
Artemia cyst	kg	56.7	41.0	2324.7
Egg custard	kg	3.7	52.9	195.7
Repair and maintenance				293.7
Administrative and communication				200.0
Depreciation for fixed costs (10 years)				870.5
Total operating cost				7653.5

All hatchery managers experienced disease and mortality of larvae caused by mid cycle disease, bacterial necrosis, protozoa fouling (*Zoothamnium*) and exuvia entrapment disease.

The constraints of *M. rosenbergii* hatchery operation include shortage of skilled technicians, unreliable quality of chemicals (bleaching powder, formalin), increased cost of production due to shortage of electricity supply, higher price of *Artemia* cyst, higher price and scarcity of berried female from river origin, loosening ripe egg during transportation, farmers' complaints for the lower growth and survival and higher proportion of female in hatchery-produced PL, cheaper price of PL (e.g., $US17.5/ 1000 PL compare to $US27/1000 PL of wild origin), and marketing PL in cash. Also, late rainfall reduced the demand of PL for stocking by farmers and prolonged rearing period.

Discussion

This study, overviewed the present status of *M. rosenbergii* hatcheries in Bangladesh, particularly PL production, larval rearing, feeding, cost benefit, disease and constraints. A large number of hatcheries (48%) were not in operation could be explained by poor investment plan, improper site selection and unavailability of skilled technicians. Most of the hatcheries were medium (38%) or small (39%) in production could be due to (i) the fact that commercial hatchery operation is relatively new activity, (ii) hatcheries owned by DoF and NGOs were built to demonstrate technology and training and (iii) high investment cost to build a hatchery.

Dependency and limited supply of wild berried female, less metamorphosis rate (20-30%), longer duration for metamorphosis (up to 45 days), application of antibiotics, incidence of disease suggests to improve hatchery management including broodstock domestication, larval rearing, better quality feed and *Artemia* cyst, *Artemia* enrichment with better nutritional products and application of probiotics. Increase electricity supply is required to reduce the energy cost.

Finally, the present study increased our knowledge on hatchery operation practices, suggests to improve skills of hatchery managers, application of better technology such as feed and probiotic, improve energy supply (e.g., electricity) for the sustainable *M. rosenbergii* culture in Bangladesh.

Acknowledgements

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LOCALISATION OF GFP-LABELLED *LISTONELLA (VIBRIO) ANGUILLARUM* IN THE GI-TRACT OF GERM-FREE SEA BASS (*DICENTRARCHUS LABRAX* L.) LARVAE

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Introduction

The portal of entry of *Listonella anguillarum*, the causing agent of vibriosis in sea bass is not clarified yet. In order to localise the pathogen inside the host, we used the recently developed germ-free model system of *Dicentrarchus labrax* sea bass larvae (Dierckens et al., 2009; Rekecki et al., 2009). This system is a powerful tool to investigate microbial interactions in the larval fish gut and is suitable to study the beneficial effect of potential probiotics. Localisation of pathogens in the gastrointestinal (GI) tract in such a germ-free host is of uttermost importance. To visualise bacterial adherence and colonisation with a confocal microscope, bacteria can be labelled with Green Fluorescent Protein (gfp) via conjugation. The aim of this study was to establish a powerful approach to study bacteria *in situ* during colonisation of the host as the exact location of adhering bacteria in the gut can be determined under germ-free conditions.

Materials and methods

In order to obtain germ-free conditions, sea bass eggs were disinfected according to a standard protocol (Dierckens et al., 2009). Axenity was tested by using marine broth (10%). Hatched larvae were stocked in vials (12 larvae.vial^{-1}) and placed on rotor on day after hatching (DAH) 0. In experiment A, either fluorescent green beads of 1µm size at a concentration of 10^6.ml^{-1} or gfp-labelled *Es-*

cherichia coli (10^8.ml^{-1}) or gfp-labelled *Comamonas testosteroni* (10^8.ml^{-1}) bacterial strains were added to germ free sea bass larvae on DAH3 and DAH4. In experiment B, germ-free sea bass larvae were challenged with gfp-labelled *Listonella anguillarum* in three different concentrations (10^3.ml^{-1}, 10^5.ml^{-1}, 10^7.ml^{-1}) on DAH4. During experiment A, sampling was carried out after the first and second incubation, on DAH4 and DAH5. During experiment B, larvae were sampled after 24 and 48 hours of challenge, on DAH5 and DAH6. During sampling, larvae were killed with an overdose of benzocain, the external body surface was disinfected with benzalconium chloride and finally the larvae were fixated with paraformaldehyde and stored at 4°C. The whole larval body was individually mounted on glass slides. Image series of larvae containing gfp-labelled *Listonella anguillarum* were acquired using a LEICA TCS SP2 confocal microscope and an Argon 488nm excitation laser line (Leica Microsystems GmbH, Heidelberg, Germany).

Results

Experiment A

After 24 hours of incubation, bacteria were mostly localized in the hindgut of DAH4 larvae whereas the green beads were seen in both the mid- and hindgut. After 48 hours from the first incubation (repeated incubations took place on DAH3 and DAH4), both bacteria and beads were visible all over the digestive tract of DAH5 larvae, mostly in the gastric region, mid- and hindgut.

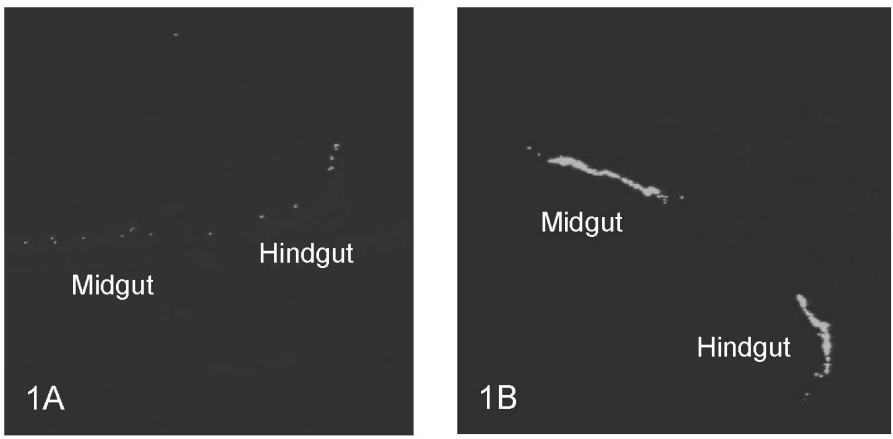

Fig.1. (A). Individually visible green beads in mid- and hindgut in DAH4 sea bass larva, (B) green beads visible in clusters in DAH5 larva.

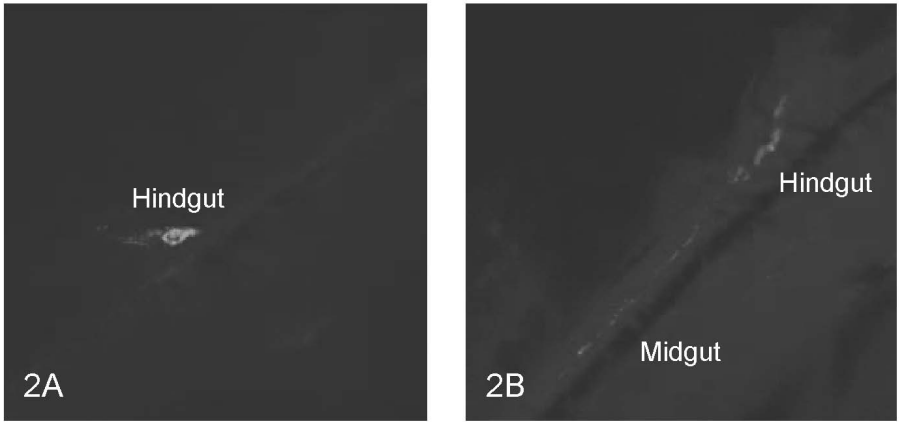

Fig. 2. (A) *E. coli* visible in the hindgut of DAH4 larva; (B): *E. coli* visible in midgut and hindgut in DAH5 larva.

Experiment B

The lowest concentration (10^3CFU.ml^{-1}) of *L. anguillarum* was used to see whether the initial adhesion place could be detected. However, only very few bacteria were seen inside the GI-tract on DAH5 and DAH6. In larvae challenged with *L. anguillarum* in higher concentrations (10^5CFU.ml^{-1} and 10^7CFU.ml^{-1}), bacteria were clearly visible in mid- and hindgut (Figure 3). In none of the tested treatments were bacteria detected outside the GI-tract.

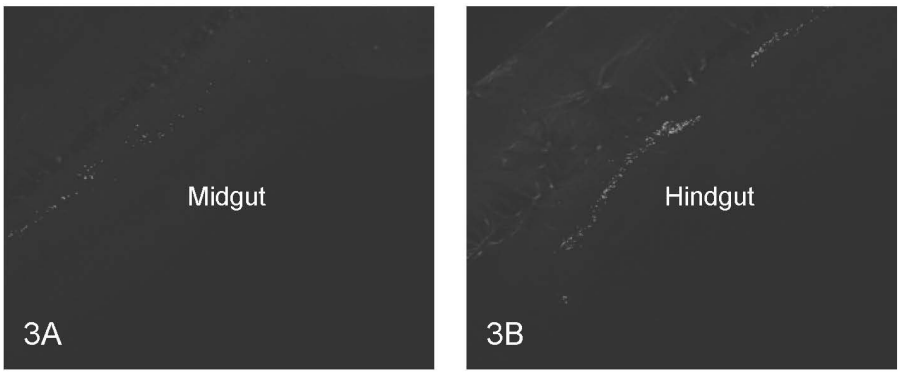

Fig. 3. *Listonella anguillarum* (105 CFU.ml^{-1}) in the midgut and hindgut of DAH6 larva.

Discussion

According to our results, gfp-labelled bacteria can be tracked in the GI tract of sea bass larvae by the use of confocal microscopy. Therefore, future tests on gfp labelled *Listonella anguillarum* in germ-free fish larvae can significantly contribute to our knowledge on host-microbe interactions. Furthermore, by the use

of Image J software, it may be possible to determine the intensity of the fluorescing bacteria, thereby allowing a semi-quantitative measurement of the presence of bacteria in the GI tract. Therefore, this method can become a powerful tool to study pathogenic/probiotic adherence and colonisation in the larval gut. Furthermore, as the exact location of bacterial colonisation can be determined, complementary studies by Transmission Electron Microscopy can investigate putative translocation of bacteria on cellular level.

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RAPID DETECTION OF ZOOPLANKTON AND MICROPARTICULATE UPTAKE BY LARVAL MARINE FINFISH – IMPROVEMENTS ON THE METHOD

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One of the bottlenecks to aquaculture of marine fish species is the requirement of live feeds at an early life stage. While rotifers and *Artemia* are common prey substitutes, copepods are still considered to be a superior prey item. Until now, a lot of guesswork has gone into determining whether larval fish are eating one prey item or another. Visual observations are not adequate in many instances to determine true fish behavior. The use of inert metal oxides to detect very small amounts of copepods and other prey items can be a useful tool when tailored to specific hatchery conditions.

This paper expands on previous work presented at Larvi 2005 by researchers at the Northwest Fisheries Science Center (Cook, Johnson, and Rust) on the use of inert metal oxides to determine the uptake of various types of zooplankton (rotifers and *Artemia*) and microparticulates by larval marine fish. This method has been improved by calibrating the incubation of the zooplankton with the marker, and testing the method with coldwater and warm water species.

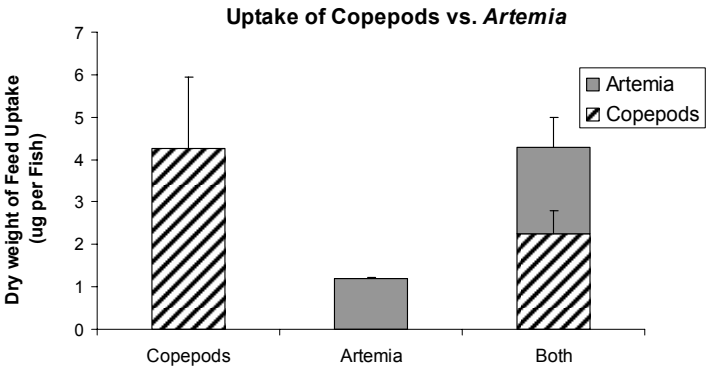

Fig. 1 Dry weight of feed uptake for day 18 brown rockfish (*Sebastes auriculatus*, Girard) larvae given copepods alone, *Artemia* alone, or a combination of copepods and *Artemia*. All error bars are standard error.

This method has been successfully utilized to determine the feeding preferences and relative uptake of live prey in comparison to microparticulates by larval rockfish (*Sebastes auriculatus*, Girard) and larval pompano (*Trachinotus carolinus*) during early feeding (Fig. 1). Once refined, this method will be useful for any aquaculture activity that requires the determination of feeding preferences and weaning windows of larval fish, as well as in the development of alternative live feeds such as copepods. In these two studies, visual observations were enhanced by analysis of inert metal oxide consumption.

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SENEGALESE SOLE (*SOLEA SENEGALENSIS*) LARVAE REARED IN RECIRCULATION SYSTEMS DEVELOP GOITRE WHEN IODINE IS NOT ADDED TO THEIR DIETS

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Introduction

Experiments with Senegalese sole show that rearing it in a recirculation system can result in the development of hyperplasia of the thyroid follicles (goitre) when larvae are fed commercially enriched rotifers and *Artemia*.

Iodine is very low in all types of commercial live feed (Solbakken et al., 2002; Hamre et al., 2008). Being an essential part of thyroid hormones (TH), iodine in the right concentration is crucial to assure sufficient level of TH, consequently assuring a successful metamorphosis and normal fish development (Leatherland, 1994). An experiment was conducted in order to determine the role of iodine in the development of Senegalese sole larvae reared in the recirculation system.

Material and methods

The experiment was conducted at University of Algarve. Larvae were given diets without extra iodine from 2 days after hatching (DAH) until 15DAH. At this age all larvae had adopted a benthic life style and were randomly distributed in 6 tanks in a recirculation system (700 larvae per tank). Larvae in three tanks received *Artemia* enriched with iodine in addition to Super Selco (Inve Aquaculture NV, Belgium) until 34DAH. Larvae in a second set of three tanks were fed control *Artemia*, enriched only with Super Selco. During the experiment, water temperature was 19.7±0.1°C, salinity was 33±0.2‰, and oxygen saturation was 96±0.4%. Samples of fish larvae were collected at 15, 27, and 34DAH to determine dry weight (dry wt.), iodine levels and thyroid status.

The density of each tank was of 3000 larvae.m^{-2} in 8 cm water column (20 l). Photoperiod was of 12:12h dark/light, with an intensity of 900lux at water surface. All flat-bottomed tanks used, were linked to one common 1700-l recircula-

tion system with 24 tanks, a protein skimmer and an additional use of ozone injection. The flow for each tank was 1400ml.min^{-1}. A maximum of 10% of new seawater was added to this system during experimental time.

Larvae were analysed for total iodine concentration using inductively coupled plasma mass spectroscopy, after alkaline decomposition as described by (Julshamn et al., 2001).

All statistical analyses were preformed with Statistica software (Statsoft Inc., Tulsa, OK. Ver.7). Data were subjected to a factorial ANOVA, to test potential difference between two groups over a period of time. Fishers LSD post hoc test was used to test for significant differences between group means in an analyses of variance. Effects and differences were considered significant at $P < 0.05$ for all tests.

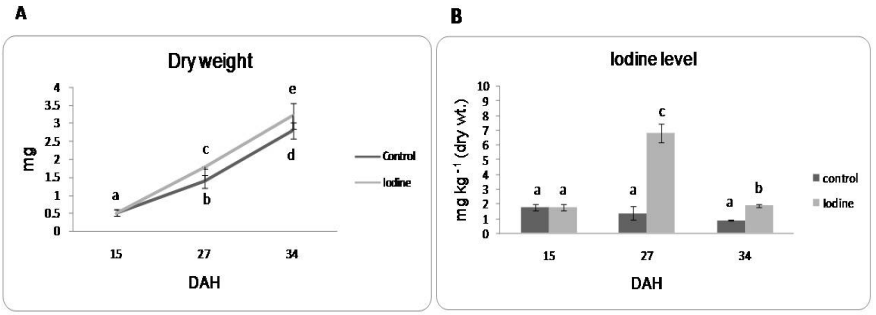

Fig. 1. (A) Dry weight (mg ±STD) of Senegalese sole larvae fed either control live feed or iodine enriched live feed from 15DAH to 34DAH. Values are means of 15 individual larvae at 15DAH and 34DAH and of pooled, taken form each tank (n = 3); (B) -Iodine levels (mg.kg^{-1} dry wt. ± STD) in Senegalese sole larvae, fed either control live feed or iodine enriched live feed from 15DAH to 34DAH Values are means of pooled larvae (100mg wet weight), taken form each tank (n = 3). a-e Mean values with unlike superscript letters are significantly different ($P < 0.05$ two-way ANOVA).

Results and discussion

In this experiment, dry wt. was significantly higher in larvae fed iodine enriched live feed, when larvae were in the recirculation system (Fig. 1A), as seen before in other work (Ribeiro et al., 2009, submitted). Iodine levels were higher at all sampling points in larvae fed iodine enriched live feed, as already seen in other study of Moren et al. (2006) (Fig.1B). No differences were found in through time and between treatments in deiodinase activity (Fig. 2A).

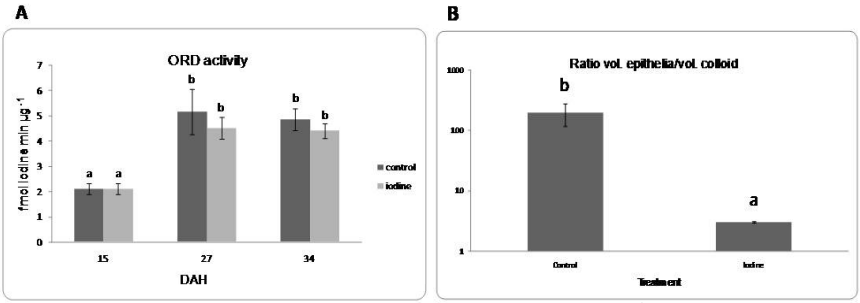

Fig. 2. (A) Outer ring deiodinase activity (fmol iodide min.μg^{-1} of protein ± STD) in Senegalese sole larvae, fed either control live feed or iodine enriched live feed from 15DAH to 34DAH. Values are means of pooled larvae (50mg wet weight), taken form each tank (n = 3). (B) - Ratio between the volume of thyroid epithelia and the volume of thyroid colloid (± STD) in Senegalese sole larvae, fed either control live feed or iodine enriched live feed from 15DAH to 34DAH. Values are means of three larvae per tank (n = 3). Y axis in logarithmic scale. a,b Mean values with unlike superscript letters are significantly different ($P < 0.05$ one-way ANOVA).

When fish were being reared in the recirculation system, control larvae showed thyroid follicles with hyperplasia, while iodine-treated larvae exhibited normal thyroid follicles, given by the ratio between the volume of thyroid epithelia and the volume of thyroid colloid (Fig. 2B).

Conclusion

At the end of the experiment, larvae from the control treatment suffered from hyperplasia of the thyroid follicles (goitre) whereas iodine treated larvae did not. Lower growth rate in fish larvae from the control treatment was probably a consequence of the hyperplasia. Iodine enrichment prevented Senegalese sole larvae form developing hyperplasia. The enrichment of live feed with iodine seem to be crucial for fish reared in a recirculation system using ozone injection, as it seems to sustain normal thyroid development and larval growth, although the iodine requirement is still to be determined.

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CO-FEEDING IN SENEGALESE SOLE AT MOUTH OPENING: CONSEQUENCES ON DIGESTIVE PHYSIOLOGY

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Introduction

Live food might present some attributes that enhance digestive activity together with some stimulatory effect towards digestive hormone secretions (Kolkovski 2001). Therefore, the aim of this study was to analyse the influence of different co-feeding regimes on *Solea senegalensis* digestive physiology by analysing the ontogenetic development of cholecystokinin (CCK) and vasoactive intestinal peptide (VIP) immunoreactive (IR) cells together with quantitative assessment of CCK-8.

Materials and methods

Solea senegalensis larvae were reared from hatching until 35 days after hatching (dah). Water parameters were measured daily and were kept at 22±1°C, at 32±1ppt, and above 90% of oxygen saturation.

Treatments consisted of: Standard (StD) – sole larvae and post-larvae were fed sequentially with enriched live food (rotifers, *Artemia* nauplii, and metanauplii) according to each developmental stage until 20dah, when they were fed with frozen *Artemia* metanauplii; Low replacement (LowR) – from mouth opening live food (*Artemia* nauplii and metanauplii) was gradually replaced by inert diet, during the first 5 days live food was at 100% decreasing to 50% at 20dah); High replacement (HighR) – from mouth opening live food (*Artemia* nauplii and metanauplii; 70% to 13% at 20dah) was abruptly replaced by inert diet. The inert diets used were Proton 1 and Aglonorse 1 diet according to larval developmental stage. Treatments were run in triplicate.

Fish larvae were sampled before feeding for dry weight determination and 30 minutes after feeding for CCK analysis; by immunohistochemical (10 larva at 2, 6, 15, 26, and 35dah) and radioimmunoassay techniques (individually at 15, 26, and 35dah).

Immunohistochemical analysis was done as described by Pinto et al. (2009). Specific antisera dilutions were 1:5000 and 1:2700, respectively for CCK and VIP.

CCK was determined on head (neural) and body (gut), of individually freeze-dried larvae. CCK was quantified by radioimmunoassay (EURIA-CCK RIA kit; CCK-8) as described for Atlantic halibut by Rojas-Garcia et al. (2001), using ethanol for extraction.

Results and discussion

Differences in growth between treatments were only observed at 26dah ($P<0.05$), with fish larvae from StD treatment exhibiting higher growth, followed by larvae from LowR treatment and with fish larvae from HighR exhibiting a lowest dry weight (Table I). At 35dah fish larvae from LowR grew to values identical to fish larvae from StD treatment, presenting significantly higher growth ($P<0.05$) when compared to fish larvae from HighR treatment. Similar observations were observed by Engrola et al. (2008) where a low replacement of *Artemia* by microdiet resulted in growth rates similar to control treatment suggesting that the amount of *Artemia* in the initial stages were sufficient to supply larval energetic requirements. However, when *Artemia* content of diets was lower than 100% during the first days after mouth opening (HighR treatment) growth was affected at later stages of development, despite the higher quantity of microdiet.

Table I. *Solea senegalensis* growth (dry weight, mg) and CCK content (fmol.larva^{-1}), when fed at different feeding regimes.

		dah	StD	LowR	HighR
Dw		6	0.05±0.01	0.05±0.01	0.05±0.01
		15	0.50±0.22	0.43±0.21	0.35±0.16
		26	2.75±0.77^{a}	1.83±0.25^{b}	1.34±0.24^{c}
		35	4.43±0.87^{a}	4.32±0.95^{a}	2.02±0.48^{b}
CCK	**neural**	15	0.59±0.19	0.87±0.08	0.57±0.24
		26	3.25±1.82	1.96±1.23	1.34±0.25
		35	2.21±0.46	1.65±0.16	1.95±0.67
	gut	15	1.30±1.04	1.62±0.54	0.72±0.24
		26	3.44±1.45	3.09±1.23	4.05±2.27
		35	2.87±0.25	3.79±0.41	2.30±0.34

Values are presented as mean ± s.d. (n=5)
Different letters in each row indicate significant differences.

Regardless of the type of feeding regime CCK- and VIP-IR cells were detected in the digestive tract of sole larvae at the same age and stage of development, 2 and 6dah, respectively (Fig. 1). Since morphological and physiological changes appear to be more dependent on size than age (Ribeiro et al., 2005), fish larvae

of similar size was sampled among treatments to avoid a misleading dietary effect on the appearance of IR cells. CCK-IR cells were initially observed in the anterior portion of intestine, but CCK IR cells were later also observed in the posterior intestine. VIP immunoreactions were observed in the submucosa and muscular layer of digestive tract at the end of some nerve fibers, similarly to that reported by Holmgren (1993), initially in the anterior intestine and later also in the stomach. These observations support that digestion is regulated by hormonal factors also in stages of development, since stomach develops at later stages during metamorphosis.

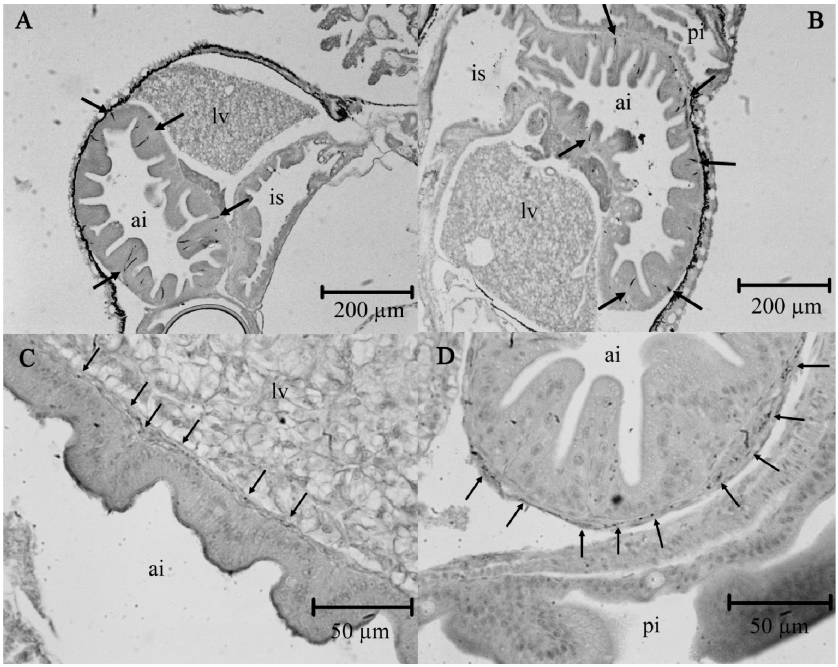

Fig. 1. Distribution of immunoreactive CCK and VIP in intestine sections (5µm) *Solea senegalensis:* CCK – IR cells in 15dah sole larvae fed live food (A) and which live food was highly replaced by microdiet (B); VIP immunoreactive nerve fibers of 35dah of sole larvae fed live food (C) and which live food was highly replaced by microdiet (D). ai – anterior intestine, is – incipient stomach, lv- liver, pi – posterior intestine.

In this study, a higher fraction of live food did not enhance CCK secretion, and consequently a similar influence on CCK related functions like bile and pancreatic enzyme release were expected among treatments. In fact, CCK content of larvae exhibited a similar pattern of variation between neural and gut portion (Table I), it increased between 15 and 26dah and remained rather stable afterwards. Moreover, the CCK located in the gut compartment comprised between 54% (HighR) to 70% (LowR) of whole body CCK at 35dah, a range of values

that compares well to 62% described for halibut 4 weeks after first feeding (Rønnestad et al. 2007).

Conclusions

A low quantity of live food at early life stages of development had a negative impact on development. However, independent of the quantity of live food, all groups of larvae had similar indicators of a neuro-endocrine regulation of digestion at the same stages of development.

Acknowledgements

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IMPROVEMENTS IN THE LARVAL CULTURE OF ROCK LOBSTERS: A REVIEW

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The farming of rock (or spiny) lobsters is of growing interest worldwide. The current relatively small-scale of farming, predominantly in south-east Asia (e.g. in Vietnam at 2 000 mt p.a.) depends on and is constrained by the unsustainable catch of post-larval (puerulus and juvenile) seedstock from wild fisheries. Hatcheries would support a considerable expansion in aquaculture to meet market demand. However, the technical problems of larval rearing are yet to be surmounted before the economic issues of industrial production of seedstock from eggs can be considered. The recent success with larval rearing through to metamorphosis as a result of progress in the research, particularly in health, feeding, and systems, is now attracting industry to plan the commercial hatchery production of seedstock to supply the growout operations. This also has application in enhancing the wild fishery by re-seeding with hatchery-reared juveniles.

The major problem that needed to be overcome with hatchery rearing of phyllosoma larvae was their high susceptibility to disease, most notably the common enteric *Vibrio* spp. This problem is exacerbated by the long larval phase, typically 6-12 months and 15-23 instars for most Palinurids. In the wild, phyllosoma develop in a pristine marine environment almost devoid of pathogens. Reducing the exposure to infection in the hatchery can be achieved by anti-microbial treatments of the culture water, the live and inert feed, or the larvae. The initial success in rearing larvae through to metamorphosis in Japan was attributed mainly to the use of broad-spectrum antibiotics, particularly chloramphenicol. Antibiotics appear to offer protection even in the presence of high microbial loads. However, wide-scale application of antibiotics is regarded as inappropriate because of environmental residues and the possible development of resistant organisms.

Chemical disinfectants are an attractive alternative to control microbial infection as they are easily and economically administered. Formalin and chlorine-based disinfectants appear to be the most suitable when used in continuous culture and cause disruption of metabolic processes in the micro-organisms. However,

chronic use over the long larval phase may have adverse effects, such as inducing abnormalities and deformities, which occur during the moult.

Recently, the ozonation of seawater was used for the improvement of water quality and minimization of bacterial pathogens to optimize the survival during phyllosoma culture. Unlike in freshwater, where the half-life of dissolved ozone may be 20min, seawater constituents quickly react with ozone to form highly reactive oxidants. These are mainly the products of reactions with the bromine in seawater, some of which have beneficial disinfective properties at low levels while others are toxic to larvae. The highly oxidative residues persist in the culture water and react with the surface membranes of micro-organisms.

The research in our laboratory showed that when ozonated seawater was used for culture, larvae could be reared from egg throughout larval development and metamorphosis. This is a technique that has produced consistently repeatable results. It can be applied on a large scale for the industrial production of viable post-larval seedstock. The next step is to develop a procedure that accurately measures the low levels of oxidative residues in the culture water that can then be used in a feedback mechanism to control the level of ozonation. This process, whereby a low level of residuals is retained in the ozonated seawater to optimise larval health, may have wider application for the culture of other crustaceans.

The feeding regimes for phyllosoma typically incorporate live *Artemia* and mussel gonad, and are the only food items that have been used successfully for complete larval development. As phyllosoma grow, the ingestion rate of *Artemia* diminishes and mussel gonad increases, while the preferred size of both increases. However, live *Artemia* and mussel gonad may be regarded as expensive because of the high labour input for the first and the high cost on a dry matter basis for the second. Alternatives have been sought, including inert fresh foods and formulated diets, but none have yet shown any similar prospects in larval performance. Nevertheless, alternative foods are needed for the efficient and economic commercial production of post-larvae.

For the production of large numbers of seedstock, culture systems need to accommodate the unique requirements of phyllosoma, including minimising their contact with tank surfaces that harbour high loads of pathogens, ensuring that larvae have continuous access to food, and yet avoiding vigorous movements that may damage the appendages. The small-scale vessels that were successfully used for experimental purposes are mostly unsuitable for mass culture and new tanks and systems need to be developed.

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GENETIC RESPONSE TO DIETARY MANIPULATIONS DURING TRANSFER TO EXOGENOUS FEEDING IN ATLANTIC COD LARVAE

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Introduction

The onset of exogenous feeding represents an ecologically, physiologically, and developmentally important transitional event for fish larvae. During this time, the digestive system is activated and nutrient acquisition changes from endogenous yolk-sac to exogenous feed which is captured and ingested. Larvae continue to undergo numerous developmental changes before they are fully transferred into juveniles and these processes are well-known to be strongly impacted by diet. The digestive system is responsible for digestion and absorption of ingested food and is exposed to all dietary (+ other biotic) components which enter the gut lumen. There is scarce knowledge on the changes in gene expression occurring in the digestive system in response to the onset of exogenous feeding. Absorbed nutrients which enter the systemic circulation have profound effects on developmental and cellular processes, but very little is known how dietary components affect ontogenetic patterns of gene expression during critical stages of larval development. Therefore, our aim of the present study was to examine the transcriptomic response of Atlantic cod, *Gadus morhua*, larvae to the onset of exogenous feeding and to different diets.

Materials and methods

Newly hatched larvae were supplied from Parisvannet, Institute of Marine Research, transferred to Bergen High Technology Center at 2 days post-hatch (dph), and divided in 4 stagnant tanks (water volume of 160 l) with a density of 31 larvae.l^{-1}. Larvae were reared at 8°C and under a 16:4h (light:dark) photoperiod with 2h dusk and dawn. Feeding began at 2dph referred to as 0 day post feeding (dpf). Prey densities were 5000 prey.l^{-1} and adjusted accordingly twice daily. In a linear regression design, one tank remained unfed (A), one tank was fed wild-captured zooplankton, mainly copepod nauplii (B), and one tank was

fed zooplankton with algae from 1dph (C). Larvae from each tank were sampled at -1, 0, 1, 2, 4, and 6dpf. Triplicate samples containing 125 pooled larvae were collected on dry ice and flash frozen. Automated RNA isolation was used. RNA quality and quantity were determined using the Agilent Bioanalyser 2100 and Nano Drop systems (A260/280 and A230/280). Total RNA was subjected to separate direct cDNA synthesis protocols using Cy3 and Cy5 labelling and hybridised to the 16K cod cDNA microarray before subjected to standard scanning procedures. Microarray data analysis was performed using J-Express data analysis software (MolMine, Bergen, Norway). Unpaired rank product analysis using 400 permutations on $\log_2$ values were performed to identify gene candidates to be used for further analysis and validations.

Results and discussion

Onset of exogenous feeding resulted in transcriptomic responses in Atlantic cod larvae in all dietary treatments. Correlation analysis clearly identified the influence of feeding regime at all specific time points (0, 2, and 6dpf) (Fig. 1).

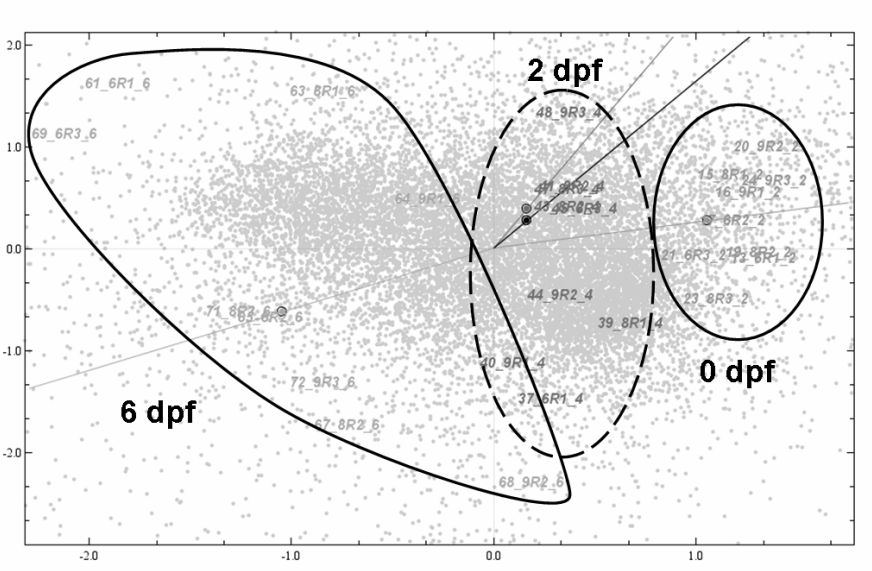

Fig. 1. Correlation analysis of Microarray Spot-pix data. Individual data are grouped according to days post feeding (dpf).

Rank product analysis identified groups of genes which were differentially expressed (Table I), and these results clearly indicated that more genes were differentially regulated in larvae fed zooplankton only than those in larvae co-fed

zooplankton and algae, whereas the number of differentially expressed genes in the unfed larvae were lower.

Table I. Number of differentially expressed genes between feeding regimes indicated by rank product analyses. (A) Unfed larvae, (B) zooplankton fed larvae, and (C) larvae fed zooplankton with algae. Unfed larvae at 0dpf were used as the reference group).

Reference group			Treatment group			
Group ID	**dpf**	**N**	**Group ID**	**dpf**	**N**	**No. of probe hits**
A	0	9	A	2	3	969
A	0	9	A	6	3	1553
A	0	9	B	2	3	821
A	0	9	B	6	3	2028
A	0	9	C	2	3	803
A	0	9	C	6	3	1418
A	0	9	B and C	2	6	345
A	0	9	B and C	6	6	723
A	0	9	B and C	2, 6	12	N.A.

Conclusion

These results show that Atlantic cod larvae alter gene expression rapidly in response to the onset of exogenous feeding and to the different dietary components.

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EFFECT OF REARING SYSTEM INTENSIVENESS ON BIOLOGICAL FEATURES, CULTURE PERFORMANCE, AND LARVAL QUALITY OF MEAGRE (*ARGYROSOMUS REGIUS* ASSO, 1801) LARVAE

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Introduction

Meagre, *Argyrosomus regius* A., is a new species for aquaculture in south Atlantic and Mediterranean countries, which can reach a mean fresh weight of 8.02±2.51g at 95dah. However, limited information about the optimal culture techniques and biological performance of meagre are available. Thus, larval rearing of this species is usually performed adapting gilthead sea bream culture techniques (Pastor et al., 2002; Estevez et al., 2007; Roo et al., 2007). In order to establish a standard rearing protocol and better understand its biological performance, a semi-intensive system was used as a model system for fry production in comparison with intensive culture system.

Materials and methods

Meagre (*Argyrosomus regius)* eggs obtained from hormonally (GnrHA injection) induced spawning, were distributed into the rearing tanks provided with filtered and UV sterilized seawater. Two rearing techniques, mainly differing in tank capacity and larval density, were compared: semi-intensive system (in two 40-m^3 tanks) stocked with 7.5 eggs.l^{-1} and intensive system, (in three 2-m^3 tanks) stocked with 125 eggs.l^{-1}. Larval rearing protocols include the use of live *Nannochloropsis sp* at 250±100×10^3 cells.ml^{-1} added once a day from day 2 after hatching (dah) until day 15, enriched rotifers *Brachionus plicatilis,* from 2-20dah, adjusted to 5-10 rot.ml^{-1}in the intensive system and 4-5 rot.ml^{-1} in the semi-intensive system twice a day. Enriched *Artemia* was introduce at 12dah Instar I (25-250 A_0.l^{-1}) (AF type, INVE Aquaculture, Dendermonde, Belgium) and quick changed to enriched *Artemia* Instar II by day 15. Commercial diet (Genma Micro, Skretting, France) was introduce from 20dah (Gemma Micro, Skretting, France) and manually distributed four times a day for 5 days and every hour by means of automatic feeders afterwards. Fish were completely weaned by day 35.

Larval growth in total length (TL) and dry weight (DW) was assessed measuring 25 larvae at 30dah according to the methodology described by Fernández-Palacios et al. (2007). Larval survival after the activity test (120 seconds air exposure) at 30dah and survival were obtained according the methodology described by Roo et al. (2009b). The incidence of skeletal anomalies was evaluated following the protocol of Roo et al. (2009a).

Results and discussion

The culture intensiveness affected larval growth, with the semi-intensive system reared larvae being significantly higher in total length and dry body weight, (Table I). These results are in concordance with data reported with other species such as *Diplodus puntazzo* and *D. sargus* sargus (Papandroulakis et al., 2004) or *Pagrus pagrus* (Roo et al., 2009b).

Table I. Effect of culture intensiveness on growth and survival at 30dah.

Treatment	TL (mm)	DW (mg)	% Activity test	Survival 30dah %
Semi-Intensive	19.08±2.30[a]	13.09±2.43[a]	75.06±13.83[a]	25.67±2.51[a]
Intensive	16.00±1.54[b]	6.46±0.52[b]	53.33±11.54[b]	18.0±2.00[b]

Note: Different letter in the same column denotes significant differences ($P<0.05$).

The survival rate in the intensive system was similar to those reported by Estévez et al. (2007) and Rodriguez-Rua et al. (2007) for this species, and was significantly improved with semi-intensive system (25.67%; Table I). In addition, survival at 30dah could be improved reducing the cannibalistic behaviour observed from 20dah at it was observed as the main cause of mortality, as occurs in other species from early stages such as *Seriola* sp. (Moran, 2007; Papandroulakis et al., 2005) suggesting that early grading might prevent aggressive behaviour over smaller individuals. The results of the activity test confirm that meagre resists handling from early stages, when cultured under semi-intensive system. The poorer resistance of intensively reared larvae could be related to the lower growth obtained as suggested by Liu et al. (2002). Regarding larval quality in terms of skeletal deformities, there was no significant interaction between the rearing system and the number of deformed fish ($\chi^2 = 1.970$; $p=0.160$) and only 4.3% of fry produced showed some skeletal deformities, with fusion affecting vertebrae 10 to 15 the most frequent anomaly (Fig. 1).

In addition, the use of intensive systems was also suitable and cost-effective for larval rearing of this specie (Table II). Thus, the use of phytoplankton, *Artemia* , and microdiet per fry produce at the end of the weaning period (35dah) were significantly lower under this type of system.

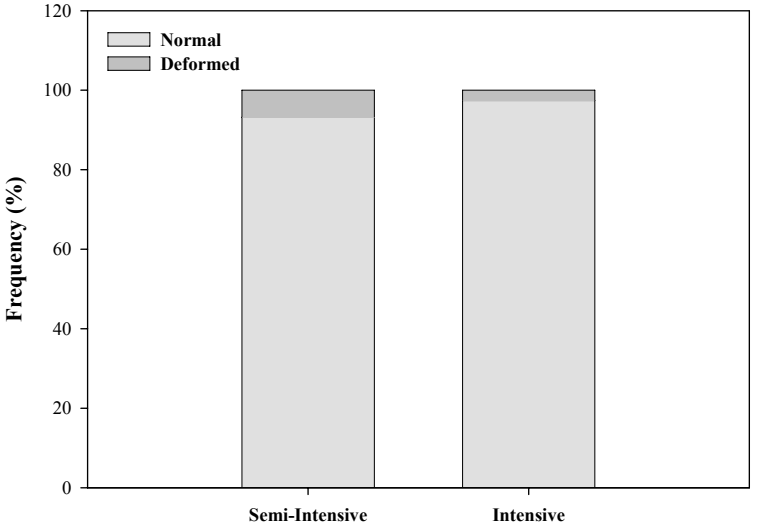

Fig. 1. Fry quality according to the rearing system.

Table II. Comparison of feed quantities (per fry produce) and feeding costs according to the rearing system at weaning (35dah).

Treatment	Microalgae (ml)	Rotifers (millions)	*Artemia* (millions)	Microdiet (g)	Cost (€)
Semi-Intensive	37.07[a]	18.2	8.2[a]	0.051[a]	0.020[a]
Intensive	26.28[b]	21.4	5.1[b]	0.025[b]	0.015[b]

Note: Different letter in the same column denotes significant differences ($P<0.05$).

Conclusions

Meagre is one of the most promising species for Mediterranean and South Atlantic coastal aquaculture; however, fry availability is one of the most important bottlenecks. These results suggest that larval rearing of meagre can be performed under intensive or semi-intensive system conditions, the latter producing better survival and growth. However, rearing system intensiveness has no effect on fry quality and the intensive larval rearing of this species results in a higher cost-affectivity for fry production at commercial scale.

Acknowledgements

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PRESENCE OF *HLYA*, *FLAC*, AND *TOXR* GENES OF VIBRIOS IN *VIBRIO HARVEYI*

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Introduction

Vibrio harveyi is a luminous gram-negative marine bacterium widely distributed in the marine environment and it is a major pathogen of wide variety of hosts including cultured penaeid shrimp and has also been associated with fish diseases. Several virulence factors have been associated with pathogenicity of *V. harveyi* such as proteases, phospholipases, hemolysins, cystein proteases, bacteriophages, and bacteriocin- like substance (BLIS) (Austin et al., 2006). It is not fully elucidated which factors are responsible for the virulence towards organism, especially in vivo. It has been proposed that the virulence genes could be horizontally transferred among *Vibrio* spp. (Waldor and Mekalanos, 1996), probably contributing to a wide variety in virulence.

This study aimed at verifying the presence of the virulence genes *zot*, *toxR*, *tcpA*, *flaC*, *ctxA*, *hlyA*, *trh*, *tdh*, and *vvh* in the genomes of *Vibrio harveyi* or the closely relates species *V. campbellii* by PCR amplification.

Materials and methods

Fifty one *V. harveyi* strains and 1 *V. campbellii* strain from a diverse range of hosts and geographical locations were used in this study. The cultures were obtained from the *Vibrio* collection in the Laboratory of Aquaculture and Artemia Reference Center, Ghent University, and were previously identified and confirmed as *V. harveyi* and as *V.campbellii*.

Genomic DNA was extracted and purified according to Ausubel et al. (1995). Primers used for the amplification of *trh*, *tdh* (Tada et al., 1992), *vvh* (Lee et al., 1999) *toxR*, *zot*, *tcpA*, *ctxA*, *hlyA* (Saravanan et al., 2007), and *flaC* (Bai et al., 2008) were taken from the literature. Other specific primers were designed using the Primer 3 software (details not shown). All the primers were designed based on consensus sequences deposited in GenBank and were blasted against GenBank.

PCR was performed according to Bai et al. (2008). Annealing was done at 50°C for *luxR, toxR, srp*, and *vhpA*; 55°C for *vhml, flac, chiA*, *vhh*, *vvh*, *trh*, and *tdh*; and 60°C for *ctxA, toxRvc, tcpA, hlyA*, and *zot*. PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide and the results observed using a Herolab gel doc system. To confirm the PCR amplificons, some of the PCR products; i.e., the *toxR,* the *flaC, hlyA,* gene of *V. harveyi,* were sequenced by Chromous biotech, Bangalore. In addition, the presence of those atypical *Vibrio harveyi* genes was confirmed by dot blot hybridization. Probes were made according to Roche Diagnostics (Germany).

Challenge experiments were performed with high quality cysts of *Artemia franciscana* (INVE Aquaculture, Baasrode, Belgium), according to Marques et al. (2004). *Artemia* were challenged with bacteria at the dose of 10^6 CFU.ml^{-1} of *Artemia* culture water. The survival of *Artemia* was counted 48h after the challenge. Each treatment was done in triplicate and the sterility of the control treatments was checked at the end of the challenge.

Results and discussion

All the reported virulence associated genes of *V.harveyi vhh*, *chiA*, *vhpA*, *toxR*, *luxR,* and gene for serine protease were detected in all the isolates by PCR. *V. cholerae* specific *hlyA*, and *toxR, V. anguillarum* specific *flaC* genes were detected in some of the *V. harveyi* isolates (Fig. 1). Out of 52 strains, 11 (21%), 8 (15%), and 33 (63%) were positive for the *toxR*, *hlyA* and *flaC* genes respectively. *tcpA*, *ctxA*, *zot* of *V. cholerae*, *tdh*, and *trh* of *V. parahaemolyticus* and *vvh* of *V. vulnificus* was not detected by PCR. All the isolates were negative for the *vhs* gene indicating the absence of siphovirus. One isolate of *V. harveyi* was positive for *vhml* gene thus confirming the presence of the myovirus like phage. All genes, positive in PCR, developed visual hybridization in dot blot confirming the presence of those genes in *V. harveyi.*

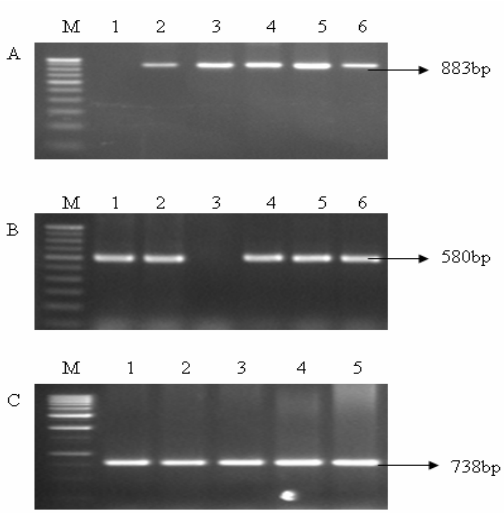

Fig. 1. Presence of *toxR* (A), and *hlyA* (C) gene of *V. cholerae* and *flaC* (B) of *V. anguillarum* in *V. harveyi*. A: M.100bp marker, lane 1. negative control, lane 2. positive control, lane 3. STD30949, lane 4. STD30953, lane 5. STD30983 and lane 6. LMG 07890. B: M. 100bp DNA ladder, lane 1. STD30949, lane 2. STD30953, lane 3. negative control, lane 4. positive control, lane 5. STD30983 and lane 6. LMG 07890. C: M: 1kb DNA ladder, lane 1. positive control, lane 2. STD30953, lane 3. STD30983, lane 4. STD30949 and lane 5. LMG 07890.

Virulence genes, *toxR*, *hlyA* and *flaC* were widely distributed in *V. harveyi* isolates from various sources, whereas the virulence genes, *zot, ctxA* and *tcpA*, *trh*, *tdh* and *vvh* which are important in *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* respectively, did not exist in *V. harveyi*. The *hlyA*, *flac* and *ToxR* gene of *V. harveyi* are more than 97% identical to *V. cholerae*. The present study reinforces the findings of previous study that have shown that virulence genes of *V. cholerae*, *V. mimicus*, *V. alginolyticus* and *V. parahaemolyticus* (Sechi et al., 2000; Shinoda and Nakagawa, 2004; Xie et al., 2005; Bai et al., 2008) are atypically present in other *Vibrio* species. The highest mortality was found in *Artemia* challenged with *V. campbellii*. Yet none of the atypical *V. harveyi* genes is present in this isolate. On the other hand a strain (LMG10947) that caused low mortality (2%) was shown to be positive for *toxR*, *flaC* and *hlyA* gene. This is in agreement with the findings of Bai et al. (2008).

Conclusion

The findings of this study showed that there is no correlation between the presence of atypical *V. harveyi* virulence genes and the virulence towards *Artemia* in the investigated collection of *V. harveyi* strains.

Acknowledgments

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AMINO ACIDS HAVE IMPORTANT ROLES IN LARVAL DEVELOPMENT OTHER THAN GROWTH

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Introduction

Amino acids are the most important energetic substrate during fish larval stages and therefore there is a high amino acid (AA) requirement (Rønnestad et al., 1999). AA composition in larval diets affects growth (Fauconneau et al., 1992) as well larval quality and performance (Aragão et al., 2007).

Tyrosine is a semi-indispensable AA, precursor of dopamines and the adrenocortical hormones norepinephrine and adrenaline. Dopamines regulate central and peripheric nervous system activity and can therefore be related to the control of stress in the fish (Lehnert and Wurtman, 1993).

Material and methods

Two different trials using *Diplodus sargus* larvae were run to test the effect of an AA balanced diet and the effect of an AA balanced diet supplemented with tyrosine. Each experiment lasted 25 days and larvae were held in 200-l conical cylindrical fibreglass tanks at a density of 80 larvae.l^{-1}. In the first trial three dietary treatments were tested: a control diet consisting on live feed, a diet with an AA profile similar to the AA profile of the larval carcass (balanced diet) and an AA unbalanced diet. On the second trial the balanced diet was used as a control and a balanced diet supplemented with tyrosine was tested. On the first experiment the AA balanced diet was given in the form of a casein microencapsulated diet whereas on the second experiment, besides the microencapsulated diet, rotifers were given liposomes incorporating free AA (FAA) in order to balance their AA profiles.

On the first experiment, feeding protocol for balanced and unbalanced diet treatments was the same as the control until the 11DAH (5 rotifers.ml^{-1}). From 12 to 14DAH the live food was decreased to half of the control and afterwards *Brachionus plicatilis* and *Artemia* were only given at 10% of the control. The microencapsulated diet was introduced at 8DAH (from 0.5g to 1.5g at the end of the experiment). On the second experiment rotifers were boosted with liposomes filled with FAA one hour before given to the larvae (5 rotifers.ml^{-1} until 14DAH and 2.5 rotifers.ml^{-1} from 15DAH). The microencapsulated diet was introduced at day 15 (1.5g to 2g). Liposomes were filled with free amino acids according to Barr and Helland (2007), using the free amino acid quantities detailed in Table I.

Table I. Amino acid quantities added to the liposomes used to enriched rotifers from the control and tyrosine treatment (Tyr) for one meal. Data are expressed as g of AA *per* million of enriched rotifers.

	His	**Lys**	**Arg**	**Thr**	**Met**	**Phe**	**Tyr**
Control	0.016	0.061	0.033	0.017	0.008	0.017	0.001
Tyr	0.047	0.183	0.100	0.050	0.023	0.052	0.003

On the first experiment ammonia excretion trials were done in fed and fasted larvae using 10 fish larvae per tank enclosed in 45 ml spherical glass vials for two hours. Five replicates from each treatment were used. Ammonia concentration was determined according to Berthelot (Grasshoff, 1983). Deformities at the vertebral column were also analysed in 90 larvae per treatment, at 25DAH. Cartilage was stained with Alcian blue (40 minutes) and ossified bone was stained with Alizarin Red (2 hours), according to Gavaia et al. (2000). Vertebral column was divided into three regions: trunk (1 to 11 vertebrae), caudal (12 to 20), and preurostyle (remaining three and urostyle).

On the second experiment, a temperature stress test consisting on a sudden drop of temperature (21ºC to 10ºC for five minutes). Stress resistance was measured in terms of survival ten minutes after the temperature was risen. Ten larvae were used from each tank and trials were repeated three times for each tank (nine replicates per treatment).

Results and discussion

On the first experiment, significant differences in ammonia excretion were found between control and balanced diet treatments in fed larvae. Balanced diet seemed to present almost no difference between fed and fasted larvae and registered the lowest ammonia excretion of all treatments. On the same experiment, the frequency of deformed larvae at 25DAH was approximately 40% in the control group, 30% in the unbalanced diet group and 20% on the balanced diet group (Fig. 1). A significant high number of vertebrae fusions were found in the control treatment. Lordosis was found in control and unbalanced diet groups but not in the balanced diet group.

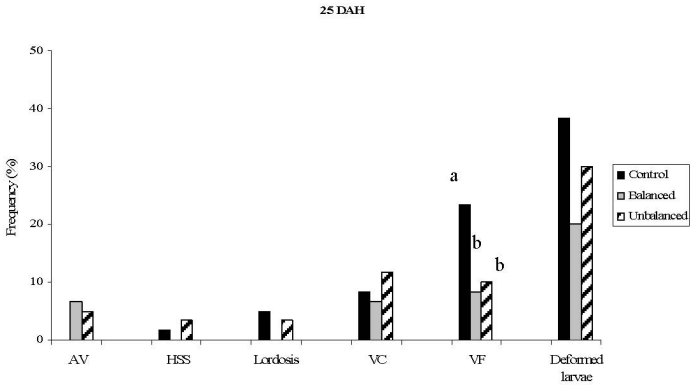

Fig. 1. Deformities observed at the dorsal column in *Diplodus sargus* fed on control, a balanced and unbalanced diets (n=60 larvae *per* treatment). AV- Abnormal shape vertebra, HSS- Supranumeric haemal process, VC- Vertebral compression, VF- Vertebral fusion. Different letters represent significant differences for $p < 0.05$.

On the second experiment the stress test showed significant differences between treatments (Fig. 2).

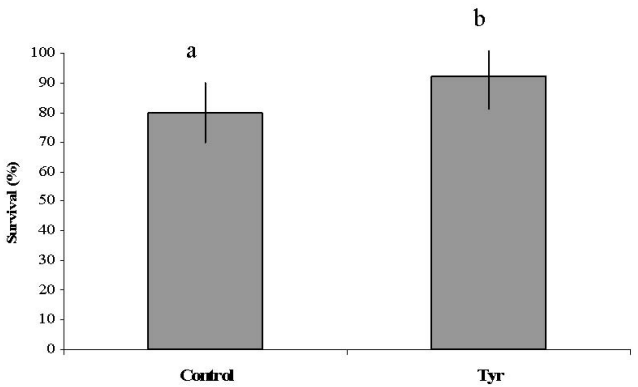

Fig. 2. *Diplodus sargus* larval survival rate after being subjected to a temperature stress test. Values are mean and standard deviations (n=9). Different letters represent significant differences.

When submitted to a sudden drop of temperature at 25DAH, larvae fed a diet supplemented with tyrosine had significantly higher survival rate when compared with the other treatments. There is lack of information on this matter related to fish but laboratory studies strongly suggest that tyrosine supplementation may serve to reduce cognitive and behavioural effects of exposure to stress (Deijen et al., 1999).

Conclusions

An AA-balanced diet seems to reduce nitrogen excretion in fed larvae and decrease the incidence of skeletal deformities at the vertebral column. Using the same AA-balanced diet with a supplement of tyrosine there seems to be a higher survival rate when larvae are submitted to a stress test such as a sudden drop of temperature. The present study confirms the idea that there are AA requirements for metabolic processes other than growth.

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ONTOGENY OF LIPID DIGESTION IN ATLANTIC COD (*GADUS MORHUA*)

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Lipase ontogeny in marine fish larvae has been investigated at different levels (mRNA, protein and activity) in a number of species, but often with only one of the mentioned parameters at the time. The ontogeny of lipase activity has been described in cod, but only until approximately 3±50 days post hatching. The aim of the present study was to describe the ontogeny of bile activated lipase (BAL) and phospholipase A_2 (PLA_2) in larvae of Atlantic cod (*Gadus morhua*).

Homogenates of larvae from first exogenous feeding until day 62 post hatch (dph) and GI tract dissections from 34 until 97dph were analyzed.

Larvae were sampled immediately after ingestion of food. This was done because food triggers lipase production. BAL mRNA (EX741858) expression was measured with RT-qPCR and enzymatic quantity assessed. PLA_2 was investigated with RT-qPCR (EX726814), Western blot, and activity (Cayman Chemical secretory PLA_2 ($sPLA_2$) Assay kit).

As found in other species, enzymatic activity for both BAL and PLA_2 is relatively high during early stages only to decrease later. This decrease in activity coincides with the switch from live prey (rotifers) to formulated diet. BAL activity per wet weight proved to be 10 times as high in rotifers compared to activity in homogenates of whole fish fed rotifers. Considering this, larvae would

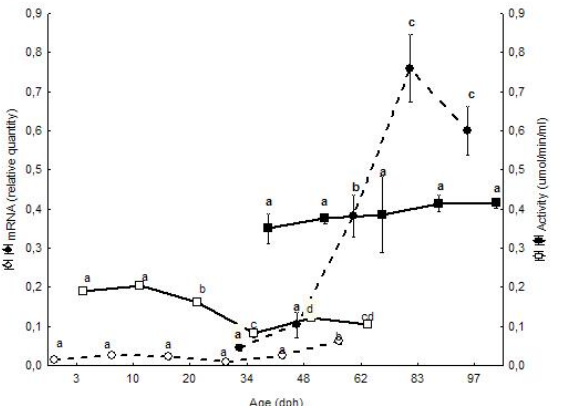

Fig. 1. BAL activity (boxes) and mRNA expression (circles), in larvae homogenates (open symbols) and GI-tract (filled symbols).

only have to fill up with 5% of its own body weight with rotifers to double the total body enzyme activity. In the dissected GI-tracts BAL activity, as well as PLA_2 activity, does not change from 34 to 97dph.

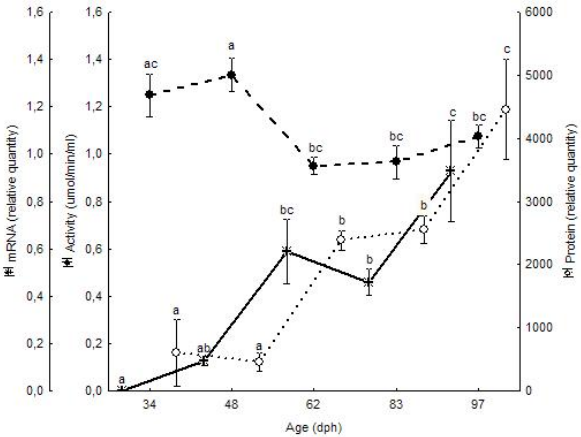

Fig. 2. Relative quantity of PLA_2 activity (open circles), protein expression (filled circles) and mRNA expression (cross) in dissected GI-tract.

On the contrary, mRNA levels are stably low during the live feed stage, the weaning period and onward until the larvae are 48dph. BAL mRNA shows a dramatic increase in quantity from 34 to 83dph. The same pattern is seen in PLA_2 in both protein and mRNA. PLA_2 mRNA expression and protein expression in GI-tract dissections correlate significantly during development ($p=0.004$, $r^2=0.52$). Thus, we have a situation where the production and amount of an enzyme does not say anything about the apparent activity of the same enzyme. In the case of PLA_2 it is important to keep in mind that the activity assay does only discriminate between the secretory and cellular forms.

In summary, cod larvae have low production of BAL and PLA_2 from hatching until 15mm standard length (48dph). Enzymatic activity on the other hand seems quite high. However, the contribution from live pray (rotifers in our case) is probably high.

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EFFECTS OF WATER FLOW, SALINITY GRADIENT, AND LIGHT INTENSITY ON THE LARVAL PERFORMANCE OF THE DEVIL STINGER *INIMICUS JAPONICUS*

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Introduction

Physical environments in the rearing tanks are important factors for larviculture. Of these factors, water temperature and salinity are relatively easy to control and are well studied. On the other hand, little attention has been paid to the water flow in rearing tanks which has the great impact on stable production for larviculture (Sakakura et al., 2007).

Devil stinger *Inimicus japonicus* is a commercially valuable demersal fish in Japan and seedling production has been conducted in many hatcheries. However, rearing results fluctuate by mass mortality during larviculture, even though using the eggs from the same batch and rearing in the same temperature and tank proportions (Kadomura et al., 2007; Ruttanapornvareesakul et al., 2007).

We hypothesized that this fluctuation is due to the differences in the rearing environment, such as water flow and light intensity in the rearing tanks, and examined the different flow field and light intensities on the larval performance of this species.

Materials and methods

Newly hatched larvae were stocked into 1-m^3 cylindrical black tank (130cm in diameter and 70cm in depth) at a density of 12 000 larvae.$tank^{-1}$. Three different rearing experiments were conducted from hatching to settlement (juvenile), and growth and survival of fish were compared.

Fish were reared at the same temperature (25°C), water exchange rate (100%.day^{-1}). Feeding regime was also set at the same conditions following the previous study on the ontogenetic changes in size preference of prey in this species (Olsen et al., 2003), where rotifer *Brachionus plicatilis* were fed from mouth opening (day 1 after hatching) to 5.2 mm in standard length (SL) and *Artemia* nauplii were offered thereafter. Rotifers were enriched with HUFA-enriched freshwater *Chlorella vulgaris* and *Artemia* was enriched with commercial product (Super capsule A-1, Chlorella Industry, Japan).

Experiment 1: Effects of water flow in the rearing tank on larviculture of devil stinger
A total of 10 rearing tanks were used and 5 different aeration rates (0, 50, 300, 600, and 1200ml.min^{-1}) were set with duplicate. Growth and survival until settlement (day 21) were monitored.

Experiment 2: Effects of salinity gradient in the rearing tank on larviculture of devil stinger
Salinity gradient in a rearing tank was formed by pumping brackish water (22ppt) from the surface and seawater (34ppt) from the bottom at the same time (n=2). Control rearing tanks were aerated at 300ml.min^{-1} (n=3). Fish were reared until day 23

Experiment 3: Effects of light intensities on larviculture of devil stinger.
A total of 9 rearing tanks were put under the natural light condition and different light intensities in the daytime were set using shade. Average light intensities in the daytime varied from 0.2 to 4700lux among rearing tanks. Fish were reared until settlement (day 26).

Results and discussion

In the Experiment 1, There was a positive relationship between aeration rate and the final survival rates (n=10, r=0.7477, $p<0.05$). High aeration rate tank (>300ml.min^{-1}) showed stable survival, but body length (8.5-9.0mm) were not significantly different among aeration rates. It is noteworthy that survival rate became stable at the environment with stronger water flow as ever reported (Sakakura et al., 2007), since devil stinger larvae with long pectoral fins had been believed as fragile (Fig. 1).

For the Experiment 2, growth and development was synchronized in the salinity gradient tanks with little standard deviation, although average survival at settlement in the salinity gradient tank (47.3%) had lower trend than the aeration tank (68.2%).

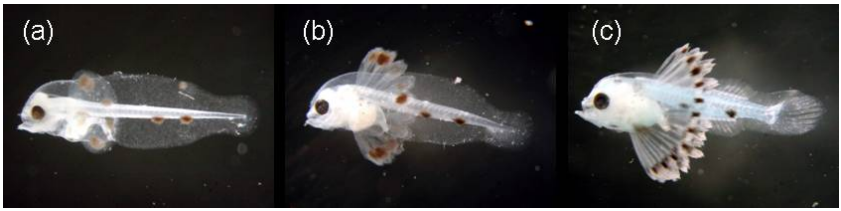

Fig. 1. Development of devil stinger *Inimicus japonicus* larvae: (a) day 1, 3.2mm SL; (b) day 6, 4.6mm SL; (c) day 7, 5.6mm SL. Note the well developed pectoral fin.

In the Experiment 3, survival rates of devil stinger juveniles reared at more than 6 Lux (37.2-65.6%) were higher than those from under 2lux (0-15.3%). Negative correlation between survival rate and body length was found. Feeding of larvae was observed even in low light intensity (0.2lux) and positive correlation was found between light intensity and number of feed in larval gut.

Conclusions

From these results, the optimal environmental conditions for larval rearing of devil stinger can be at the light intensity of over 6lux and the aeration rate of over 300ml.min^{-1}. Introducing salinity gradient can be a novel approach for enhancing larval performance.

Acknowledgements

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EXPRESSION OF DIGESTIVE ENZYME PRECURSORS UNDER DIFFERENT FEEDING CONDITIONS IN *SPARUS AURATA* LARVAE

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Introduction

The onset of feeding is a crucial step during the development of marine fish larvae. As with most marine species, the digestive tract is not totally mature at mouth opening, but it develops over the whole larval period (Sánchez-Amaya et al., 2007). The digestive capacity during early development of the digestive system is provided by the pancreatic enzymes (proteases, lipases and glucosidases) in conjunction with alkaline proteolitic enzymes secreted by the intestine, which are important prior to stomach development. Most studies examining the ontogeny of fish digestive system has been performed in hatchery-reared species, in order to achieve an understanding of their nutritional needs. These studies have been focused in designing formulated microdiets for replacing live prey during the first days of feeding (Kolkovski, 2001).

Adequate nutrition, one of the main factors affecting larval survival, depends on the effective ingestion, digestion and assimilation of diets containing the required essential nutrients. Digestion is a key process in animal metabolism, since it determines the availability of nutrients needed for all biological functions. The study of digestive enzymes expression is a useful tool when studying the nutritional condition and adaptation of the organism to dietary change.

In the present study, full length cDNAs from α-amylase, trysinogen and bile activated lipase (BAL) have been cloned and sequenced in gilthead seabream, and their expression analyzed by *in situ* hybridization and Q-RT-PCR during the first weeks of larval development and under different feeding conditions (starvation, live prey and inert microdiets). The aim of this work was to study the expression onset and ontogeny of these enzymes in *Sparus aurata* in order to better understand the sequence of events occurring during the alkaline digestion in larval development and to gain insights into the digestive physiology from hatching stage onwards.

Materials and methods

Larvae were reared in 300-l tanks, 19.5±0.5°C temperature, and 12L:12D photoperiod. All experiments were performed at least in triplicate. The following four experimental time-course designs with specific feeding conditions were tested: (a) starvation from 4 to 11 days after hatching (DAH); (b) live prey (4-20DAH with rotifers, 21-25 with rotifers plus *Artemia* and 26-54DAH with *Artemia*); (c) microdiets from first feeding (4DAH) to 12DAH; and (d) rotifers from first feeding to 8DAH and microdiets from 9 to 15. Larvae fed on rotifers were considered as control treatment.

Four types of microencapsulated diets with different macronutrients composition (proteins-P-, carbohydrates-C-, and fat-F-) were prepared by internal gelation with alginate (Yúfera et al., 2005). These inert diets were formulated according to theoretical balanced macronutrient composition (PCF), and with low content in fats (PC), carbohydrates (PF), and proteins (CF).

Larvae for in situ hybridization and Q-RT-PCR were sampled periodically in starvation conditions at 0, 3, 4, 5, 7, 8, 9, 11 DAH (Fig. 1a); in larvae fed on live prey at 4, 5, 10, 15, 25, and 54DAH (Fig. 1b); and in experiments with inert diets, from 4 to 12DAH, at 4DAH (before start feeding) and at 12DAH for all treatments -CF, PC, PF, PCF and rotifers- (Fig. 1c) and in larvae fed on microdiets from 9 to 15, at 9DAH (before adding the microdiets) and at 15DAH for all treatments -CF, PC, PF, PCF and rotifers- (Fig. 1d).

Full length cDNA from α-amylase, trysinogen, and BAL have been cloned after screening a digestive tissue library in gilthead sea bream *Sparus aurata* larvae and they were totally sequenced. The amount of targets was normalized to an endogenous reference and relative to a calibrator. Its relative expression was analyzed for Q-RT-PCR using the housekeeping gene β-actin. The ΔΔCT method was employed for relative expression quantification.

BAL, α-amylase, and trysinogen mRNA was localized using in situ hybridization on 6-µm sections of paraffin embedded samples. The same riboprobe batch and reaction time of alkaline phosphatase-NBT/BCIP was used for all samples to avoid any difference due to methodological variations.

Results and discussion

Trysinogen, α-amylase, and BAL transcripts were localized specifically by in situ hybridization in the exocrine pancreas and the signal started to detect with very low intensity from hatching, when it started to increase. Sections incubated with the sense probe did not show any hybridization signal.

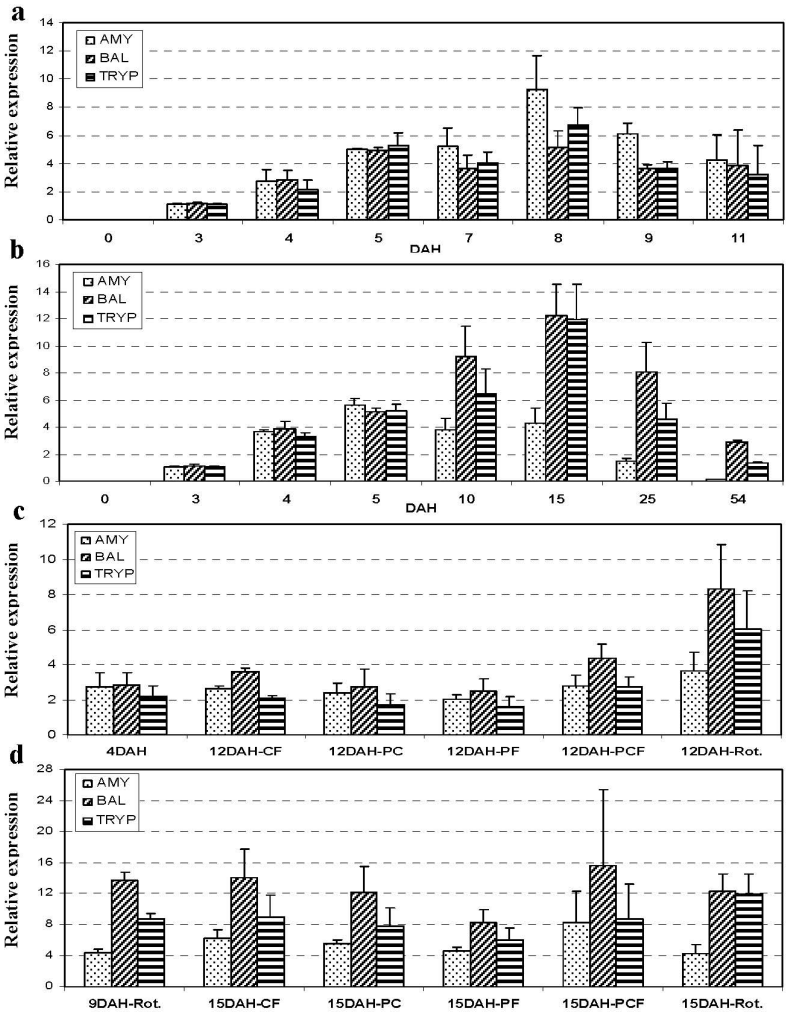

Fig.1. Relative expression and SEM (error bars) for α-amylase (AMY), trysinogen (TRYP) and bile activated lipase (BAL) in *Sparus aurata* larvae under different feeing conditions: a) Starved larvae; b) larvae fed on live prey; c) larvae fed on microdiets from 4 to 12DAH; d) larvae fed on microdiets from 9 to 15DAH.

Main results with Q-RT-PCR showed that in starved larvae the expression of these enzymes increased from 0 to 8DAH, and decreased until 11DAH, just before 100% mortality (Fig.1a). This is in agreement with moment of irreversible starvation (7-8DAH) determined in this species (Yúfera et al., 1993). In larvae fed on live prey expression increased till 15DAH, and started to decrease afterwards. Expression at 54DAH was almost negligible for α-amylase (Fig.1b). In

larvae fed on microdiets from 4 to 12DAH, gene expression profiles at 12DAH were very similar in all treatments and to 4DAH larvae just before first meal, but not to 12DAH larvae fed on rotifers (control), which exhibited higher expression (Fig. 1c). In the experiment with microdiets from 9 to 15DAH, gene expression profiles and levels at 9 and 15DAH were very similar in all treatments (inert diets or live prey) without significant differences between them (Fig. 1d).

In conclusion, α-amylase, trysinogen and BAL, involved in the digestion of carbohydrates, lipids and proteins, were present in *S. aurata* larvae before the onset of exogenous feeding. Moreover, patterns of expression were different between fed and fasted larvae, as well as between both microdiets time-courses, indicating the importance of macronutrients composition and quality and set-point in the beginning of the digestive system development.

Acknowledgements

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THE EFFECT OF DIETARY PHOPHATIDYLCHOLINE/ PHOSPHATIDYLINOSITOL RATIO ON MALFORMATION IN LARVAE AND JUVENILE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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Introduction

Malformation in commercially raised fish, such as cranial, skeletal and gill cover deformities, is a major factor reducing their market value (Koumoundourous et al., 1997). Although these deformities are most apparent in the juvenile and adult stages they may originate from suboptimal nutrition during the critical larval rearing stage. Previous research hypothesized that dietary phosphatidylinositol (PI) was more effective in reducing deformities than the main membrane phospholipid phosphatidylcholine (PC) (Geurden et al., 1997; 1998). Consequently, the aim of this study was to test the effect of different dietary ratios of PC and PI fed to the gilthead sea bream (*Sparus aurata*) larvae, on developmental performances in juvenile fish in terms of survival, growth, and malformation rate.

Materials and methods

Four microdiet (MD) treatments that differed in their PC/PI ratio and replaced 75% of the normal *Artemia* ration (wt/wt), were fed to 20-34dph (days post hatching) sea bream larvae. In addition to the high PC/PI or low PI containing MD control, a commercial reference treatment (100% *Artemia* ration) was given. The dietary PC/PI ratio in the experiment treatments is outline in Table I.

At 40dph, the fish were graded in all treatments into small (<1.3mg.dw^{-1} larva) and large (>2.9mg.dw^{-1} larva) larvae, in order to test if growth rate influenced treatment effect throughout development to 141dph.

The experiments were carried out in four replicates and results analyzed by a one-way ANOVA followed by the Tukey-Kramer Honestly Significant Difference (HSD) multiple range test ($P<0.05$).

Table I. The dietary PC/PI ratio in the experiment treatments

	A	B	C	D	Art
(g.100g^{-1} dry diet)	25% *Artemia* +75% MD	25% *Artemia* +75% MD	25% *Artemia* +75% MD	25% *Artemia* +75% MD	100% *Artemia*
Phospholipids					
Phosphatidylcholine	5.7	4.54	4.42	3.88	4.42
Phosphatidylinositol	1.86	1.95	2.76	3.04	1.78
PC/PI in total diet	3.07	2.32	1.6	1.28	2.48

Results

There was no marked (P>0.05) treatment effect on growth rate in 40dph fish although larvae fed the MDs were significantly (P<0.05) smaller then the commercial reference treatment (Art) larvae. On the other hand, in later juvenile development (67dph), decreasing dietary PC/PI ratio contributed to a significantly (P<0.05) better growth and non-significant (P>0.05) higher survival (Fig. 1).

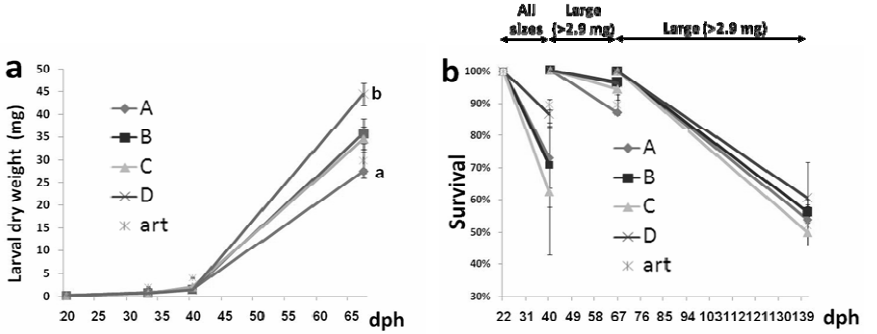

Fig. 1. The effect of dietary PC/PI ratio on large (>2.9mg.dw^{-1} larva) larvae (a) dry weight (20-67dph) and (b) survival (22-141dph).

Moreover, reducing dietary PI markedly (P<0.05) increased jaw (cranial) deformity in both size groups at 67dph which may have adversely affected their feeding once weaned completely on to a dry hard starter feed. This is suggested as fish fed the high PC/PI ratio (low PI) diet demonstrated poorer growth at 67dph. Conversely, increasing dietary PI (reducing PC/PI ratio) showed a non-significant trend of increased skeletal deformity which was markedly (P<0.05) higher in faster growing larvae in all MD treatments (Fig. 2). Interestingly, both Cranial and skeletal malformations increased throughout development (67-141dph) in most of the treatments suggesting that they were not deleterious. A possible explanation for the jaw-skeletal contradiction is by the Osteocalcin (BGP) mRNA levels. High level of PI contributed to higher BGP levels which

reduced significantly ($P<0.05$) the jaw deformity levels while simultaneously elevated the skeletal deformities due to over mineralization. Although there was no clear affect of PC/PI ratio on gill cover deformity rate, there was a size dependent susceptibility to this deformity where smaller larvae showed the highest incidence of this malformation. Moreover, throughout development (67-141dph), gill cover deformity generally decreased suggesting the possibility of operculum regeneration or that the exposure of the gills was deleterious with age. This work has demonstrated an effective dietary PC/PI ratio of 1.28 for the sea bream larvae, effecting positively on jaw (cranial) deformity rate, growth, and survival in juvenile fish.

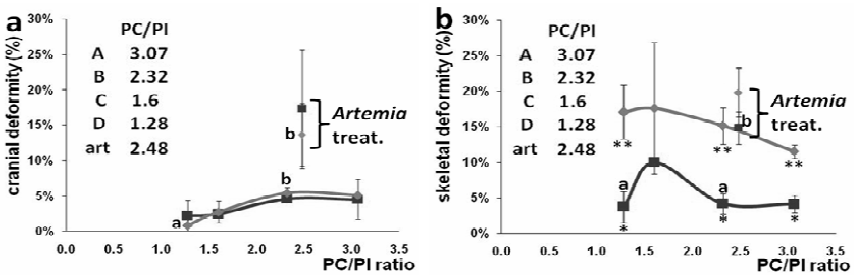

Fig. 2. The effect of dietary PC/PI ratio on (a) cranial and (b) skeletal deformities (%) in small (<1.3mg.dw^{-1} larva; squares) and large (>2.9mg.dw^{-1} larva; diamonds) juveniles at 67dph.

Conclusion

Decreasing the dietary PC/PI ratio (5% total phospholipid) during larval rearing significantly increased juvenile growth. This may have resulted from a reduced incidence of jaw deformity which leads to more effective pellet feeding. Moreover, the decreased rate of cranial deformity may be due to PI enhancing osteocalcin synthesis and normal jaw development. The results conclude that a PC/PI ratio of 1.28 or a PI level of 3.04g.100g^{-1} DW diet gave the best larval and fry performance.

Acknowledgments

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IS *VIBRIO SPLENDIDUS* PATHOGENIC TO ATLANTIC COD LARVAE?

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Introduction

Vibrio splendidus is known to cause disease to a variety of marine species including bivalves (Sandlund et al., 2006; Sugumar et al., 1998) and fish (Thomson et al., 2005; Sedano et al., 1996), especially during the larval stages. The background for studying virulence of *V. splendidus* to Atlantic cod (*Gadus morhua*) larvae was the observation of a massive infection with *V. splendidus*-like strains associated with enteritis and mass mortality among tank reared cod larvae (Sandlund, 2008). Recently, Reid et al. (2009) were able to demonstrate mortality of cod larvae fed rotifers, following challenge with *V. splendidus*. It was therefore of interest to study the ability of different *V. splendidus* strains to cause disease and mortality to cod yolk sac larvae by means of a challenge experiment and immunohistochemical examinations.

Materials and methods

Atlantic cod eggs were provided by the commercial hatchery Sagafjord SeaFarm AS, Stord, Norway. Eggs were randomly sampled and individually transferred to a 24-well multidish system where each well contained 2 ml of aerated sterile seawater. Each larval challenge group consisted of three identical multidishes, a total 72 larvae. The virulence of each bacterial strain was tested using two separate challenge groups exposed to either a high or a low challenge dose, approximately 10^6 and 10^4CFU.ml^{-1}, respectively. The experiment included an unchallenged control group.

The eggs were exposed to bacteria at the day of arrival (10 days post fertilisation) and they hatched within 96 hours. Mortality reading was done daily.

The seven *V. splendidus* isolates tested in this experiment included five isolates isolated from diseased cod larvae; HI 22094, HI 22095, HI 22099, HI 22107, HI 22019 (Sandlund, 2008), one isolate pathogenic to scallop *Pecten maximus* larvae, LT 06 (Torkilden et al., 2005; Sandlund et al., 2006) and one isolate pathogenic to turbot *Scophthalmus maximus* larvae, DMC-1 (Thomson et al., 2005). All bacterial strains were grown as described in Sandlund and Bergh (2008).

In addition to the challenge groups used for mortality readings, extra challenge groups were included and exposed to both high and low bacterial doses of the six strains LT 06, HI 22094, HI 22095, HI 22099, HI 22107, HI to provide larvae for immunohistochemical sampling. This sampling started two days post hatch (dph) and three to four larvae were samples each day. The larvae were fixed in 4% phosphate-buffered formaldehyde, dehydrated in ethanol, cleared in xylene, and infiltrated in paraffin and embedded in histowax. Larval samples were sectioned at 3 and 2µm. Immunostaining of larval sections was performed as described in Sandlund et al. (2006) using the same polyclonal antiserum, anti-LT 06.

Statistical analysis of the mortality readings were performed using a 2×2 contingency table ($p < 0.0055$ Bonferroni correction).

Results and discussion

The statistical analysis of the mortality readings showed no significant difference in mortality when comparing the negative control group and the larval groups challenged with the various *V. splendidus* strains. The results were similar in both the high and low challenge dose groups.

However, the preliminary immunohistochemical examinations show presence of bacteria in the gastrointestinal tract and signs of pathology. Sloughing of epithelia and necrotic mucosal cells was observed. In conclusion, despite the absence of significant changes in mortality, the immunohistochemical observations indicate that *V. splendidus* could cause disease problems in cod hatcheries.

Acknowledgements

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EFFECTS OF HYPOXIA AND HYPERCAPNIA ON THE EMBRYONIC DEVELOPMENT OF RED SEA BREAM, *PAGRUS MAJOR*

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Introduction

Hypoxic and hypercapnic conditions sometimes occur in the high density layer of fish eggs during egg collection and transportation in aquaculture, and in the red tide in the open sea. This study investigated the effects of hypoxia and hypercapnia on the red sea bream *Pagrus major* embryos during somitogenesis.

Materials and methods

Experiment I: Effects of hypoxia and hypercapnia on somite segmentaion during embryonic development
Hypoxic and hypercapnic exposure: 10-somite stage eggs were exposed to hypoxia and hypercapnia. After the exposure, eggs were incubated in the water of 100% DO and 0mg.l^{-1} DCO_2.

Analysis: Eggs were investigated the hatching rate, and 10-15 newly hatched larvae were investigated the incidence of somitic disturbance.

Experiment II: Centrum defect induction in juveniles by exposure to hypercapnia
Hypoxic and hypercapnic exposure: The experimental groups were set as follows: Control: 100% DO, 0mg.l^{-1} DCO_2; Hypoxia: 10% DO, 0mg.l^{-1} DCO_2; Hypercapnia: 100% DO, 120mg.l^{-1} DCO_2; Hypoxia and Hypercapnia: 10% DO, 120mg.l^{-1} DCO_2. All the exposures were done for 90min. Resulting larvae were raised to juveniles.

Analysis: The juveniles of 45DAF were examined the incidence of centrum defects by radiography.

Experiment III: Effects of acidity and hypercapnia on somite segmentaion during embryonic development
10-somite stage eggs were incubated in seawater with pH 4, 5, and 6 for 30 to 180 minutes.

Analysis: Newly hatched larvae were examined the somatic disturbances.

Results

Experiment I: Total and normal hatching rates of treated eggs were 90.1 and 6.0% and above for each experiment. Somitic disturbances did not occur in the control group (100% DO and 0mg.l^{-1} DCO_2). They occurred in the hypoxia group (0% DO, 0mg.l^{-1} DCO_2) and hypercapnic group (100% DO, 60 and 120mg.l^{-1} DCO_2). Abnormal somitic segmentation; disappearance and/or bifurcation of somite boundary, and unequally-spaced somites, were observed in larvae of these groups. In addition, concurrent exposure to hypoxia and hypercapnia had the synergetic effect in somitic disturbance induction.

Experiment II: Centrum defects were induced in the groups of hypoxia, hypercapnia, and their simultaneous conditions at high incidence rates.

Experiment III: The incidence of somitic disturbances was below 0.7% for embryos exposed to any acidic condition.

Discussion

This study elucidated that the hypoxic and hypercapnic conditions during fish embryogenesis have teratogenic effects. In addition, the teratogenetic effect of hypercapnia is caused not by the acidity of hypercapnic seawater but by the toxicity of carbon dioxide itself.

In concrete terms, the somitic disturbances in newly hatched larvae were induced by the extreme hypoxia of the exposure to 0% dissolved oxygen concentration for 30 and 60 minutes. Extreme hypercapnia of 120mg.l^{-1} dissolved carbon dioxide concentration for 120minutes also induced them. Embryos exposed to both these hypoxic and hypercapnic conditions during somitogenesis developed to juveniles with the centrum defects.

This study demonstrated the teratogenetic effect of hypercapnia is not caused by the exposure increase of hydrogen-ion concentration (low pH) but by the physiological toxicity of carbon dioxide itself.

Conclusions

Hypercapnia, along with hypoxia, during somitogenesis was elucidated to induce somitic disturbances in fish larvae and centrum defects in juveniles. This suggests that hypercapnia and hypoxia in aquaculture production (caused by inappropriate handling of fertilized fish eggs) and in the aquatic environment (caused by human-induced excess organic matter loading, red tides, and the planned carbon dioxide disposal into the sea) are the possible causes of the reduction of production efficiency in aquaculture and causes of the reduction of wild fish population by the induction of developmental defects in their early life history.

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THE IMPACT OF DIETARY SUPPLEMENTATION WITH ARACHIDONIC ACID ON EGG QUALITY IN ATLANTIC COD BROODSTOCK (*GADUS MORHUA*, L.)

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Introduction

Cod hatcheries rely to a large extent on eggs produced by wild-caught broodstock since egg quality from farm-reared broodstock tends to be poor. Differences have been identified in concentrations of arachidonic acid (20:4n-6; ARA), an essential fatty acid of the n-6 series, between eggs from wild and farmed cod (Salze et al 2005). Pickova et al. (1997) showed that ARA concentration was correlated with hatching success and other egg quality parameters in different stocks of wild cod. Supplementation of broodstock diets with ARA has been shown to improve egg quality in halibut, sea bass and Japanese flounder (Bruce et al. 1999; Furuita et al. 2003; Mazorra et al. 2003; Alorend 2004). This project investigated the impact of dietary supplementation with ARA on egg quality in cod. The experiment investigated the effect of feeding a diet supplemented with ARA, for 1, 2, or 3 months prior to peak spawning, on egg quality in wild cod in order to determine the optimum period of supplementation for best reproductive performance.

Materials and methods

The experiment used four treatment groups which contained 16 males and 8 or 9 females. Group A was fed an unsupplemented control diet throughout the spawning period and Groups B, C, and D were fed a diet supplemented with 3% ARA for 1, 2, or 3 months prior to peak-spawning. Fish were fed to satiation twice daily with feed (Vitalis® Marine Broodstock Mix), supplied by Skretting UK). Each day during spawning, egg batches were collected and quality assessed to measure total and floating egg production and fertilisation rate. The fatty acid composition and total lipid content of feed and egg samples was measured using standard laboratory procedures. Analysis of variance or Kruskal-Wallis tests, were used to identify differences in egg quality or biochemical parameters. Where differences were identified, multiple comparison tests were used to identify differences between the group means. Spearman's rank test was used to detect any correlation between fatty acid composition and egg quality.

Results and discussion

Table I. Egg production and egg quality indicators.

Group	A	B	C	D	B-D
Treatment	No ARA Control	+ARA 1 month	+ARA 2 months	+ARA 3 months	+ ARA pooled
Total no of eggs produced.kg^{-1} female	590 185	738 132	356 707	572 813	555 884
No of batches produced	61	62	50	55	
Mean no per batch of eggs produce.kg^{-1} female	9675	11 905**	7134**	10 415	9818
Total weight of eggs collected (g)	24 660	34 220	20 729	26 537	27 162
Total no of collected eggs.kg^{-1} female	376 339	556 097	320 337	442 727	439 720
Mean no per batch of eggs collected.kg^{-1} female	6169*	8969*	6407	80 450	7809
Total wt of floating eggs (g)	13 118	17 352	12 161	13 877	14 463
Mean no per batch of floating eggs.kg^{-1} female	3282*	4623	3835	4283	4247*
Mean fertilisation rate % floating eggs	55%*	56%	70%*	60%	62%
Mean no per batch of fertilised eggs/kg female	2122*	3013	2934	3045	2997*
Mean hatch rate (% floating eggs)	24	18	20	16	18
ARA (%)	1.8 ± 0.4*	2.1 ± 0.7	3.0 ± 1.2 *	2.2 ± 0.5 *	2.4 ± 0.5
EPA (%)	14.5 ± 2.9	12.1 ± 1.7	12.1 ± 2.5	13.2 ± 1.7	12.5 ± 0.7
DHA (%)	31.5 ± 5.9*	26.8 ± 3.8	25.3 ± 5.0*	29.1 ± 4.3	27.1 ± 1.9
DHA/EPA ratio	2.2 ± 0.2	2.2 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	2.2 ± 0.13
EPA/ARA ratio	8.4 ±1.2***	6.3 ± 1.7 ***	4.4 ±1.1***	6.1 ± 0.8	5.6±1.5***

Egg numbers are expressed as numbers per kg female. Differences in mean weights or numbers per batch are shown as * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

Supplementation of the diet with ARA resulted in an increase in the ARA content of the eggs. Groups fed the ARA supplement produced higher numbers per batch of floating eggs per kg female and fertilised eggs per kg female than the control group. Groups fed the ARA supplement produced higher numbers per batch of floating eggs per kg female and fertilised eggs per kg female than the control group. The mean ARA concentrations were lowest in the control group and highest in eggs from Group C fed the supplemented diet for 2 months. This suggests that two months prior to peak spawning is an adequate feeding period to enable manipulation of ARA concentrations in eggs. There was no consistent evidence that ARA supplementation increased the total number of eggs spawned, but larger numbers of eggs were collected in batches produced by fish fed the supplement. Pooled data from Groups B-D indicate that, in fish fed the ARA supplement, the mean number per batch of floating eggs/kg female was 29% higher, and the mean number per batch of fertilised eggs was 41% higher

than in the control group (Table I). There was no correlation between egg quality and duration of supplementation or between mean ARA concentrations, or EPA/ARA ratios and egg quality in individual batches.

Conclusion

Short term supplementation of the diets of cod broodstock with ARA for a period of two months prior to peak spawning increased its concentration in their eggs. Higher numbers per batch of floating eggs/kg female and fertilised eggs/kg female were measured in groups of fish fed the ARA supplement. No correlation between egg production or egg quality and the duration of ARA supplementation was identified.

Acknowledgments

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HIGH DIETARY PROTEIN:LIPID RATIO IMPROVES GROWTH OF *OCTOPUS VULGARIS* PARALARVAE

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Introduction

The rearing of *Octopus vulgaris* paralarvae during its planktonic stage is a major challenge in the culture of this high-value species, as mass mortalities are often observed, possibly due to nutritional imbalances of the diet. The improvement of the biochemical composition of *Artemia* has been pointed out as one of the key issues to overcome this bottleneck (Iglesias et al., 2007). In this study *Artemia* juveniles were enriched with different diets in order to feed octopus paralarvae.

Material and methods

One-day post hatch (dph) *O. vulgaris* paralarvae were distributed into nine conical fibre glass tanks containing 50 l of seawater (34ppt) at an initial density of 10 individuals.l^{-1}. Rearing temperature was 19.5±0.5ºC. Three groups of paralarvae were set, each receiving a different combination of *Artemia* juveniles (1.6-2.8 mm), which were distributed twice a day at equal proportions with a total ration of 0.05 *Artemia*.ml^{-1}. The first meal was common to all groups and consisted of *Artemia* juveniles enriched with a mixture of *Rhodomonas lens* and *Isochrysis galbana* (70%:30% dry weight basis), cultured semi-continuously in nutrient saturated conditions and with a daily renewal rate of 30%. The proportion of *R. lens* and *I. galbana* was chosen on the basis of previous works related with the enrichment of *Artemia* with different microalgal species (Seixas et al., 2008). The second meal differed according to each treatment: group AR+I was offered *Artemia* juveniles enriched with *R. lens* and *I. galbana*; group AGOLD was fed juveniles enriched for 6h with Ori-Gold® (Skretting); and group AGOPEL received juveniles enriched for 6h with a manually prepared diet consisting of grinded pellets for turbot (Sorgal, Portugal) supplemented with 10% of Ori-

Gold® (wet weight basis) to increase docosahexaenoic acid (DHA, 22:6n-3) content. The biochemical composition of *Artemia* is shown in Table I. The dry weight (DW) of paralarvae was determined individually (n=10 per replicate).

Table I. Gross biochemical composition (% of dry weight) and major polyunsaturated fatty acids (PUFA, % of total FA) of *Artemia* juveniles (1.6-2.8mm) used to feed *Octopus vulgaris* paralarvae.

	AR+I	AGOLD	AGOPEL
Protein (Nx6.25)	63.8 ± 0.9^{b}	63.4 ± 0.4^{b}	65.5 ± 0.3^{a}
Lipid	11.8 ± 0.8^{b}	16.2 ± 1.2^{a}	14.6 ± 1.2^{a}
Carbohydrate	9.9 ± 0.5^{a}	6.8 ± 0.2^{b}	8.9 ± 0.8^{a}
Protein:Lipid ratio	5.4 ± 0.2^{a}	3.9 ± 0.3^{b}	$4.5 \pm 0.2^{a,b}$
PUFA (% of total FA)			
20:4n-6	0.4 ± 0.1^{a}	1.8 ± 0.8^{b}	1.4 ± 0.0^{b}
20:5n-3	13.8 ± 0.6^{a}	11.2 ± 0.3^{b}	15.4 ± 0.2^{c}
22:6n-3	1.6 ± 0.2^{a}	8.0 ± 0.7^{b}	5.7 ± 0.1^{c}

Data are means±S.D. (n=3). Different superscript letters within the same line indicate significant differences among groups ($P<0.05$).

Protein content was derived from the total amount of nitrogen (Nx6.25), determined by combustion using an autoanalyzer Fisons Model EA 1108. Total lipid was extracted with chloroform/methanol according to Bligh and Dyer (1959), and calculated gravimetrically. Fatty acid composition was determined through GC-MS (Fisons Instruments, MD-800) as described elsewhere (Seixas et al., 2008). Data were compared by analysis of variance (ANOVA) followed by Tukey-Kramer HSD tests for post-hoc multiple comparisons, at a significance level of 0.05, after log-transformation of DW and arcsine-√ transformation of biochemical composition and survival percentages.

Results and discussion

The highest survival of 15-dph paralarvae was observed in group AR+I (52±9%), followed by group AGOPEL (40±21%) and finally group AGOLD (35±11%). Despite this same tendency for 25-dph paralarvae from group AR+I (21±15%) to display a higher survival rate, in comparison with the remaining groups (12±5% and 7±2% in groups AGOPEL and AGOLD, respectively), no statistically significant differences were found. Regarding the dry weight (DW) of paralarvae (Fig. 1), individuals from AGOLD had a significantly lower DW than paralarvae from the other groups ($P<0.05$).

A positive linear correlation ($P<0.01$) was found between dietary protein:lipid (P:L) ratio and paralarval DW for both 15-dph and 25-dph paralarvae (Fig. 2), whereas no correlations could be established between DHA or eicosapentaenoic acid (EPA, 20:5n-3) levels in the diet and any of the growth parameters.

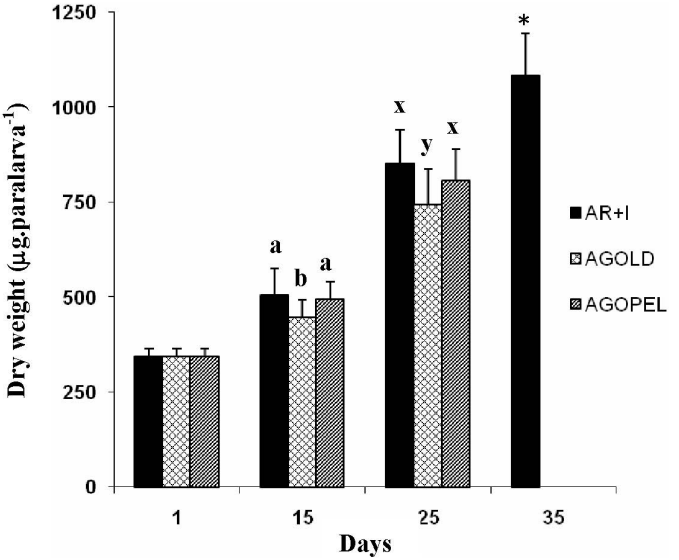

Fig. 1. Dry weight of octopus paralarvae fed on three different diets. Data are means±S.D. (n=3, 10 paralarvae per replicate). Different superscript letters indicate significant differences among groups ($P<0.05$). *The dry weight of 35-dph paralarvae from AR+I are from a single tank (n=15 paralarvae).

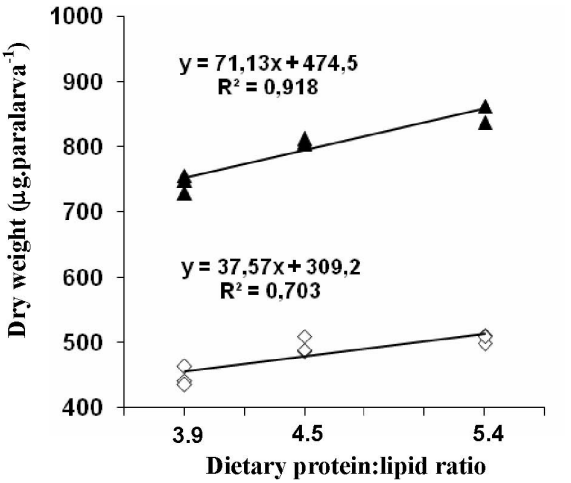

Fig. 2. Correlations between the dry weight of 15-dph (◇) and 25-dph (▲) paralarvae from the three dietary treatments and the protein:lipid ratios found in *Artemia* juveniles (AGOLD=3.9; AGOPEL=4.5; AR+I=5.4).

Regarding the biochemical composition data, octopus hatchlings contained nearly 68% protein (% of DW), which decreased slightly to 64-66% in 15-dph and 25-dph paralarvae from all groups. Despite no significant differences were found in the lipid content of 15-dph paralarvae, in 25-dph individuals from group AGOLD the lipid content was higher (11.8%) than in paralarvae from the remaining groups (10.7-10.9%, $P<0.05$). As for the fatty acid (FA) composition, EPA increased from an initial value of 13% in octopus hatchlings, to 20-22% in 25-dph paralarvae from all groups. In contrast, a remarkable drop of DHA was observed from hatchlings (20% of total FA) to 25-dph paralarvae from all groups. Moreover, individuals from groups AGOLD and AGOPEL contained significantly higher levels (circa 10%) than paralarvae from AR+I (7%, $P<0.05$).

Despite paralarvae from AR+I were fed *Artemia* juveniles with the lowest DHA content, this group showed the highest DW and tended to have higher survival rates than the other groups, being the only group attaining the 35-dph stage.

Conclusions

The high correlations found between paralarvae dry weight and *Artemia* P:L ratio demonstrated that this indices could be more important to sustain a good performance of paralarvae than the FA composition of *Artemia* per se.

Acknowledgments

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EVALUATION OF COMMERCIAL PROBIOTICS IN PRAWN LARVICULTURE (*MACROBRACHIUM ROSENBERGII* DE MAN, 1879)

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Introduction

Currently, probiotics are drawing attention in Bangladesh to promote prawn's disease resistance, maintain a healthy microbial environment, and as an alternative to antibiotics. This study attempted to investigate the efficacy of commercial probiotic Ecomax (*Bacillus subtilis*) to the rearing water of freshwater giant prawn, *Macrobrachium rosenbergii* larviculture in comparison with antibiotic Oxytetracycline (as control).

Materials and methods

The experiments were carried out in the commercial freshwater prawn hatchery facility of A.G. Aqua Hatchery, Chakaria, Cox's Bazar, Bangladesh (21°46'N and 92°04'E). Newly hatched larvae were stocked at 80 individual.l^{-1} into PVC tanks of 200 l capacity and reared up to postlarvae (PL) under two experimental groups by adding probiotic Ecomax (FishTech, Bangladesh) and Oxytetracycline in tank water. The larvae were offered identical diets with a combination of *Artemia* and egg custard, and weighed weekly to adjust the amount of daily food requirements. Larvae from day 2 to first week were given 4-5 nauplii ml^{-1} of tank water, and gradually decreased to 3-4 nauplii on second week and 2-3 nauplii in the subsequent weeks. While, custard was broadcasted into tank water four-times daily (3.00, 6.00, 9.00, and 12.00h) at the rate of 0.5, 1.5, 2.5, 3.0 and 4.0g.$tank^{-1}$ in the weeks of 2, 3, 4, 5, and 6, respectively. About 40-60 and 60-80% volume of the water was exchanged every evening during first and last 3 weeks. The water was well aerated throughout the experiment.

The larvae were sampled weekly until 42-days of larviculture to record larval survival, metamorphosis, mean larval stage and development stages. Water quality parameters; i.e. water temperature, salinity, pH, and dissolved oxygen (DO), ammonia (NH_3), nitrite-nitrogen (NO_2-N), total alkalinity, and hardness ($CaCO_3$)

were recorded twice daily using electronic apparatus, standard method and test kits. Whereas, and postlarval stress tolerance (stressors: salinity and dissolve oxygen) were examined at the end of the experimental period.

All treatments were tested in duplicate and the results were statistically analyzed by one-way analysis of variance (ANOVA) and Duncan's comparison of means. Percentage data were transformed to square-root arcsine values to homogenize variance. Statistical tests were performed using SPSS software (SPSS, Release 14.0, SPSS, Chicago, IL, USA).

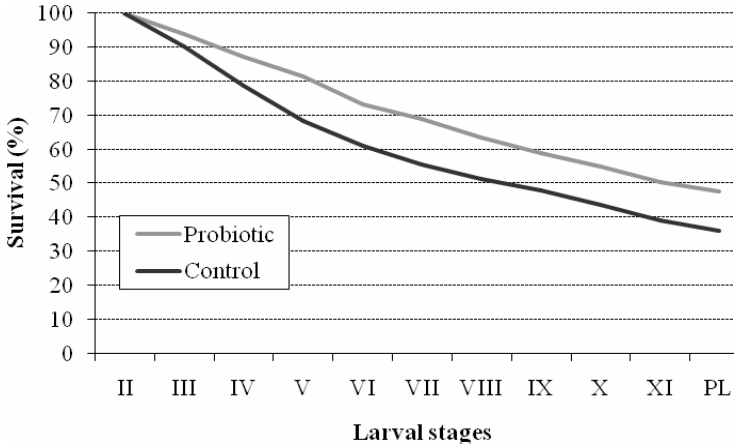

Fig. 1. Survival of *M. rogenbergii* larvae in the hatchery. The performance of Ecomax was compared with the antibiotic Oxytetracycline (control).

Results and discussion

Results revealed that larvae survival after 42-days was higher ($P>0.05$) in probiotic treatment (48%) as compared to the control (36%; Fig. 1). Moreover, the probiotic group was found to have significantly faster larval development and metamorphosed to postlarvae in 36 days ($P<0.05$), whereas it was 42 days in control (data not shown). The postlarval tolerance to stress (salinity and dissolve oxygen) was also greater with probiotic (Table I). Furthermore, NO_2-N and NH_3 values in rearing water of probiotic treatment were lower (Table I).

Mono- or mix-culture of probiotics *B. subtilis*, *B. mycoides*, *B. licheniformi*, and *B. megaterium* introduced to water was successfully colonized to the rearing water of shrimp and fish (Ringo et al., 1996; Rengpipat et al., 1998; Chen and Chen, 2001; Ziaei-Nejad et al., 2006; Lallo et al., 2007), and effectively reduced pathogen load (Lallo et al., 2007). Therefore, improved survival of prawn larvae might be attributed to control of potential pathogens through a mechanism of competitive exclusion by probiotics (Lallo et al., 2007). Alternatively, coloniza-

tion of probiotics to the gut of turbot and *M. rogenbergii* was also reported by water-diluter probiotics (Ringo et al., 1996; Ziaei-Nejad et al., 2006). Rengpipat et al. (1998) found that probiotic *Bacillus* colonized in shrimp *P. monodon* digestive tract was able to replace *Vibrio* spp. in the gut and thus an increased shrimp survival. Generally *Bacillus* spp. added to culture water can effectively reduce the NH_4-N, NO_2-N, and NO_3-N [(Chen and Chen, 2001); Taoka et al. (2006) used a mixture of *B. subtilis*, *Lactobacillus acidophilus*, *Clostridium butyricum*, and *Saccharomyces cerevisiae*; Wang et al. (2005) used probiotics composed of *Bacillus* sp. and photosynthetic bacteria as major species together with yeast, *Nitrosomonas* sp., *Nitrobacter* sp.], and improve aquatic environment for fish and shrimp. According to Gatesoupe (1999), bacteria which improve water quality may be beneficial to animal health. So, we expected that the significant reduction of metamorphosis time (to postlarvae) in this study was related to larval growth improvement caused by probiotics. Keysami et al. (2007) reported significantly faster development rate of metamorphosis in *M. rosenbergii* larvae treated with *B. subtilis* ascribed to nutritional effect, a source of micro- and macroelements as feed. By now a number of studies have demonstrated the ability of probiotics to improve shrimp and prawn growth rates (Rengpipat et al., 1998, Ziaei-Nejad et al., 2006). In this study, probiotics-treated larvae showed an improved tolerance to stress artificially induced by salinity and dissolve oxygen than the control group. Taoka et al. (2006) reported greater stress tolerance of fish treated with probiotics exposed to heat shock and air stress. Moreover, the lower cortisol response in probiotics treated fish was observed during a pH-induced stress (Rollo et al., 2006). These facts suggest that probiotics might have lessened the stress stimulus or affected the physiology of stress response in prawn larvae, thereby an enhanced stress tolerance.

Table I. Water quality parameters in larval rearing tanks, and the 50 and 100% lethal time for *M. rogenbergii* postlarvae exposed to salinity and dissolved oxygen shock test. Row with different superscript is significantly different at $P<0.05$.

Parameters/stressors	Probiotic	Control	Remarks
NO_2-N ($mg.l^{-1}$)	0.06 ± 0.03^a	0.10 ± 0.01^b	Optimum range < 2.0
NH_3 ($mg.l^{-1}$)	0.04 ± 0.0^a	0.11 ± 0.02^b	Optimum range < 0.3
Salinity stress			
a) 30ppt: [1]LT50 (h)	24.0 ± 0.5^a	20.0 ± 0.5^b	-
[2]LT100 (h)	48.0 ± 0.4^a	36.0 ± 0.3^b	
b) 90ppt: [1]LT50 (h)	0.45 ± 0.05^a	0.15 ± 0.05^b	
[2]LT100 (h)	1.10 ± 0.26^a	0.30 ± 0.04^b	
Dissolve oxygen stress			Initial DO: ~6.6 $mg.l^{-1}$
[2]LT100 (h)	8.0 ± 0.5^a	6.2 ± 0.15^a	DO at LT100: ~1.9 $mg.l^{-1}$

[1]LT50: lethal time to 50% mortality; [2]LT100: lethal time to 100% mortality

Conclusions

On the whole, the results of this study reinforce the view that probiotics can enhance number of fry production and reduce the length of hatchery phase that can cut production cost of *M. rosenbergii* larviculture, and simultaneously may be an appropriate alternative to the prophylactic use of antibiotics.

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ONTOGENETIC EXPRESSION OF NANOG AND HSP70 IN ATLANTIC COD EGGS UNDER AMBIENT AND THERMALLY STRESSED CONDITIONS

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Introduction

The timing of the transition from maternal to embryonic gene control varies among diverse taxa. In mammals this transition occurs as early as the 2-cell stage (Telford et al., 1990), whereas in teleosts zygotic transcription is delayed until the 1k-cell stage, a period known as the mid-blastula transition (MBT) (Kimmel, 1995). The MBT marks the beginning of gastrulation, a profound phase that organises the embryonic axis and subsequently determines the cell fates of the germ layers. Recent studies have demonstrated that incubation temperature can have long-term effects on the somatic cell lineages of teleosts so that future growth potential is partially determined in the embryo (Johnston, 2006). Much less is known concerning the influence of temperature on the germline, a pluripotent cell lineage that will determine the future reproductive capacity of the adult. Nanog is a homeobox transcription factor, a germ cell marker that works together with other key regulators such as Oct4 and Sox2 to maintain pluripotency of cells during early development (Pan and Thomson, 2007). Heat shock protein (Hsp70) is a molecular chaperone that facilitates protein folding, transportation and assembly during normal cellular functions and during stress situations. Here we have studied the expression of *nanog* and *hsp70* during embryonic stages of Atlantic cod (*Gadus morhua*) to elucidate the expression pattern during normal and thermally stressed conditions.

Materials and methods

Three independent egg batches were obtained from three wild caught Atlantic cod (*Gadus morhua*) brood stocks, spawning naturally within the tank. Floating eggs were collected at the tank outlet and transferred to incubation tanks with constant air and water flow in a cold room (6.2 ± 0.5°C and 10 ± 0.5°C). Triplicate samples of approximately 50 eggs were collected from each egg batch and rearing temperature during the first five days of development and further at 8,

13, and 16 days post fertilisation (DPF). Experiments were terminated on day 16 PF when the larvae hatched. The stages of development analyzed were set according to Hall et al. (2004) (Fig. 1). At sampling the eggs were immediately homogenized in Trizol (Invitrogen) and RNA was extracted. The quantity and quality of RNA were approved. The RT-qPCR protocols were run using TaqMan reverse transcription reagents containing Multiscribe reverse transcriptase (50U.μl^{-1}) (Applied Biosystems). Pooled samples from all the RNA samples were run in six serial dilutions (100-3.1ng.μl^{-1}) for efficiency calculations. The standards were run in triplicate, whereas the samples (diluted to 50ng.μl^{-1}) were run in duplicate. The program for reverse transcriptase reactions was: 25°C for 10 min, 48°C for 60min, and 95°C for 5min. To quantify the gene expression, the cDNA (2µl) was mixed with 2.8µl ddH_2O, 0.1µl of forward and reverse primers, and 5µl TaqMan® Universal PCR Master Mix. *hsp70* and *nanog* were analysed as representatives of zygoticly and maternally activated genes, respectively, Elongation factor 1α (*ef1*α), heat shcok protein 90β (*hsp90*β) and Tubulin 2 (*tub2*) were run as reference genes. The mRNA levels were analysed using real-time qPCR on Roche Applied Sciences LightCycler® 480. All the reactions were set up using 5min at 95°C, followed by 45 cycles of 15s denaturing step at 95°C, 60°C annealing temperature, and 30s synthesis step at 72°C. The reaction efficiencies were calculated from the standard curve, and the software package of GeNorm was applied to calculate the normalisation factor used to quantify the mean normalised expression (MNE) during embryonic development. The results were analysed in Statistica by repeated measures ANOVA and significant differences determined by a Tukey multiple comparison test.

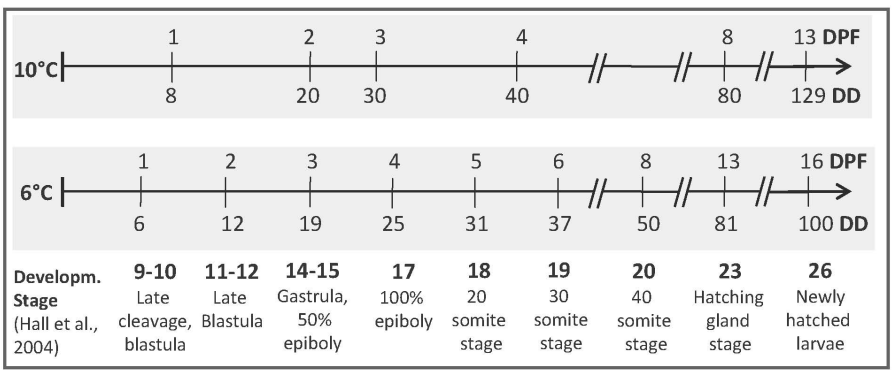

Fig. 1. Embryonic development of Atlantic cod (*Gadhus morhua*) staged after Hall et al. (2004), given in days post fertilisation (DPF) and day degrees (DD) at 6°C and 10°C water temperature. Samples from the indicated time points were collected for stage determination and RNA extraction with subsequent real-time qPCR analysis to elucidate the embryo's capacity for transcription of *hsp70* and *nanog*.

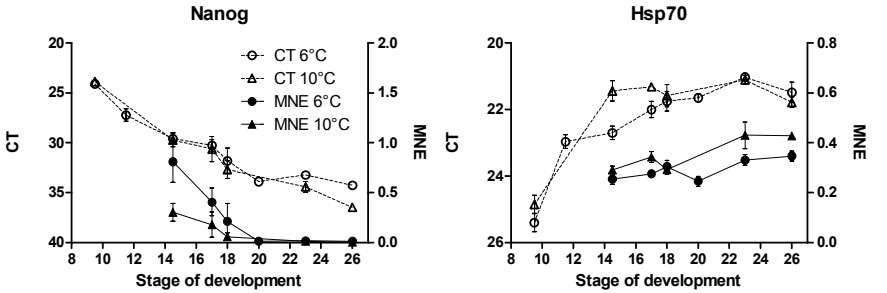

Fig. 2. Raw cycle threshold (CT) values (left y-axis) and mean normalised expression (MNE) values (right y-axis) determined by real-time qPCR of *nanog* and *hsp70*. Gene expression profiles at 6°C and 10°C are given during stages of embryonic development shown in Fig. 1. Ct values are quantified with input RNA of 50 (±1) ng/µL. MNE are normalised against *tub2*, *hsp90*β and *ef1*α as reference genes. Data points represent mean ± SD of N = 3 independent groups of approximately 50 eggs.

Results and discussion

During embryonic development of Atlantic cod, the gene expression pattern of *nanog* decreases while the expression of *hsp70* increased. For *nanog* there was a decrease in expression during the first 20 stages of development (until the 40 somite stage) for both the samples incubated at 6°C and at 10°C. The most intense decrease in gene expression of *nanog* was seen from developmental stage 9 to 14-15 (late cleavage to early gastrula). When normalising the gene quantities of *nanog* from stage 14-15 (early gastrula) the results indicate a downregulation, however the embryos incubated in 10°C appear to have a lower expression of *nanog* than the embryos incubated at 6°C. RNA from the homeobox transcription factor, *nanog*, is believed to be maternally deposited into the eggs during the final maturation and its response to temperature might be due to an unknown mismatch between embryos developmental stage rather than an effect of temperature. In summary, *nanog* was highly expressed at stage 9-10 when most of the cells are still pluripotent, and was downregulated when the cells were differentiating during early gastrulation (stage 14-15).

The CT values for *hsp70* indicate a rapid and significant increase in expression from stage 9 to 17 (100% epiboly). After stage 17 the expression stabilised. However, when analysing the normalised expression of *hsp70*, continuous rearing at 10°C did not affect the gene expression pattern compared to 6°C. The upregulation of *hsp70* seen in cod eggs in the present study coincides with the MBT, and is thus likely to be of zygotic origin.

The present data show that rearing Atlantic cod eggs at 10°C compared to 6°C does not influence the degradation of germ cell markers, such as *nanog* at the

MBT. Nor did it affect the zygotic transcription pattern of stress genes, such as *hsp70*. During early development when maternal genes are degraded at the same time as zygotic genes show increased expression, it can be problematic to normalize the raw CT-values, since many traditionally used reference genes are of zygotic origin. However, after the MBT gene expression of zygotic genes stabilise and makes normalisations of the RT-qPCR data using reference genes possible.

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INFLUENCE OF HATCHING TIME ON TIME OF FIRST FEEDING AND SUBSEQUENT GROWTH AND CANNIBALISM IN PIKEPERCH (*SANDER LUCIOPERCA*)

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Introduction

High potential growth rates and temperature preference (Hokanson 1977) make pikeperch (*Sander lucioperca*) a promising candidate for intensive aquaculture. One problem identified that may hamper the expansion of intensive culture of this species is cannibalism (Kestemont et al., 2007)

In salmonids, individual timing of the shift to exogenous feeding has been shown to predict social dominance, growth, and life history strategy. Larvae reaching first feeding early may possess competitive advantages (McCarthy et al., 2003; Metcalfe and Thorpe 1992; Metcalfe et al., 1995). In pikeperch, it is not known whether such a developmentally-based differentiation takes place. If it does, a hypothesis could be that a large temporal variation in time of first feeding could lead to increased cannibalism, caused by the higher size variation within the batch.

Hatching of a batch of pikeperch eggs can span several days, giving rise to speculations on the effects of mixing early and late hatched larva during subsequent larval rearing, and possible implications of this on later observed cannibalism. It is not known though whether early hatching larvae reach first feeding before later hatched individuals.

The present study evaluates effects of intra batch hatching time on size, ontogenetic development, stress sensitivity and cannibalism in pikeperch.

Materials and methods

Wild mature pikeperch breeders were obtained from Mossø, Skanderborg, Denmark. To induce ripening of eggs, LHRH hormone was injected at a concentration of 20µg.kg^{-1}.female. Ripe eggs were obtained by stripping females within a few days. Eggs were mixed with semen from several mature males. To avoid

sticking of eggs after stripping, alcalase from Novo Nordisk, Denmark was added at a concentration of 0.5.ml.l^{-1} for 2 minutes. Eggs were incubated in 6-l McDonald type up welling units until spawning. When hatching began, the larvae were divided into 4 groups in triplicate; i.e., early hatched, medium hatched, late hatched, and a mixed group with equal numbers of larvae from each group. Larval rearing took place in a 12 tank recirculation system, each larval tank holding 150 litres.

The larvae were fed AF *Artemia* the first 4 days followed by enriched EG *Artemia* thereafter. *Artemia* were enriched with 0.6g.l^{-1} DHA Selco emulsion for 24h. *Artemia* were harvested once a day in the morning, and were administered continuously for 3 periods of 6 hours by automatic dispensers holding a suspension of live *Artemia* in seawater. Total prey administration of *Artemia* to the rearing tanks was calculated to 3 *Artemia*.ml^{-1} at each feeding.

All samples of pike perch were collected in duplicate on DPH 0, 1, 2, 3, 8, 10, and 20. Larval standard lengths were measured by photographing 4 samples of 10 larvae with a digital DFC 320 Leica camera connected to a dissecting microscope (MZ6).

The larval dry weight were then estimated on two pools of 20 larvae after transfer to pre weighed and pre combusted Whatman Ø 25 0.7µm GF/F glass fibre filters and exposure to 105°C for 24h before weighting on a Mettler Toledo MT5 (d =0.1µg).

Larval mortality throughout the experiment (excluding type II cannibalism) was monitored by counting dead larvae siphoned from the bottom of the rearing tanks on a daily basis. Type 2 cannibalism was estimated at the end of the experiment.

Stress tolerance experiments were conducted on DPH 10 and 20. Approximately 20 larvae from each tank; i.e., early, intermediate, and late hatched larvae were transferred to beakers holding 2 litres of 10ppt seawater or freshwater from the rearing system. Survival after 30 minutes was used to evaluate stress performance. Statistics were mainly based on a nested ANOVA design: measurements nested in tanks nested in treatments.

Results and discussion

Larval growth estimated as larval length or dry weight did not significantly depend on time of hatching. Time of yolk sac depletion, time of oil globule depletion, and time of first feeding also did not differ significantly between the different hatching times. It seems as if larval development takes place at the same rate in pre- and post hatched larvae until all larvae have hatched.

In salmon it has been speculated if earlier hatched individuals within a batch differed from later hatched ones. It seems not to be the hatching time, but rather the time when larvae reach first feeding that correlates with behavioural indicators such as post metamorphic growth and tolerance to hypoxic conditions (Cook et al., 2000; Farrell et al., 1997; Stevens et al., 1998).

Larval mortality spanned from 39.7% in late hatched larvae to 51.0% in medium hatched larvae but the differences between treatments were not significant.

Stress tolerance by confinement in freshwater or seawater did not have any effect on the fish. Full survival was observed in both treatments on both dates (DPH 10 and 20).

The stress imposed on the fish may have been insufficient to induce an effect. In an earlier experiment on pikeperch a clear effect of similar stress treatments was observed on fish fed high inclusions of olive oil containing mainly oleic acid (C18:1n-9) (Lund and Steenfeldt, accepted Aquaculture Nutrition, July 2009). In salmon, a negative relation between time of first feeding and stress tolerance has been found (Cook et al., 2000; Farrell et al., 1997 and Stevens et al., 1998). Sorting of pike perch larvae based on hatching time could easily be managed in the typically used Zuger or McDonald-type egg incubators. In salmonid fish, a procedure for selecting early first feeders has been developed. It is based on the behaviour of prefeeding larvae that will hide on the bottom and only after having reached the ontogenetic stage of first feeding will leave the bottom and swim up in the water column, possibly in search of food. By selectively catching these free swimming individuals it is possible to separate early and late first feeders. In most fish species including pikeperch no such separation is possible. Since early and late hatched individuals reached time of first feeding at the same time, hatching time can not be used to separate early and late first feeders. Whether or not alternative methods can be used to separate early and late feeders is yet unknown and this limits progress in this type of behavioural management of pike perch.

Conclusions

Early or late hatched fish seemed to develop simultaneously. No effects of hatching time was identified on growth, time of yolk sack depletion, time of oil globule depletion or time of first feeding. Sensitivity to stress by confinement in 2 litres of fresh water or 10ppt seawater also did not differ between the early and late hatched larvae.

Consequently there seems not to be any beneficial effect of differentiating between larvae within a batch or of force hatching them to hatch within a shortened time interval.

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APPLICATION OF ^{1}H-NMR METABOLOMICS TO STUDY COD LARVAE (*GADUS MORHUA* L)

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Introduction

Metabolomics is the comprehensive study of naturally occurring, low molecular weight metabolites within a cell, tissue, or biofluid. Based on nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS), and coupled with multivariate statistics, it has become a powerful technique to study a wide variety of medical issues such as differences in metabolic profiles between healthy and cancerous tissue in humans (Sitter et al., 2009). In environmental toxicology NMR based metabolomics has particularly been used to study effects of different toxins and stress on organisms in the marine environment, such as Japanese Medaka (*Oryzias latipes*) and the marine mussel (*Mytilus edulis*) (Tuffnail et al., 2009). For the former, NMR metabolomics has been used to study metabolic developmental trajectories; i.e., how the metabolic profiles change during embryogenesis and hypoxia (Pincetich et al., 2005). NMR metabolomics applied to plasma has been used to study stress in salmon (Solanky et al., 2005).

The high resolution magic angle (HR MAS) NMR technique obtains high quality metabolic profiles directly from tissues and cells with very little sample preparation. In the reported study we investigated the applicability of this technique for analysis of cod larvae (*Gadus morhua*, L.) by assigning the ^{1}H-NMR spectra obtained by HR MAS NMR directly on whole larvae ranging between 8.2 and 42.6mg in wet weight. Multivariate analysis was used to study differences in the metabolic profiles from larvae of different weights.

Materials and methods

Metabolic profiles in cod larvae were obtained by analyzing directly on the whole larvae using a Bruker DRX 600 spectrometer (BRUKER GMBH, Rheinstetten, Germany) fitted with a 4mm HR-MAS probe at 4°C. Sample preparation

consisted of transferring the frozen larvae (obtained from an experiment where larvae were subjected to different water treatments; e.g., UV or ozone, larvae killed by anesthesia with metacain) to a 4mm HR-MAS rotor with an internal volume of 50µl and adding D_2O with an internal chemical shift reference, and closing the rotor. The time elapsed from taking the sample from the freezer until the sample was in the magnet was ~5min.

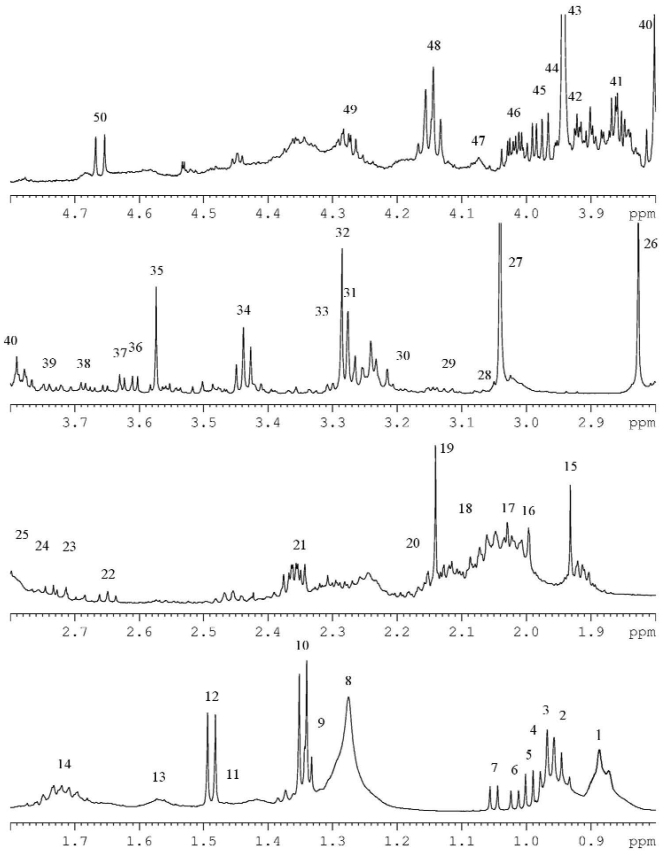

Fig. 1. The 4.8-08ppm region of a typical 1H-NMR HR MAS NMR spectrum. NMR observes nuclei, and as such may give more than one resonance (signal) per compound. 1) Fatty acid (FA) -CH_3, 2) Ile, 3) Leu, 4) FA n-3 CH3, 5) Val, 6) Ile, 7) Val, 8) FA -CH_2-, 9) Thr, 10) Lac, 11) Ile, 12) Ala, 13) FA, 14) FA β-CO, 15) Acetate, 16) Pro, 17) FA, 18) Pro, 19) Met, 20) Met, 21) Pro, 22) Met, 23) Asp, 24) FA double allylic, 25) Asp, 26) Dimethylglycine, 27) Creatine, 28) Tyr, 29) Phe, 30) Tyr, 31) Glycine betaine, 32) TMAO, 33) Phe, 34) Taurine, 35) Glycine, 36) Thr, 37) Val, 38) Ile, 39) Leu, 40) Met, 41) Ser, 42) Asp, 43) Creatine, 44) Tyr, 45) Ser, 46) Phe, 47) Glyceride, 48) Pro, 49) Glyceride, 50) β-Glucose.

Two experiments were performed to obtain normal ^{1}H-NMR spectra (not presented here), and T2-filtered CPMG spectra (suppress lipid resonances) in ~30 minutes. The recorded spectra where preprocessed in TOPSPIN v1.3 software. Spectra were exported as ASCII files and imported into R for partial least squares regression (PLS) using the PLS-library of Mevik and Wehrens (2007). The wet weight of the larvae was correlated to the metabolic profile using this method. Linear discriminant analysis (LDA) (Discrim Toolbox 0.3 by Michael Kiefte, downloadable from www.mathworks.com/matlabcentral) in MATLAB 7.8 was used to discriminate the two water treatments by the score values of the two first latent variables (LVs) from PLS.

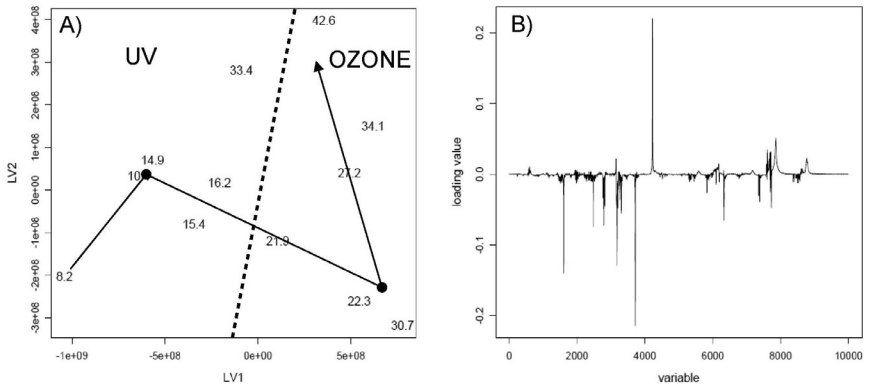

Fig. 2. (A) The metabolic developmental trajectory (solid line) described by the score plot of the two first LVs of the PLS-analysis. Linear discriminant analysis of the scores gave decision regions as separated by the dotted line. (B) Loading plot showing the differences in the metabolic profile along the first LV.

Results and discussion

The high-field region of a typical CPMG-spectrum obtained in this study is shown in Figure 1 with assignments. 50 peaks in this region were assigned comprising 22 compounds. Lipids are suppressed by the CPMG pulse sequence, but are still visible in this spectrum. The score plot from the PLS analysis of the metabolic profiles of the larvae and wet weight is shown in Figure 2A. The metabolic developmental trajectory from a wet weight of 8.2 mg to 42.6mg for cod larvae is indicated. The best correlation is seen for wet weights of 10-30 mg. The loading plot for the first principal component is shown in figure 2B and gives the metabolites which vary along the first latent variable and account for 64.6% of the variation. The PLS analysis shows that the changes in the metabolic profile of cod larvae are correlated with the weight of the larvae. The HR MAS NMR method may as such, prove simple, rapid and highly useful in studies to describe larval metabolic development.

Conclusion

It has been shown that HR MAS NMR may provide information on a number of metabolites by analysis directly on cod larvae with very little sample preparation, and that changes in the metabolic profile during development are observable. The metabolites assigned in this study are only the starting point of the characterization of the metabolome of cod larvae by NMR based metabolomics. The number and type of metabolites assigned will define the problems and hypotheses that may be studied by the technique by way of the compounds that are observable. Equally important, in addition to hypothesis driven research, NMR metabolomics is an ideal tool for question driven research. The non-selective nature of sample preparation and broad range of different metabolites that are observed at the same time holds potential to discover hidden and unthought-of changes in metabolic profiles, some of which may lead to new knowledge on its own and to new hypotheses to be investigated.

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CURRENT STATUS OF CHINESE MITTEN CRAB *ERIOCHEIR SINENSIS* LARVICULTURE IN CHINA

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The Chinese mitten crab *Eriocheir sinensis* is an indigenous species in East Asia with a native distribution along the eastern pacific coast of China. Yet it has been recognized as an invasive species in Europe and Northern America, probably translocated through ballast water of ships over the past hundred years. Mitten crabs are considered as a most nutritious and delicious crustacean by Chinese consumers, and thus have high economic value in China.

Before the 1980s, aquaculture production of mitten crab relied on the provision of wild megalopa and the restocking of captured megalopa in lakes. However, natural recruitment became severely exhausted in the late 1980s due to overfishing and the construction of dams and irrigation works. The dramatic decline in availability of wild-caught megalopa has greatly stimulated the development of controlled production of seed to stock ponds. A breakthrough in hatchery techniques for mitten crab was achieved in the beginning of the 1980s. The booming of mitten crab aquaculture however only emerged ten years later, especially since penaeid shrimp culture in China was hit by severe virus outbreaks in the early 1990s. The total production of megalopa in hatcheries doubled from 277mt in 2001 to 638mt in 2005. Meanwhile, the annual yield of market-sized mitten crabs increased from 17 500mt in 1993 to 570 000mt in 2005.

As a catadromous species, the hatchery phase of mitten crab is conducted in seawater. Brood crabs mate and spawn at a water salinity of 17-20g.l^{-1} and temperature of 9-13°C. A ratio of female to male of 2-3:1 is normally employed. Mating is done either in late autumn or early spring. In the first case, the berried females carry the eggs over winter, which is believed to result in higher spawning rates and better egg quality. The brood crabs are usually fed fresh or frozen squid, fish, mussels and annelid worms. The production window for megalopa is narrow (usually from March to May), although an extension of the reproduction period can be achieved (from January to June) by temperature control. The eggs hatch as zoea larvae at a salinity of 20-25ppt and temperature of 18-21°C. Larval

development includes five zoeal stages and one megalopa stage. In China, two models of larviculture techniques are currently performed in the hatchery production of mitten crab.

Indoor intensive larval rearing is carried out at high stocking density in indoor concrete tanks. Aeration and temperature (usually increased 1°C before each molt up to 24-25°C at megalopa stage) are closely controlled in the rearing tanks and sufficient food (e.g., algae, egg yolk, live rotifers and *Artemia*, as well as frozen rotifers, copepods and *Artemia* biomass) is supplied. Yields are up to 0.15-0.5kg megalopa per cubic meter at stocking densities of 0.2-0.5 million Z1 per cubic meter, and 0.5-0.9kg at stocking densities of 0.5-1 million Z1 per cubic meter. The disadvantages of this technique are mainly related to microbial interference and the high production cost. Therefore a more economical and ecological-friendly mesocosm system has been developed for mitten crab larvae rearing earlier this century.

Outdoor extensive larval rearing (mesocosm system) is usually managed at low stocking density in outdoor earthen ponds. The larvae are considered to have better resistance to disease and extreme culture conditions, and thus they have a higher market price. Early larval stages feed on natural food (e.g. microalgae, rotifers and other zooplankton) available in the ponds; and later larval stages are fed minced fish and *Artemia* biomass. The yield of megalopa can be 2-10 kg per hectare. The less complicated management and the more simple facilities required make this technique widely adopted by local farmers, although the yield is usually low and unstable due to the fluctuations of water quality parameters (mainly temperature and salinity).

Successful batches in the mitten crab hatchery can reach an average survival of 10-15%, with a maximum of 30-50% survival at the megalopa stage; but total mortality before Z2 or megalopa stage also frequently occurs. The variable yield is attributed to several factors, such as inferior larval quality caused by genetic degradation and nutritional unbalances of the broodstock, cannibalism at metamorphosis from Z5 to megalopa due to asynchronous molting, and unstable microbial conditions in the rearing water, etc.

With the aim to improve hatchery production of mitten crab and increase its sustainability, many researches have been conducted by Chinese scientists during the last decades. These studies have focused on the nutritional requirements of the broodstock (e.g. HUFA, phospholipids and vitamins) in relation to ovary development, egg quantity and quality; genetic diversity of the different broodstock origins; larval feeding strategies and larval nutritional requirements (e.g. for HUFA and phospholipids); and microbial control in larval rearing, etc. Some achievements have been applied in large-scale hatchery production and resulted in improved hatchery output.

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EFFECT OF PROTEIN LEVELS IN DIETS ON GROWTH, SURVIVAL RATE, AND MOLTING FREQUENCY OF YOUNG SWIMMING CRAB (*PORTUNUS PELAGICUS*)

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Introduction

Portunus pelagicus is commercially important in Thailand due to its great demand and high market value. Development of an effective diet is essential for successful rearing. Optimization of protein level and feeding regime will improve growth and production cost. This study aims to evaluate the effect of protein levels in diets on its growth, survival rate, and molting frequency.

Materials and methods

Young swimming crab obtained from Samut sakorn Fisheries Research and Development Center with an initial weight of 0.45-0.46g were reared in four 300-l tanks, each divided into 20 compartments. Rearing water was exchanged daily in semi-closed system. The bottom of each culture tank covered with sand as a substrate. Aeration was supplied via 4 air stones per tank. Culture water had total ammonium 0.173-0.232mg.l^{-1}, nitrite 0.125-0.277mg.l^{-1}, pH 8.1-8.2, alkalinity 139-144mg.l^{-1}, and salinity 25-26ppt. Crabs were stocked individually in each compartment and fed practical test diets at satiation level 2 times a day at 9:00 am and 4:00 pm for 12wk. There were 2 formulated diets (high (42.8%) and low (34.3%) protein) and 2 feeding regimes (T1=34.3%:12wk; T2=42.8%:12wk; T3=42.8%:4wk and 34.3%:8wk; and T4=34.3%:4wk and 42.8%:8wk).

Practical test diets were prepared by mixing dry ingredients before oily ingredients were added. The resulting dough was pelletized using a meat mincer fitted with a 1.9-mm die. All test diets were then dried in an oven at 60°C and stored at -20°C. Crude protein was analysed by Truspec CN Carbon/Nitrogen Determina-

tion (LECO), lipid by Fat Extractor TFE 2000 (LECO), and ash, moisture, and fiber were analysed according to AOAC (1984).

Crabs were monitored every day and week to obtain data on molting frequency, survival rate and growth respectively. At termination, crabs were freeze-dried and subjected to biochemical analysis. Results were analyzed by a one-way ANOVA and significant differences determined by a Tukey multiple comparison test by Systat version 5.0 (Wilkinson et al., 1992)

Table I. Proximate analysis of practical tests diets.

Diet	Moisture %	Biochemical composition (% as fed basis)					Gross energy Kcal.100g^{-1}
		Protein	Lipid	Fiber	Ash	NFE	
1	9.6±0.2^{a}	34.3±0.1^{b}	13.5±0.2^{a}	2.6±0.2^{a}	13.4±0.1^{b}	22.1±0.5^{a}	412±1^{a}
2	10.2±0.1^{a}	42.8±0.0^{a}	11.4±0.4^{a}	2.4±0.1^{a}	15.2±0.1^{a}	15.8±0.3^{b}	413±2^{a}

The different superscript in the same column were significantly difference ($p<0.05$)

Results and discussion

Growth (weight, width, and length gain and molting frequency) of crabs were significantly affected by diets ($p<0.05$). Due to high variation in survival rate, differences were not significant among treatments ($p>0.05$). At week 4, crabs of treatments 2 and 3 had highly significant weight gain over those of treatment 1 and 4 ($p<0.01$). Changing new diets in treatments 3 and 4 after week 4 up to termination had a highly significant effect on crab growth ($p<0.01$) and the best performance was obtained from treatments 2 and 4, with mean body weights 11g and 11.6g, respectively. Growth of treatment 3 (9.2g) was in the second order and was not significant different from treatments 1, 2, and 4, while treatment 1 (8.2g) was the poorest and was significantly lower than those of treatments 2 and 4 ($p<0.01$). Additionally, treatments 2 and 4 had molting frequency significantly higher than those of crabs from treatments 1 and 3 ($p<0.01$).

Table II. Growth, survival rate, and molting frequency of *P. pelagicus* at 12 wk.

Treatment	SVR (%)	Growth rate (%)			Molting frequency Days/time
		Weight gain	Width gain	Length gain	
1 L P	85±37^{a}	1627.8±762.3^{b}	154.5±31.0^{c}	172.5±34.4^{c}	23±4^{b}
2 H P	85±37^{a}	2317.4±744.2^{a}	180.0±37.3^{ab}	201.6±35.1^{ab}	18±3^{a}
3 H:L P	100±0^{a}	1912.0±414.0^{ab}	166.9±20.2^{bc}	185.5±24.0^{bc}	22±3^{b}
4 L:H P	90±31^{a}	2444.1±633.0^{a}	191.9±32.4^{a}	208.3±32.6^{a}	19±4^{a}

The different superscript in the same column were significantly difference ($p<0.01$)

Dietary protein levels and feeding methods were significantly effect on bio-chemical composition of crabs ($p<0.01$). Crabs in treatments 2 and 4 contained significantly higher protein (39.7-41.9%) lipid (3-3.2%) and gross energy (260-267kcal.100g^{-1}) than those of crabs from treatment 1 and 3 which were in the range of 32-34.3%, 1.6-1.7%, and 208-216kcal.100g^{-1}, respectively ($p<0.01$). Crabs in treatment 2 and 4 accumulated long chain fatty acids 18:2n-6, 20:4n-6,

20:5n-3, 22:6n-3 including total n-3, total n-6, n-3 HUFA and n-3/n-6 ratio significantly higher than those of crabs in treatment 1 and 3 ($p<0.01$). In the present study, swimming crab fed high protein (42.8%) for a longer period (8-12 weeks) had better growth, molting frequency, and higher protein and lipid accumulation in their tissue. Crab tend to be carnivorous and highly cannibalistic, which requires high dietary protein like other crustaceans such as penaeid shrimp, whose protein requirement is high (43-57%) (Akiyama et al., 1988).

Table III. Proximate analysis results of *P. pelagicus* reared under different treatments for 12 weeks.

Treatment	Dry matter %	Biochemical composition (% dry matter)					GE kcal.100g^{-1}
		Ash	Fiber	Lipid	Protein	Carbon	
1 L P	25.3	52.8±1.1^{a}	10.6±0.2^{a}	1.6±0.1^{b}	32.0±1.3^{b}	3.0±0.1^{a}	208±8^{b}
2 H P	25.8	44.7±0.1^{b}	9.8±0.5^{a}	3.0±0.0^{a}	41.9±0.7^{a}	0.5±0.0^{b}	267±3^{a}
3 H:L P	25.5	52.5±0.9^{a}	10.0±1.1^{a}	1.7±0.0^{b}	34.3±0.6^{b}	1.5±0.4^{ab}	216±1^{b}
4 L:H P	29.2	45.0±0.2^{b}	10.6±0.3^{a}	3.2±0.2^{a}	39.7±0.1^{a}	1.6±0.1^{ab}	260±2^{a}

The different superscript in the same column were significantly difference ($p<0.01$)

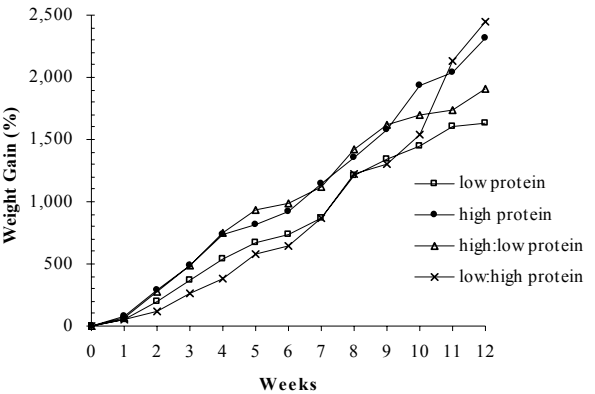

Fig. 1. Weight gain (%) of *P. pelagicus* reared under different treatments for 12 weeks.

Table IV.Fatty acids profile (%area of total lipid) of *P. pelagicus*

Fatty acids	Treatments			
	1 low P	2 high P	3 high:low P	4 low:high P
C 18:1n-9	37.96	31.94	34.56	30.66
C 18:2n-6	7.33	16.13	10.86	14.37
C 18:3n-3	ND	1.25	ND	0.87
C 20:4n-6	ND	1.74	0.57	1.27
C 20:5n-3	1.51	4.58	1.38	3.00
C 22:6n-3	2.05	6.86	2.72	7.31
Total n-3	3.56^{b}	12.70^{a}	4.09^{b}	11.68^{a}
n-3 HUFA	3.56^{b}	11.45^{a}	4.09^{b}	10.82^{a}
Total n-6	7.33^{b}	18.99^{a}	12.16^{ab}	16.53^{a}
n-3/n-6	0.49^{b}	0.67^{a}	0.34^{b}	0.71^{a}

The different superscript in the same column were significantly difference ($p<0.01$)

Protein plays an important role in the molting frequency of crustaceans (Guillaume, 1997) by reducing intermolt period (Jones et al., 1996).They accumulate protein in hepatopancreas prior to molt and used this preserve for growth at postmolt. Beside that, protein plays an important role in shell formation by being a constituent of chitin in the form of protein-polysaccharide complex (Horst, 1989; Sarac et al., 1994). Additionally, Dall et al. (1990) stated that crustaceans used a considerable amount of body lipid prior to molting, as was found in this study where high total lipid as well as long-chain fatty acids accumulated in the tissue of crab fed on high protein for 8-12wk.

Conclusions

It can be concluded from the results that fed crabs with protein level of 42.8% as fed basis in diet with gross energy of 413kcal.$100g^{-1}$ for either 12wk or later 8wk contributed to the best growth and molting performance in young swimming crabs (*P. pelagicus*) which resulted in body weight higher than 10g within 12wk.

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FAMILY AND PLOIDY EFFECTS ON LARVAE SURVIVAL, DEFORMITIES, AND PERFORMANCE IN ATLANTIC SALMON

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The Atlantic salmon (*Salmo salar*) farming industry is under increasing pressure to eliminate the impact of escapees, particularly potential inbreeding between wild and farmed stocks. Furthermore, greater public awareness and the need to protect natural resources require the development and implementation of new or alternative environmental regulations and operating standards. One option is the production of sterile stocks by triploid induction. Triploidy is not a new concept. Unfortunately, poor performance, higher mortalities, and deformities led to the industry abandoning triploidy in favour of photoperiod control of pre-harvest maturation. However, although photoperiod reduces maturation in culture, such stocks remain reproductively competent and the threat of escapes persists. Triploid induction is the only method that can produce sterile fish. Furthermore, significant advances in selective breeding, husbandry, diet formulation, operating procedures, and an overall recent greater knowledge of salmon physiology suggest that some of the problems previously associated with triploidy may no longer be an issue and the industry now looks to re-explore this option.

We established a series of experiments as part of the EC FP7 "Salmotrip" project to examine the interaction of family and ploidy on survival, deformity prevalence, and growth performance from egg to smolt in Atlantic salmon. In two year classes (2007, 2008) 10 full-sib experimental families were created. Family egg batches were divided in two at fertilisation with one group subjected to a hydrostatic pressure shock of 9500psi for 5min at 300°min post-fertilisation to induce triploidy. A third trial produced 90 full-sib families for commercial field trials using the same protocol. In the 2007 year class stocked in 2008, parr were subjected to one of two photoperiod regimes to produce S1+ or S0+ smolts. The 2008 year class was monitored from egg to pre-smolt regimes of the summer 2009. A series of trials were also conducted in Norway to examine triploid performance under sub-optimal conditions. Three full-sib groups, with half of each group subjected to triploidization by hydrostatic pressure, were made in 2007 and 2008 to explore the effect of sub-optimal nutrition and environmental condi-

tions in triploid compared to diploid salmon during on-growth in seawater under semi-commercial conditions. In addition, fertilized eggs of 43 females (with half of the eggs of each female subjected to triploidization by hydrostatic pressure) were incubated and subsequently start fed under commercial conditions at Marine Harvest in 2008. These fish are used to study triploid performance compared to diploid salmon under commercial conditions from fertilization until harvest size. In all trials, during egg incubation, hatchery, and smolt grow-out, mortality, growth, gill ATPase activity, deformity prevalence, and triploid yield were recorded and the interaction between ploidy and family examined.

In most year classes, survival to hatch did not differ between ploidy but was significantly affected by family. However, reduced family survival was found to strongly correlate with gamete quality. At hatch, 100% triploid induction was confirmed using nuclear RBC length. During the hatchery phase ploidy significantly affected size at hatch (530°d), with diploids generally larger than triploids. A significant effect between families on weight was also evident. Weight advantage of diploids over triploids was only maintained for 6 weeks post-first feeding. Deformity prevalence in first feeding and freshwater stages was generally low (mean <2%), with no overall effect of ploidy, but there was a significant family effect on occurrence of deformity (0.5-4%).

During freshwater grow out to smolting, significant family effects on size were maintained irrespective of ploidy. Unlike the hatchery phase the growth advantage diploids had over triploids was reversed. Within 2 months triploids were significantly heavier than diploids and this was maintained from the onset of smolt regimes throughout grow-out in both S0+ and S1+ populations. Importantly, production of S0+ triploid smolts is reported for the first time. ATPase activity and completion of smoltification was affected by time and ploidy in S0+ populations, but not in S1+. This correlated strongly with fish size at onset of smolt regimes and subsequent growth performance.

Our findings show that with correct broodstock selection, triploids can perform as well – if not better – than diploid siblings. The low deformity is a significant improvement over previous reports in triploid salmon stocks; however, long-term sea monitoring will be required to determine whether previously reported deformities are due to environmental change or genetics. It will also be important to determine if families that perform well in freshwater continue to do so in saltwater, which will aid in improving selective breeding programs. Furthermore, out-of-season triploid smolts can be successfully produced without the poor performance reported in previous trials. Our results show important criteria to consider when manipulating smoltification time in triploid salmon. Overall, our results provide promising knowledge for the salmon industry, as they indicate year-round production of salmon can be maintained and genetic/ecological threats reduced through the potential adoption of triploid salmon.

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COMPARISON OF EARLY-LIFE STAGE STRATEGIES IN 65 EUROPEAN FRESHWATER FISH SPECIES

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Introduction

Arguably the most striking characteristic of fish is their seasonality, with the timing of developmental and maturational events dominated by and, in parallel, synchronized with seasonal changes in climate, water chemistry, temperature, photoperiod and food supplies (Wootton, 1999). This coordination and the associated internal processes of control ensure that larvae are produced when environmental conditions are most suitable for their survival, i.e. when the size spectrum and abundance of prey are appropriate. These most favorable conditions generally occur in spring and early-summer in mid and high latitudes.

Egg size and temperature both significantly influence larval size upon hatching. Egg and larval sizes are positively correlated, while larvae hatching from eggs incubated at higher temperatures generally being shorter. It has also been demonstrated that large larvae have generally more energy reserves at hatching than small larvae and are more resistant to starvation. More importantly, large larvae have a greater flexibility (window of opportunity) in first feeding times (Miller et al., 1988).

From an analysis of twelve egg and larval variables, temperature and spawning season for 65 teleost freshwater fish, the main objectives of the present study were to reassess previous conclusions on the possible relationships between egg size, larval size, temperature and degree-days and explore further the different trade-offs during early-life stages ensuring that larvae start feeding at the most suitable conditions.

Materials and methods

All data were obtained from a comprehensive literature search (1900 references) and are currently hosted within a new database called STOREFISH, acronym for STrategy Of REproduction in FISH (Teletchea et al., 2007). The target fish are freshwater teleosts inhabiting Europe and, to a lesser extent, North America, among which 65 species are used in the present study. Bivariate relationships

among variables were analyzed for all significant comparisons using Pearson's correlation coefficient and the fits of five different models were compared using the coefficient of determination (r^2). Principal component analysis (PCA) was used to investigate the associations between the ten quantitative variables as well as the ordination of species.

Results and discussion

Time or degree-days in yolk-feeding fishes (i.e. during incubation and/or endogenous feeding of larvae) was weakly related to egg size and strongly to temperature. These results are chiefly because egg size and yolk reserves are weakly related and temperature strongly increases tissue differentiation rate, activity of hatching glands and embryo motility. Strong positive relationships were found between larval size and egg size and degree-days for incubation.

No relationship was found between larval size and degree-days from hatching to mixed feeding and between degree-days for incubation and degree-days from hatching to mixed feeding. These last two results are chiefly because the developmental stages at hatching and at the onset of exogenous feeding are not fixed in ontogeny and are not directly related to either larval size or degree-days for incubation, but more probably are species-specific.

Five major groups of species were found based on the PCA analysis of ten quantitative variables. It appeared that whatever the spawning season is, occurring almost all year long, the different trade-offs at the early-life stages ensure that most larvae are first-feeding during spring, when food size and abundance are the most appropriate.

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MODELLING THE DIGESTIBILITY OF *ARTEMIA FRANCISCANIS* 'IN VITRO' DURING THE EARLY LARVAL STAGES OF MARINE FINFISH: A NOVEL APPROACH

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Establishing feeding strategies that match the nutritional needs of marine finfish larvae are essential to ensure normal development and optimal growth under culture conditions. Co-feeding strategies remain the most common practice, despite attempts to rear larvae exclusively on artificial diets. Understanding the digestibility of live food organisms is important for successful larviculture and could contribute towards designing improved replacement diets.

Various studies have shown that determining the digestibility of diets in vitro can complement, and in some instances precede or substitute, in vivo digestibility techniques, largely due to biochemical techniques that offer a wide range of practical applications and are quick, simple, and relatively inexpensive in comparison to standard digestibility studies. The aim of this study was to design an in vitro protocol to estimate the digestibility of *Artemia franciscanis* in the larvae of three warm temperate, marine fish species: *Sarpa salpa* (Sparidae), *Diplodus sargus capensis* (Sparidae), and *Argyrosomus japonicus* (Sciaenidae).

An in vitro approach begins with the characterisation of the activities for selected digestive enzymes under different environmental conditions, followed by the determination of the specific enzyme activity (U.mg protein^{-1}) under optimal conditions. Characterisation of enzyme activities was achieved by altering pH and temperature during enzyme-substrate incubation. Specific enzyme activity was determined for alkaline proteases, lipase and amylase using enzyme specific substrates and standard colourometric techniques. In the absence of a completely developed digestive system these enzymes are responsible for digestion during the early larval stages.

The activity for each enzyme at the different pHs were plotted for each species and resultant models compared with a log likelihood ratio test. The optimal pH for alkaline proteases, lipase and amylase was 7.67 (A=0.4, p=0.55), 8.03 (A=2.1, p=0.54) and 7.69 (A=0.5, p=0.46), respectively. No significant difference was found between species. All the enzymes showed optimal activity

around a temperature of 50ºC. This high temperature has no biological meaning in digestive studies and all trials were run at a standard temperature of 20ºC, the average natural temperature experienced by warm temperate species.

In vitro trials (n=64) were performed by digesting newly hatched and enriched (DHA Selco, INVE) *Artemia franciscanis* nauplii at a pH of 7.7 for 15, 30, 45, 90, 120, 150, 210, and 270min for each fish species. A defined number (1828±487) of *Artemia* nauplii was used, while the source of digestive enzymes was obtained from crude larval extracts. The degree of protein, lipid and carbohydrate digestion in the digesta was determined with a modified Ninhydrin colourometric method for amino acids, the Korn method for glycerol (by-product of triglyceride digestion) and a modified Somogyi-Nelson method for reducing sugars, respectively. In vitro digestion of *Artemia* was then analysed and modelled using a generalised linear model which showed that both protein and carbohydrate digestion is strongly correlated to the time of digestion, and the preparation method of the *Artemia*. The preparation method was built into the model as a categorical predictor, where newly hatched *Artemia* was assigned a value of 1 and enriched *Artemia* a value of 2.

The degree of protein digestion (DPD) and carbohydrate digestion (DCD) can be described by the following equations, where c = constant specific to the preparation method of *Artemia*.

DPD = 0.001 × time (min) + 0.592 × Alkaline protease activity – 1.323 × Amylase activity – 0.099 × *Artemia* preparation method + c (AIC = - 136.7, $p<001$)

DCD = 0.0008 × time (min) – 1.632 × Amylase activity – 0.123 × *Artemia* preparation method + c (AIC = -316.1, $p<0.001$)

Both models demonstrated good predictive power for in vitro digestion of *Artemia* for another warm temperate species, *Dagetichthys marginatus* (Soleidae) (DPD, $\chi^2=2.8$, $p=0.83$ and DCD, $\chi^2=1.8$, $p=0.94$). The Korn method showed no detectable levels of glycerol and consequently a lack of triglycerides in both newly hatched and enriched *Artemia,* despite the reasonably high levels of triglycerides in fish oil emulsions like DHA Selco. This can partially be explained by the fact that other copepods generally store fats as wax esters and only maintain small amounts in a triglyceride form for use as a metabolic energy reserve. A more suitable biochemical assay is thus required to quantify the degree of lipid digestion.

Debate exists over the contribution by exogenous enzymes from *Artemia* towards digestion in first feeding larvae. This study showed that exogenous enzymes contributed significantly (40.0±12.7%) to carbohydrate digestion and protein digestion (14.6±7.6%). This work confirms the usefulness of in vitro studies to complement or possibly even replace in vivo digestibility studies.

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FEEDING OF CARP LARVAE (*CYPRINUS CARPIO*) WITH NEMATODES AS AN ALTERNATIVE FOR ARTEMIA (*ARTEMIA SALINA*) AND DRY FEED

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Introduction

Nematodes (Family Panagrolaimidae, "microworms") are commonly used as food for ornamental fish larvae and have been positively tested using larvae of aquaculture fish as well as crustacean species (Biedenbach et al., 1989; Schlechtriem et al., 2004; Sautter et al., 2007). Nematodes can be easily cultivated in high quantities (Ehlers and Shapiro-Ilan, 2005) and enriched with deficient nutrients. The present work evaluates the suitability of two nematode species as substitutes for *Artemia* and dry feed for newly hatched carp larvae (*Cyprinus carpio*).

Materials and methods

In a three week feeding trial, carp larvae were reared in 10-l tanks in three closed recirculation systems. The following treatments were evaluated: *Pangrellus redivivus*, NFS 30-4 (nematode, Family Panagrolaimidae), *Artemia* nauplii, dry feed (Perla Larva, Skretting), and a starved control. Each treatment was performed in triplicate and tanks were stocked with 500 larvae each. Both nematode species were raised in bioreactors in liquid culture (see Ehlers and Shapiro-Ilan, 2005 for details) and delivered suspended in water to the feeding site. All treatments were fed ad libitum. Samples of nematodes were counted and observed to ensure sufficient numbers of vital nematodes.

Eight days post hatching (dph), 200 larvae of each tank were moved to empty tanks and switched to dry feed ("ArtemiaD", "PanagrD", "NFS 30-4D"). The remaining larvae were switched to dry feed 15dph ("Artemia", "Panagr", "NFS 30-4"). Samples of about 20 individuals were taken every week for growth measurements. At the end, all remaining larvae were counted and growth pa-

rameters for all larvae were measured and calculated. Pooled samples for whole body cortisol measurements were taken to evaluate possible stress effects.

Results of survival and whole body cortisol were analyzed by a one-way ANOVA, growth analyses were analyzed by a nested ANOVA with measurements of individuals in each tank being nested within treatments. Upon significant differences ($P \leq 0.05$), the ANOVA was followed by a multiple comparison test, either by Tukey-HSD or, if heterogeneity of variances was observed despite log transformation, by Dunnett T3. Statistical analyses were conducted using SPSS 14.0.

Results and discussion

Results revealed that carp larvae fed with *Artemia* for two weeks and dry feed for one week ("Artemia") or *Artemia* for one week and dry feed for two weeks ("ArtemiaD") showed the highest growth parameters (Fig. 1 and Table I). Larvae fed *Panagrellus redivivus* for one week and dry feed for two weeks afterwards ("PanagrD") were significantly larger than larvae fed dry feed alone, but significantly smaller than larvae fed both *Artemia* treatments. Survival [%] of both *P. redivivus* treatments was not significally different to dry feed and both *Artemia* treatments. Carp larvae fed the nematode NFS 30-4 for two weeks and dry feed for one week showed the lowest growth parameters and lowest survival. However, standard deviations of growth parameters and results of the nested ANOVA showed strong tank effects and strong individual response of larvae to feeding regimes. Cortisol measurements showed no significant differences between feeding variants. Larvae in all treatments experienced comparable stress situations.

Table I. Survival [%], total length [mm], fresh weight [mg] and whole body cortisol [ng.g^{-1}] of carp larvae at 22dph (mean ± SD). "D" indicates a switch to dry feed of respective feeding variants at 8dph. Remaining larvae were switched to dry feed at 15dph. Different letters indicate significant differences ($P \leq 0.05$). "-" indicates no data.

Treatment	Survival (%)	Total length (mm)	Fresh weight (mg)	Cortisol (ng.g^{-1})
No feed	7.0 ± 1.4^{a}	8.62 ± 0.98^{a}	3.23 ± 1.82^{a}	-
Dry feed	54.3 ± 14.2^{ab}	14.27 ± 2.87^{d}	28.16 ± 29.57^{e}	4.5 ± 1.8
Artemia	86.6 ± 11.7^{b}	20.03 ± 2.36^{f}	83.03 ± 35.15^{h}	5.7 ± 2.8
ArtemiaD	88.1 ± 7.2^{b}	18.18 ± 2.22^{e}	64.53 ± 30.13^{g}	5.0 ± 1.0
NFS 30-4	31.8 ± 16.6^{ab}	11.15 ± 2.06^{b}	12.67 ± 11.01^{b}	4.1 ± 1.8
NFS 30-4D	56.8 ± 30.9^{ab}	13.23 ± 2.13^{c}	23.43 ± 15.33^{d}	4.4 ± 0.8
Panagr	69.0 ± 20.7^{ab}	12.90 ± 2.01^{c}	19.67 ± 13.19^{c}	3.8 ± 1.3
PanagrD	76.0 ± 6.0^{b}	14.74 ± 2.75^{d}	37.39 ± 29.10^{f}	4.7 ± 1.0

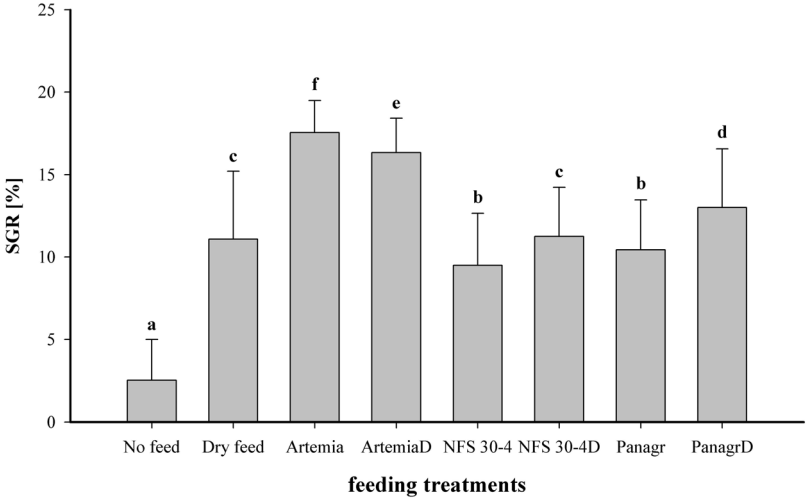

Fig. 1. Specific growth rate (SGR [%]) of carp larvae fed different treatments on 22dph (mean ± SD). "D" indicates a switch to dry feed of respective feeding variants at 8dph. Remaining larvae were switched to dry feed at 15dph. Different letters indicate significant differences ($P \leq 0.05$).

Conclusions

The present study reveals the suitability of nematodes (Panagrolaimidae) as live food for carp larvae (*Cyprinus carpio*). However, newly hatched carp larvae are large enough (6.5mm total length, 1.6mg fresh weight) to utilize *Artemia* nauplii. Further studies should focus on the suitability of nematodes as live food for fish species with smaller larvae.

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TOWARDS THE IMPROVEMENT IN SEED QUALITY OF REARED PACIFIC COD (*GADUS MACROCEPHALUS*)

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Introduction

The annual catch of the Japan Sea stock in Pacific cod (*Gadus macrocephalus*) decreased greatly in the 1990s. To increase the Pacific cod stock, Notojima station, NCSE, FRA started stock-enhancement program in 1985. In recent years, we achieved production of 500 thousand juveniles a year and survival rates between 20-40%. Improved factors of mass seed production were the utilization of Obama L-type rotifer strain (*Brachionus plicatilis*) acclimated to low temperature environment (8-10°C) and the larval acclimation technique to artificial diets.

Our further objectives are improvement in growth, survival, and seed quality of reared cod juveniles. The present study intends to develop larviculture techniques for mass production of healthy juveniles.

Materials and methods

The larvae were reared in a 50-m^3 land-based tank from day 0 to day 42, and were fed with enriched rotifers and *Artemia* nauplii. Two rearing experiments were conducted from day 42 to day 80 to examine effect of extensive method: comparison of larviculture methods (Exp. 1) and comparison of larval stocking densities (Exp. 2). Experimental design is shown in Figure 1.

The starvation resistance and the morphological normality of reared juveniles, which are prime indices of seed quality, were compared between two reared groups of cod larvae and juveniles. The experimental group was fed with only light-allured zooplankton in the sea net-cage (sea net-cage group by extensive method) and the comparative group was fed with enriched *Artemia* nauplii and artificial diets in the land-based tank (land-based tank group by intensive

method). The starvation resistance was evaluated according to tolerance test without food supply at every 10 days (days 50, 60, 70, and 80). Cod juveniles of each group at the size of approximately 30 mm in total length were sampled to compare the morphological normality examined with soft X-rays.

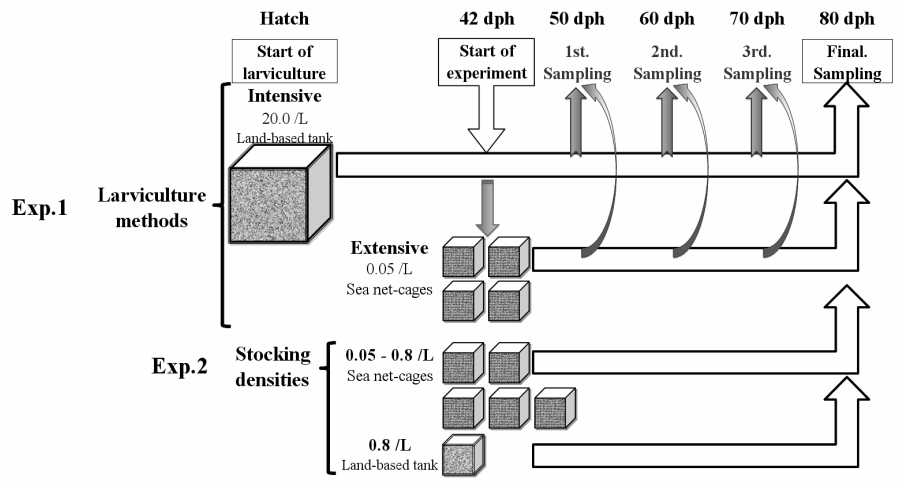

Fig. 1. Experimental design.

Results and discussion

Food organisms attracted to the net-cage by means of night lighting were chiefly composed of Cladocera (*Evadone nordmanni*, *Podon leuckarti*, etc.) and Copepoda (*Paracalanus parvus*, *Centropages abdominalis*, *Oitina similis*, etc.), which are the main food items of Pacific cod (Arai et al., 2006). The estimated daily amount of these zooplankton were ranged from 0.5 to 5.7 million ind.night^{-1} for the cage.

Table I. Statistical comparison of seed quality between two reared groups of Pacific cod. (TL ; total length, CF ; condition factor (coefficient of fatness), SR ; starvation resistance).

Land-based tank	Sea net-cage											
	50dph (10.2)			60dph (51.0)			70dph (59.7)			80dph (231.4)		
	TL	*CF*	*SR*	*TL*	*CF*	*SR*	*TL*	*CF*	*SR*	*TL*	*CF*	*SR*
50 dph (1.7)	S**	ns	S*	-	-	-	-	-	-	-	-	-
60 dph (6.9)	L***	L***	ns	S***	ns	S***	-	-	-	-	-	-
70 dph (18.5)	L***	L***	ns	L***	ns	S***	S***	ns	S***	-	-	-
80 dph (39.9)	L***	L***	L***	L***	ns	ns	ns	ns	ns	S***	ns	S***

Figures in parentheses show the values of SAI (survival activity index). Survival activity index was calculated according to Shinma and Tsujigado (1981). Starvation resistance was evaluated according to tolerance test without food supply (Mushiake et al., 1993). ns: not significant; S: sea net-cage > land-based tank; L: land-based tank > sea net-cage (Mann-Whitney's U test); *P< 0.05, **P< 0.01, ***P< 0.001. - : not compared.

In Exp.1, the condition factor of sampled juveniles in two groups at the same days post-hatch showed no significant difference, but sea net-cage group had significantly higher growth and higher starvation resistance than land-based tank group (Table I). Furthermore, small-sized cod of sea net-cage group were equal or superior to large-sized cod of land-based tank group in starvation resistance. Comparing the abnormality of vertebrae between the same-sized reared cod of two groups, sea net-cage group that had been fed with only wild zooplankton had a lower incidence of vertebral deformity ($p<0.005$).

In Exp.2, the group of higher stocking density per cage had a tendency of lower growth and lower survival activity index, but they were clearly superior to land-based tank group at the same stocking density (Table II).

Table II. Results of the experiment to examine the effect of different stocking densities in Pacific cod TL ; total length, CF ; condition factor (coefficient of fatness), SR ; starvation resistance, SAI ; survival activity index

Group	Stocking density	*TL**[1] (mm)	*CF**[1]	Survival rate*[2] (%)	*SR**[3]	*SAI*
20-m^3 sea net-cage (non-feeding, utilization of light-allured zooplankton)	1000 ind.$cage^{-1}$	40.1±3.4^{a}	6.8±0.6^{ab}	41.9	S***	291.8
	2500 ind.$cage^{-1}$	39.6±3.5^{a}	6.9±0.6^{a}	40.5	S***	251.7
	5500 ind.$cage^{-1}$	38.3±3.4^{ab}	6.9±0.5^{a}	31.3	S***	213.2
	11 000 ind.$cage^{-1}$	37.2±3.8^{b}	6.4±1.3^{b}	44.2	S***	171.4
	16 000 ind.$cage^{-1}$	34.0±4.1^{c}	6.5±0.5^{b}	53.8	ns	139.9
20-m^3 land-based tank (satiation feeding)	16 000 ind.$tank^{-1}$	26.5±2.4^{d}	7.2±0.8^{a}	48.8	-	75.9

Survival activity index was calculated according to Shinma and Tsujigado (1981). *1 Mean ± S.D. (n= 56-112); *2 Survival from 42 dph to 80 dph; *3 Statistical comparison of starvation resistance between sea net-cage and land-based tank. a,b,c,d Values within a column with different superscript letters are significantly different at $P< 0.05$. (Scheffe's F, a>b>c>d). ns: not significant; S: sea net-cage > land-based tank; ***: $P< 0.001$ (Mann-Whitney's U test).

It is inferred that the superiority of sea net-cage group was caused by the intake of highly unsaturated fatty acids and amino acids (e.g., taurine) from wild zooplankton. According to the results that land-based tank group had a higher incidence of vertebral deformity, it seems that there are inadequacies of nutritional and/or environmental conditions in the present intensive method.

Conclusions

The results from the present study suggest that cod larviculture utilizing wild zooplankton in the sea net-cage has a great possibility of providing better seed quality. In terms of detailed comparison between intensive and such an extensive rearing, more nutritional and behavioral study is required.

According to the previous reports (Ohno, 1992; Morioka, 2002), extensive larviculture can achieve higher growth performance and superior survival. The re-

sults of this study not only are supported by the previous knowledge but also newly reveal that extensive method helps increase of the starvation resistance and decrease of the deformity examined in reared juveniles. However, in order to attain high efficiency of seed production by high density of reared juveniles, larviculture method with supplementary utilization of artificial diets, namely, semi-extensive method needs to be evaluated in the mass production scale in future.

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RESEARCH ON THE LARVICULTURE OF TIGER SHRIMP *PENAEUS MONODON* WITH HIGH STOCKING DENSITIES USING RECIRCULATING BIOFILTER SYSTEM

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Introduction

In backyard shrimp hatcheries in Vietnam, recirculating aquaculture/biofilter systems (RAS or RBS) have been used in recent years with different levels of installation and technology. However, this practice is mainly based on experience and a lot of improvement could be made. This research aimed to find out the best RBS in terms of functional structure, highest stocking density, and proper initial level of rearing water for shrimp larviculture.

Materials and methods

Four experiments rearing from nauplii (N) or postlarva 1 (PL1) to PL15 were performed in Can Tho University. Experiment I consisted of three rearing systems (treatments) with the same stocking density of 120 PL1.l^{-1}: Popular RBS = only biofilter, Upgraded RBS = biofilter and protein skimmer and Improved RBS = biofilter, protein skimmer and ozonation (ozone injected into the protein skimmer with residual ozone concentration maintained at 0.1mg l^{-1}). Experiment II comprised 4 treatments on the density of stocked PL (210, 270, 330, and 390 PL1.l^{-1}). Experiment III was made up of 6 treatments on the density of stocked N (150, 300, 450, 600, 750, and 900 PL1.l^{-1}). Experiment IV was designed as a factorial experiment with two factors: Stocking N density at 3 levels (150, 300, and 450N.l^{-1}) and Initial water level at 2 levels (20 and 50% of maximum water volume). Thus, the last experiment composed of 6 treatments namely (stocking N density × initial water level): 150×20, 150×50, 300×20, 300×50, 450×20, and 450×50). The last 3 experiments were carried out in the improved RBS. Duration of the first 2 and last 2 experiments lasted 15 (from PL1 to PL15) and 21 days (from N to PL15), respectively. Water recirculation was initiated at the beginning (PL1 stage) in the first 2 experiments and at mysis II stage in the last 2 experiments.

Shrimp nauplii were obtained from the wild brooders and PL1 were collected from an experimental hatchery using a popular RBS. All experiments were stocked N or PL1 from the same female brooder. They were disinfected by 25mg.l^{-1} formalin in 15min prior to stocking. Rearing water of 30g.l^{-1} was prepared by mixing 100g.l^{-1} brine and tap water and then disinfected by chlorination (30mg l^{-1} in 48h). The full structure of a rearing system for one treatment included 3 rearing cylindrical 100-l tanks (i.e.; 3 replicates) connected to a central biofiler (200-l tank) and a protein skimmer (20cm diameter and 100cm height). All tanks were made of fibreglass. Feeding (using live *Chaetoceros calcitrans*, *Artemia* cysts from Vinh Chau, Vietnam and micro-encapsulated feeds from INVE Group, Belgium) and daily management followed the common-used protocol for backyard shrimp hatcheries in Vietnam (Thanh et al., 2006).

Environmental parameters (temperature, pH, TAN, nitrite, and nitrate) were recorded periodically. Criteria or their combination for treatment evaluation at the end of experiments (at PL15 stage) included survival rate and postlarval density at the end of experiments, total length, individual dry weight, muscle over gut index, stress mortality (% of dead PL15 after 60min exposed in 200mg.l^{-1} formalin) and larval stage index (calculated based on 8 stages: nauplii, zoae 1-3, mysis 1-3 and PL1 are assigned 1 to 8, respectively). In addition, total bacterial count was also determined. The results of experiment III and IV are evaluated based on the sum of scores of all evaluating criteria. The value of the same criterion of every treatment is scored based on the result of statistical treatment. Significantly better values are assigned higher scores (modified from Wickins and Lee, 2002). Using Statistica 6.0 software, Tukey HSD test is applied to compare means of evaluating criteria between treatments and factorial analysis is also used in experiment IV for detecting any possible interaction between factors.

Results and discussions

All environmental parameters were suitable for shrimp. In experiment I, among all evaluating criteria, only two were significantly different ($p< 0.05$), i.e. total length (highest in Improved RBS, 12.1±0.5mm) and total bacterial count (highest in Upgraded RBS, 21.67±5.51×10^3CFU.ml^{-1}). In combination, the improved RBS can be considered as the best rearing system. In experiment II, most evaluating criteria are similar, except the density of PL15 is significantly higher ($p< 0.05$) in treatment 330 and 390 PL1.l^{-1} (253±26 and 261±12 PL15.l^{-1}, respectively) compared to the remaining treatments. Therefore, it is possible to stock up to 390 PL1.l^{-1} in the improved RBS. In experiment III, most evaluating criteria are significantly different, except the muscle:gut ratio and stress mortality. The total score of treatment 450N.l^{-1} is highest (= 26, Table I). In experiment IV, most evaluating criteria are significantly different, except the muscle:gut ratio and total bacterial count. The total score of treatment 300×20 is highest and the next is that of treatment 450×20 (= 19.5 and 18, respectively; Table II).

Table I. Evaluation criteria for PL15 at the end of experiment III

Evaluation criterion	Treatment (Stocking density of N l^{-1})					
	150	**300**	**450**	**600**	**750**	**900**
Survival rate*	53.9±	60.6±	55.9±	35.9±	33.8±	21.5±
(%)	0.7^{d} [4]	0.3^{f} [6]	0.7^{e} [5]	0.4^{c} [3]	0.4^{b} [2]	0.4^{a} [1]
PL density*	81±	182±	252±	215±	254±	194±
(ind l^{-1})	1^{a} [1]	1^{b} [2]	1^{e} [5]	2^{d} [4]	3^{e} [5]	3^{c} [3]
Total length	9.9±	9.6±	9.8±	9.3±	9.3±	9.2±
(mm)	0.1^{c} [4]	0.2^{bc} [3]	0.1^{c} [4]	0.1^{ab} [2]	0.1^{ab} [2]	0.1^{a} [1]
Dry weight	0.6±	0.5±	0.6±	0.6±	0.5±	0.5±
(mg ind^{-1})	0.0^{c} [4]	0.0^{ab} [2]	0.0^{bc} [3]	0.0^{abc} [2.5]	0.06^{a} [1]	0.0^{a} [1]
Muscle:gut	5.3±	6.3±	6.7±	6.0±	6.0±	5.7±
ratio	0.58^{a} [1]	0.58^{a} [1]	0.6^{a} [1]	1.0^{a} [1]	0.0^{a} [1]	0.6^{a} [1]
Stress	10.0±	16.7±	13.3±	23.3±	23.3±	26.7±
mortality (%)	0.0^{a} [1]	11.5^{a} [1]	15.3^{a} [1]	5.8^{a} [1]	5.8^{a} [1]	5.8^{a} [1]
Larval stage	7.9±	7.9±	7.8±	7.6±	7.3±	7.0±
index	0.1^{d} [5]	0.1^{d} [5]	0.1^{cd} [4]	0.2^{bc} [3]	0.1^{b} [2]	0.1^{a} [1]
Total bacterial count	1.91±	0.24±	0.43±	1.49±	3.92±	2.61±
(10^3 CFU ml^{-1})	0.32^{bc} [2]	0.22^{a} [4]	0.37^{ab} [3]	0.90^{abc} [2.5]	2.71^{c} [1]	1.46^{bc} [2]
Total score of treatment	[22]	[24]	[26]	[19]	[15]	[11]

* Data of PL1 (due to low survival of PL15). Values (mean ± standard deviation [score]) in the same row followed by the same superscript letter are not statistically different ($p \geq 0.05$, Tukey HSD test).

Table II. Evaluating criteria for PL15 at the end of experiment IV.

Evaluating criterion	Treatment (Stocking density of N l^{-1} × Initial water volume in %)					
	150×20	**300×20**	**450×20**	**150×50**	**300×50**	**450×50**
Survival rate	43.9±	72.8±	66.2±	41.0±	49.6±	47.7±
(%)	1.3^{ab} [2]	1.2^{c} [4]	2.9^{c} [4]	2.5^{a} [1]	3.4^{b} [3]	2.8^{ab} [2]
PL density	66±	218±	298±	62±	149±	215±
(ind l^{-1})	2^{a} [1]	4^{c} [3]	13^{d} [4]	4^{a} [1]	10^{b} [2]	13^{c} [3]
Total length	9.9±	9.6±	9.8±	10.1±	9.6±	9.4±
(mm)	0.1^{ab} [2]	0.2^{ab} [2]	0.1^{ab} [2]	0.2^{b} [3]	0.1^{ab} [2]	0.4^{a} [1]
Dry weight	0.5±	0.6±	0.5±	0.7±	0.6±	0.6±
(mg ind^{-1})	0.1^{a} [1]	0.0^{ab} [2]	0.0^{a} [1]	0.0^{c} [4]	0.0^{bc} [3]	0.0^{ab} [2]
Muscle:gut	5.3±	6.3±	6.67±	6.0±	6.0±	5.7±
ratio	0.6^{a} [1]	0.6^{a} [1]	0.58^{a} [1]	1.0^{a} [1]	0.0^{a} [1]	0.6^{a} [1]
Stress	6.7±	16.7±	23.3±	10.0±	16.7±	20.0±
mortality (%)	5.8^{a} [4]	5.8^{abc} [2.5]	5.8^{c} [1]	0.0^{ab} [3]	5.8^{abc} [2.5]	0.0^{bc} [2]
Larval stage	7.2±	7.6±	7.55±	6.7±	7.1±	7.1±
index	0.1^{bc} [3]	0.0^{c} [4]	0.07^{c} [4]	0.3^{a} [1]	0.1^{b} [2]	0.1^{b} [2]
Total bacterial count	38.54±	37.86±	43.68±	32.78±	18.20±	17.95±
(10^3 CFU ml^{-1})	13.57^{a} [1]	11.08^{a} [1]	16.93^{a} [1]	1.80^{a} [1]	8.45^{a} [1]	10.81^{a} [1]
Total score of treatment	[15]	[19.5]	[18]	[15]	[16.5]	[14]

Values (mean ± standard deviation [score]) in the same row followed by the same superscript letter are not statistically different ($p \geq 0.05$, Tukey HSD test).

There was also significant ($p< 0.05$ and 0.01) interaction between two factors on the most evaluating criteria. Figure I shows a typical interaction of density of PL15 that positively and negatively relates to the stocking density and initial water level, respectively. Meanwhile, popular stocking densities in backyard hatch-

eries range from 100 to 200N.l^{-1}. Lower initial water volume resulted in algae that were fresher due to more feeding times.

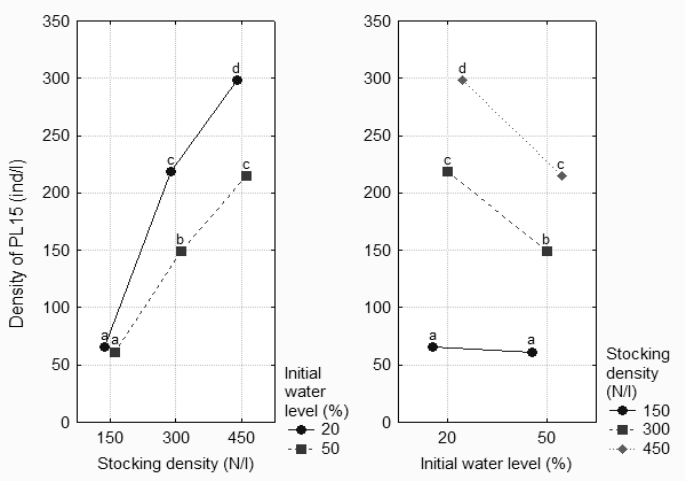

Fig. 1. Interaction between stocking density and initial water level on density of PL15 at the end of experiment IV. Different letters over plots denote significant difference (p< 0.01).

Conclusions

The improved biofilter system (biofilter, protein skimmer, and ozonation) is the best rearing system. The best stocking densities are 390 PL1.l^{-1} and 300-450N.l^{-1} for rearing to PL15 with the best initial over full water volume of 20%.

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PHYTOPLANKTON PRODUCTION IN GREEK MARICULTURE

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Introduction

During 1996-1997, a survey was conducted by the Laboratory for Aquaculture of the Ghent University in order to assess the status of phytoplankton production in the Mediterranean aquaculture farms (Tzovenis, 2001). In an effort to review the evolution of the sector during the last decade, an survey is underway and preliminary results for the Greek mariculture industry are presented in this study.

Materials and methods

Structured questionnaires were distributed to all Greek hatcheries in operation and less than half were returned before this report. Additional information was gathered through interviews of key persons of the sector.

Results and discussion

Phytoplankton production in the Greek mariculture industry is used almost exclusively to support fin-fish larvae rearing either as live-feed at the basis of a simple food-chain via zooplankton feeding (*Brachionus plicatilis*) or as "green water" for conditioning the larvae-rearing tanks. However, in comparison with a decade ago fewer strains are used nowadays namely *Nannochloropsis oculata* (Eustigmatophyceae), *Tetraselmis suecica* (Prasinophyceae), *Isochrysis* aff. *galbana* T-ISO (Prymnesiophyceae), and a Greek marine isolate tentatively identified as *Chlorella* sp. (Chlorophyceae). These strains are either kept in pure lines in the Greek hatcheries or are regularly renewed from Culture Collections abroad.

Strains of either *Nannochloropsis* or *Chlorella,* usually in combination with T-ISO are widely used for "green waters", while some hatcheries still use *Tetraselmis* for keeping rotifer stocks and T-ISO for rotifer enrichment. Few alternative products to fresh phytoplankton for "green water" were tried in Greek hatcheries during the last years with most successful the dried powder of microalgae (Sanolife ALG, INVE Belgium). This product is used in support of the live

production with a yearly turnover of 250-300kg (about 85 000€). Another product (Chroma, BernAqua, Belgium) which is an inert dye is scarcely used, normally in combination with T-ISO, to imitate the "green water" effect during transport or emergency, with an annual turnover of 200-300kg (5000€).

In the last decade a mixotrophic freshwater *C. vulgaris* strain (Chlorophyceae) produced heterotrophically in Japan (Chlorella Industry Co. Japan) has been successfully introduced in hatcheries either as alternative or as backup to the ordinary rotifer production protocol. As a consequence, less than 20% of the rotifers now produced are entirely reared on fresh phytoplankton. The rest is reared on the hyper-concentrated slurry of *Chlorella* imported from Japan in refrigerated 20-l packs, with an estimated annual turnover of about 7 tons DW or 1M€.

The mainstream production method is still the successive batch culture scale-up to grid-supported 180-300-l bags. Artificial illumination indoors is the norm for lower volumes with several hatcheries deploying the final volumes in secluded green houses outdoors. Water treatment of large volumes is normally done by combining mechanical filtering and UV while small volumes may even be autoclaved in some hatcheries. Final culture stage lasts about 4-6 days and the overall performance of the protocols is in gross terms reliable when executed dutifully. Alternative production methods, such as using raceway ponds outdoors or sophisticated photobioreactors (PBR), have failed so far to become industry's standard due to various reasons. Two hatcheries use a self-made bubble column PBR system, another two an Italian PBR system while two tubular PBR systems (BioFence, UK) exist but not in regular use. A tubular prototype in a farm near Athens is working properly at pilot-scale outdoors but has not yet been utilised in a hatchery.

During 2008 the twenty-two hatcheries operating in Greece (20 private-commercial owned by 12 different companies, 1 academic-commercial, 1 public-commercial) have produced about 420 million fry (214 million sea bream *Sparus aurata* and 195 million sea bass *Dicentrarchus labrax*; FEAP) selling at about 0.22 € including transportation. A preliminary estimate of the phytoplankton used for rearing the fish fry is between 4 to 9mg.dw^{-1} fry for "green water" and 2 to 7mg.fry^{-1} for rotifer rearing (or 11 to 19mg.fry^{-1} when reared on concentrated slurry) or a total between 6 to 25mg.fry^{-1} a value higher than that of ten years ago (12.8±7.42mg.fry^{-1}). The observed slight increase in phytoplankton demand reflects the wide acceptance of the "green water" technique for both sea bream and sea bass (40% less in sea bass according to most protocols) along with the improvement of phytoplankton production management. The large range of values found, results from the wide variety of protocols used for larval rearing, the different requirements between sea bass and sea bream, and the different characteristics of the phytoplankton strains employed by the different hatcheries.

In general the phytoplankton production in the Greek hatcheries is small and of intermediate final density (normally 2-6×10^7cells.ml^{-1} for *Nannochloropsis* sp., and 1×10^7cells.ml^{-1} for T-ISO) affecting the cost of production, which normally ranges between 150 and 500€.kg^{-1} dry weight (range 150-1000€.kg^{-1} dw). Although these values are high, in fact they are much better than those found by previous studies for the Mediterranean finfish aquaculture (1400$.$kg^{-1}$, Muller-Feuga et al., 2003) or those estimated a decade ago in the Greek mariculture (1332±900€.kg^{-1}). The improved production cost reflects the production scale-up and the better management resulting in higher densities, cost compression and minimised losses. Nevertheless, the high cost does not impair the overall production-cost per fry as it represents a rather low fraction of it (2-5%) not different from a decade ago although, it includes now the production cost for both live food and "green water" technique.

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DEVELOPMENT OF VISUALIZATION TOOLS TO IDENTIFY FOR MUSCLE AND NERVE ABNORMALITIES IN FISH

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Introduction

The incidence of malformed fish is an important problem in seed production of cultured fish species. Most of the studies conducted to date have been restricted to investigations of abnormal appearance or bone formation of adult fish with developed bones. However, here we report a method for visualizing muscle and nerve tissue in the early stages of fish development (fish embryos to juveniles) using a modification of conventional whole mount immunohistochemical staining methods. This method allows for the early detection of abnormalities in fish. Here we show how the technique can be applied to identify abnormalities in the Japanese eel, *Anguilla japonica*.

Materials and methods

Fertilized eggs of *A. japonica* were obtained as reported previously (Kurokawa et al., 2008). The fertilized eggs were incubated in nets immersed in aerated filtered seawater at 22°C in a flow-through system at the Fisheries Research Agency (FRA) in Mie, Japan. We collected six different batches of Japanese eel larva (one batch at 5 days post hatching (dph), three batches at 7dph, and two batches at 10dph). Following anesthesia with 3-aminobenzoic acid ethyl ester (Sigma-Aldrich Co.), the larvae were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) containing 0.3% tween 20 (PBST), rinsed in PBST, dehydrated in a graded series of methanol and stored at –20˚C. Thirty larvae from each batch are used for whole mount immunostaining. Briefly, after storage, larvae were rinsed in PBST and were digested with 0.05% trypsin in a saturated solution of sodium tetraborate for 20 minutes at room temperature. Specimens were then refixed with 4% PFA, washed in PBST, and submerged in 1% KOH supplemented with 3% hydrogen peroxide to remove melanin before incubation in Blocking Ace (Dainippon Sumitomo Pharma Co., Ltd.) for two hours at room temperature. Two antibodies were used for immunohistochemical

staining: anti-myosin antibody A4.1025 (1:200, Upstate) for identifying muscle myofibers, and anti-acetylated tubulin antibody (1:200, Sigma-Aldrich Co.) for identifying nervous tissue. Before commencing antibody reactions, both antibodies were labeled with Alexa Fluor 488 and 555 respectively using a Zenon Labeling Kit (Invitrogen). Following rinsing in PBST, specimens were incubated overnight at 4°C in a solution containing both labeled primary antibodies. After staining, samples were cleared in glycerol and observed using an Eclipse 90i (Nikon) microscope. The confocal z-stacks of the image obtained from whole mount-stained larvae were collected using the ECLIPSE C1si laser scanning confocal microscope system (Nikon).

Results and discussion

We observed a high incidence of disorders and partial fusion of muscle mass in the trunks of Japanese eel larvae (Fig. 1). In addition, abnormal muscle development patterns were often associated with abnormal nervous development in the trunk (Fig. 1). To our knowledge, this is the first report to investigate abnormal muscle and nerve development in Japanese eel larvae. The incidence of abnormal muscle was observed to differ between batches (Fig. 2), with the highest and lowest rates being 81% and 4%, respectively. The incidence of malformed fish is a significant problem for the aquaculture industry because malformations are often associated with depressed growth and high mortality rates (Andrades et al., 1996). Although oxygen depletion is considered to cause muscle abnormalities such as those observed in *Pagrus major* (Sawada et al., 2006), the influence of other environmental factors such as mechanical or thermal shock and salinity on muscle and nerve abnormalities need to be investigated. In addition, we also need to investigate the influence of egg quality on these abnormalities. Conversely, it may be possible to assess the impact of environmental factors, such as mechanical or thermal shock, fluctuations in salinity, and oxygen depletion, as well as that of egg quality on normal muscle and nerve development in fish using these visualization tools.

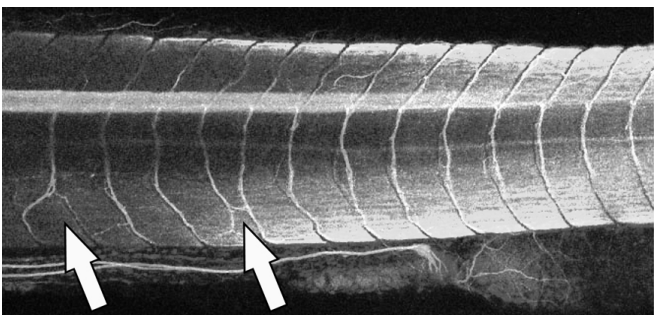

Fig. 1. The abnormal muscle and nerves pattern in the trunk of Japanese eel larva. Arrows indicate the abnormal muscles and nerves.

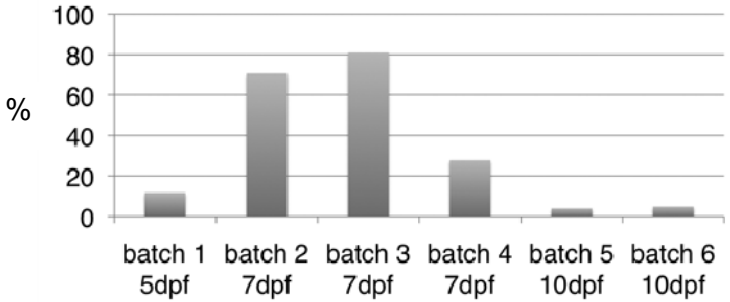

Fig. 2. The different incidence of abnormal muscle pattern (y-axis, %) in 6 different batches.

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ROBSONELLA FONTANIANA LARVICULTURE: ONTOGENIC CHANGES OF THE MORPHOLOGY AND DIGESTIVE ENZYMES

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R. fontaniana females with eggs were collected at Hueihe (41°52´S; 73°51´W), south west Patagonia, Chile and transported to the Marine Invertebrate Hatchery Laboratory of the Universidad Austral de Chile (HIM-UACH). Once in the laboratory, the animals and stones with eggs were placed individually in aerated sea water tanks kept at 11°C and 30‰ salinity and connected to a sea water recirculation system. During embryonic development, the spawn was incubated by the females and, upon hatching, the paralarvae were transferred to glass recipients and fed live king crab (*Lithodes santolla*) zoeae. At the beginning of the benthic stage, the juvenile octopuses were fed a mix of king crab zoeae and live wild juveniles of the crab *Petrolisthes laevigata*; later octopus were fed only juvenile crabs.

The same clutch was used to determine morphometric relationships: egg length (EL), egg weight (EW), embryo total length (ETL), mantle length (ML), arm length (AL), and eye diameter (ED) during the embryonic period until hatching. Samples of 30 eggs were taken from seven different clutches collected during the year to evaluate the growth rates of the eggs and relate the change in perivitelline liquid protein concentrations to the egg size and age. Paralarvae and settlements were measured during the paralarval and juvenile phase until 160 days after hatching (DAH). The total length (TL), mantle length (ML), arm length (AL), and eye diameter (ED) were measured.

To establish the maturation of the digestive system we measure the digestive enzyme activity in paralarvae after hatching. Different groups of newly hatched paralarvae were fed with *Artemia* sp. (FA), *L. santolla* zoea (FL), or were starved to serve as negative control (ST); the experimental period lasted 27DAH. A semi quantitative commercial assay was used to identify the pool of digestive enzymes present in paralarvae prior to selecting the specific enzymes acid phos-

phatase, total protease, trypsin and chymotrypsin for analysis. Larval protein content was also measured during the experiment.

The *R. fontaniana* eggs measured 3.13-3.77mm in length in the clutch used for the descriptions and 2.4-4.7mm (3.5 and 6.8% of adult ML, respectively) in the seven clutches used for gravimetric characterization. The entire embryonic development took 71-76 days, until the paralarvae hatched, the hatching period lasted an average of 4-5 days at 12ºC.

During development, the embryos rotated twice in the eggs. The cephalic organs were visible after day 22 and the eyes were well defined after day 26. The yolk decreased exponentially during embryonic development, with only 10% of the initial yolk remaining a few days prior to hatching. The protein content in the perivitelline liquid declined exponentially from 0.19 to 0.04 mg protein μL^{-1} with increased egg size and embryonic age. During posthatching stages, the morphometric measurements varied exponentially over time and did not show different tendencies before and after settlement. Mantle growth in relation to total length fit a logarithmic regression, whereas arm length and eye diameter increased linearly with respect to total length throughout the entire paralarval and juvenile periods.

Two critical moments were observed: one during the planktonic phase (9-16DAH) and another during the benthic phase (90-100DAH). In those critical moments a reduction in survival of 83 to 33% and 14 to 5%, was observed, respectively. The daily growth coefficient for *R. fontaniana*, was $4.4\%.day^{-1}$ and $3.2\%.day^{-1}$ for planktonic and benthic phases, respectively.

Total protease and acid phosphatase activity was not related to either time of development or diet ($p<0.05$). However, the activity of trypsin and chymotrypsin after 10DAH, depended on diet ($p<0.05$). Paralarvae fed with *L. santolla* zoea (FL), performed better than paralarvae in the other two groups, exhibiting significantly ($p<0.05$) higher protein content and the highest proteolytic activity for trypsin and chymotrypsin.

The present study shows that this octopus can be reared from hatching through the final paralarval stage on a diet of *L. santolla* zoeae; showing the highest proteolytic activity with this food item. After settlement, the juveniles can be reared on a crab diet obtaining baby octopus of 1.8g in 160 days. Therefore, this species could be a candidate for aquaculture diversification in cold environments.

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DIET RELEVANCE DURING REPRODUCTIVE CONDITIONING ON THE QUALITY OF THE PROGENY OF THE CHILEAN SCALLOP *ARGOPECTEN PURPURATUS*

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Introduction

It is possible to obtain mature gonads and subsequent successful spawn within four to six weeks in the Chilean scallop *Argopecten purpuratus*, if broodstock are maintained at 18°C and 15°C, respectively.

Diets (microalgae) with very low protein content (> 0.29 times over protein found in normal nitrogen conditions) used for broodstock conditioning do not allow adequate gonadal development and spawning. However, the use of diets with high protein content (> 1.3 times) used to feed broodstock has been observed to improve fecundity (Farías and Uriarte, 2001).

A. purpuratus have eggs with slight vitelum and trochophore larvae show an increment of its protein during its development may due to the incorporation of amino acid from the environment before the digestive tube aperture (Farías et al., 1998). The diet of broodstock during conditioning affects growth and survival rates until metamorphosis (Uriarte et al., 2004). The time period that larvae of *A. purpuratus* could survive without food is unknown. Our work proposes that the variation of high-unsaturated fatty acids (HUFA) affects the progenies' success in terms of eggs quality and the contribution of endogenous food during the period before incorporation of exogenous food.

Material and methods

Adult *A. purpuratus* were obtained from the harvest area of Ilque and transported to the Hatchery of Marine Invertebrates, Universidad Austral de Chile´s dependences (HIM-UACH). At the beginning of the experiment gonads, were evaluated as state 1 of development. Groups of 10 scallops were placed individually in 150-l tanks in a closed system with 1/3 recharge of water every day.

Scallops were initially weighed and tagged prior to the experiment. Conditioning of broodstocks was done at 16ºC according to Uriarte et al. (2004) and fed with 3 monoalgal diets: 1) *Chaetoceros neogracile* high protein (GH) characterized by high eicosapentaenoic acid content (EPA), 2) *Isochrysis* aff. *galbana* (T-Iso) high protein (IH) with high docosahexanoic acid (DHA), and 3) *Dunaliella tertiolecta* normal protein level (DN) without HUFAs. Microalgal diets were made based on protocols described by Uriarte and Farías (2001). The experiment was conducted at different seasons to determine any possible interaction of this factor and diets on the conditioning season. At the end of the experiment, when gonads reached state 4 and/or 5, scallops were induced to spawn. After release of gametes, we quantified the number of eggs per individual, egg size, proximal composition, and fatty acid composition. Eggs were hatched and then cultured at 18ºC until D-shaped larvae. Before being feed, larvae of all groups and replicates were sampled according to its conditioning origin and divided into two groups (A and B). The larvae in the former group (A) were not fed and their non return point was evaluated, while larvae of the other group (B) were fed with IH. Proximal composition and fatty acid composition determination followed Farías et al. (1998) and Farías et al. (2003), respectively. From fatty acid composition of eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) were statistically compared with essential fatty acid value for the larval development of scallops.

Results and discussion

Scallops were conditioned over winter with 3 diets: IH, GH, and DN reached reproductive maturity when their gonad developed to stage 3, but even a single individual was able to spawn. In broodstock maintained during spring, all showed mature gonads and those fed with GH and DN responded to spawning induction. During this period, scallops attained 518.4 (SE=0.1) accumulative thermal degree (ATD) before spawn without difference between diets. In summer ATD for broodstock was 511.1 (±0.2) and scallops of all diets responded positively to spawning induction without significant difference among them.

The isolated effect of diet did not affect proximal composition of scallop gonads. However, a significant interaction between diet type and season of the year was observed to affect gonad's soluble protein content (Table I). This was attributed to high protein value recorded in winter with individuals fed with IH, while in spring and summer high values were observed with DN, and GH and DN, respectively. In summer the highest soluble protein content was recorded. For scallop conditioned during spring and fed with IH, there was no spawning and their gonads showed a high lipid and carbohydrate content, with minor protein content. These suggest that the high soluble protein content in gonads might indicate an optimal gonadal condition for subsequent spawning success.

Table I. Proximal composition and essential fatty acids in gonads of *A. purpuratus* at the end of conditioning periods at three seasons. Percentage was estimated on dry matter. Values correspond to mean and standard error of 2-4 replicates.

Period	Diet	Soluble protein (%)	Total carbohydrates (%)	Total Lipid (%)	EPA (%)	DHA (%)
Winter	GH	26.9±0.6 [a]	4.1±0.5	14.08±1.39	0.60±0.20	0.48±0.04
	DN	28.8±1.3 [ab]	4.5±0.3	11.75±4.00	0.21±0.09	0.43±0.14
	T-Iso (IH)	31.0±3.8 [bc]	5.3±0.8	14.47±2.22	0.25±0.03	0.50±0.22
Spring	GH	28.8±2.1 [ab]	6.2±0.7	19.77±0.28	2.01±0.00	0.70±0.02
	DN	32.1±2.7 [bc]	6.4±1.6	13.80±1.91	0.34±0.00	0.44±0.06
	IH	27.3±2.8 [ab]	7.3±1.6	28.23±1.17	1.15±0.00	1.96±0.00
Summer	GH	39.0±2.0 [c]	6.4±0.9	15.03±0.35	0.35±0.00	0.26±0.12
	DN	34.6±1.7 [c]	5.1±0.3	18.28±1.17	0.44±0.03	0.00±0.00
	IH	32.9±2.2 [bc]	5.9±1.0	18.52±0.42	0.66±0.00	0.68±0.14

Superscript letters indicates significant differences within column.

After comparison among mature gonads at each season and diets, correlation analysis showed: 1) the number of eggs/individual increased with high DHA content in the diet; 2) the percentage of able individuals for spawning was correlated with high lipid content in the diet; and 3) the low percentage of spawned individuals was correlated with an increase in dietary EPA content.

Eggs collected from all diets during the summer conditioning period varied significantly in protein and carbohydrate composition (Table II). The correlation analysis showed: 1) diet's EPA content was positively correlated with egg EPA and negatively with protein, carbohydrate, and lipid content of eggs; and 2) egg DHA content presented a positive correlation with DHA on broodstock diet.

Table II. Proximal composition and essential fatty acids in eggs from summer conditioning experiment of *A. purpuratus*. Percentage was estimated from egg's dry matter. Values correspond to mean and standard error of 2-4 replicates.

Broodstock origin	Soluble protein (%)	Total carbohydrates (%)	Total lipid (%)	EPA (%)	DHA (%)
GH	24.0±1.4 a	1.8±0.2 a	14.25±0.80	1.44±0.00	0.59±0.20
DN	33.4±3.6 c	3.0±0.1 b	17.53±0.33	1.11±0.15	0.68±0.12
IH	31.3±1.2 b	2.7±0.2 b	15.24±1.28	1.11±0.11	1.06±0.08

Non return point experiments with larvae of group A showed an exponential decrease in the survival until reaching zero at the end of the first four days of initiation without difference among conditioning diets. Therefore, vitelum quality may not affect survival during the period of final endogenous reserves. High survival rates were observed in larvae of group B, especially those fed with IH, thus the effect of endogenous reserves may have a synergic contribution in addition to the reserves consumed by larvae during their first days.

Just from summer experiment it was possible to obtain eggs and larvae from all treatments used to condition scallops. Broodstock population, methodologies, and diets at each season were the same, which suggests that during winter and spring other variables might play an important role in the broodstock conditioning response. In addition, the use of monodiets as food may also influence the success of conditioning.

Acknowledgements

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INFLUENCE OF LARVAL CONDITIONING ON THE METABOLIC PROCESS OF EUROPEAN SEA BASS JUVENILES

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Worldwide supplies of fish oils and meals have reached their sustainable limits, forcing industries to look for alternative lipid sources for use in marine fish diets. As terrestrial animal products are prohibited, there is great interest in aquaculture to produce fish better able to utilise vegetable feedstuffs. Vegetable products are rich in 18 carbon fatty acids (C18 FA) but do not contain C20-22 n-3 highly unsaturated fatty acids (n-3 HUFA), such as ecosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acids (DHA; 22:6n-3). The aim of this study was to know if it would be possible to apply a metabolic programming using nutritional conditioning during early larval stages, as already described in mammals, in order to stimulate the FA desaturation pathways of n-3 HUFA synthesis in marine fish.

For the first experience (E1), four replicated groups of larvae were reared at 16 or 22°C and fed microparticulated diets from mouth opening (day 6 post-hatching d-6) to d-45. Two diets with a low (LH) or high (HH) HUFA content were tested: 0.8 and 2.2% EPA+DHA on a dry matter basis. The larval period was followed by an intermediate period of three months during which the four groups were separately held at 19°C and fed on a commercial diet with 2.7% EPA+DHA. Following this period, the capacity of juveniles to adapt to a HUFA-deprived diet (0.5% EPA+DHA) was tested during 60 days (19°C). For the second experience (E2), four replicated groups of sea bass larvae were fed XH (3.7% EPA+DHA), HH (1.7%), LH (0.7%) or XLH (0.5%) diets from d-6 to d-45. After a subsequent one-month period feeding a commercial diet (2.7% EPA+DHA), the capacity of the four initial groups to adapt to an n-3 HUFA-Restricted diet (0.3% EPA+DHA) was tested for 35 days.

Results obtained at the end of the two larval periods indicated a significantly higher Δ-6 desaturase mRNA level (Δ6D, rate-limiting enzyme responsible of the first step of the fatty acid desaturation pathways for the n-3 HUFA synthesis) in larvae fed a low dietary HUFA n-3 content (0.8, 0.7, and 0.5% EPA+DHA) than in others. This indicated a positive modulation of the Δ6D gene transcrip-

tion by low dietary n-3 HUFA levels. However, this stimulation of desaturation pathways did not allow an enough HUFA production in larvae, as a deficiency in DHA in phospholipids (PL) was observed. A lower growth rate was also observed. No significant effect of larval nutritional conditioning was observed in juveniles, but E1 showed a transient increase in mRNA level, over the intermediate period. It also showed a low but significant increase in DHA content in PL. The second experience E2 showed a persistent mRNA increase in preconditioned fish during the juvenile period, but similar DHA content in PL in all groups.

Our studies clearly showed that it was possible to influence fatty acid desaturation pathways for HUFA synthesis, using a nutritional conditioning during larval stage, even if no noticeable higher HUFA synthesis was measured in preconditioned fish. This work also brought new findings suggesting the involvement of PPARs in the Δ6D mRNA level stimulation. The enzymatic Δ6D assay was performed and gave preliminary results about the existence of post-transcriptional regulation of the Δ6D gene. The originality of results lead to better investigate mechanisms involved in desaturation pathways in response to a low HUFA dietary content, as well as the mechanisms involved in the metabolic programming in fish.

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CONTINUOUS LIGHT VERSUS PHOTOPERIOD: EFFECTS ON EYE SIZE AND DEFORMITIES IN LARVAL AND JUVENILE COD

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Introduction

The larval rearing environment often differs considerably from the natural conditions from which reared organisms have adapted their life strategy, development, and ecology. This may cause developmental constraints when organisms are cultured, particularly during early life history, as the larval stages in a very short time go through large changes in morphology, physiology, and organ development. One environmental parameter that is difficult to match with natural conditions is light, with respect to both quality and intensity. Photoperiod is also often altered in intensive rearing systems, and 24h continuous light is commonly used. This might induce peculiar effects on larval development, as recently reported by Harboe et al. (2009) with disrupted eye migration success in Atlantic halibut (*Hippoglossus hippoglossus*, L.). The present work compares the use of photoperiod versus continuous light during larval and early juvenile rearing of another cold-water aquaculture species: the Atlantic cod (*Gadus morhua*, L.).

Materials and methods

Two experiments were carried out where larval groups of cod were exposed to either continuous light (24h) or a 16h light:8h dark photoperiod cycle. The first experiment was carried out at GreatBay Aquaculture Ltd. (GBA), Portsmouth, NH, USA. Two square, 2.5-m^3 green fibreglass tanks were used, one for each light regime. The tanks were covered by black plastic with a timer-controlled light bulb inside. Light intensity was in the range of 38 and 550lux at the surface. Cod larvae were fed rotifers (*Brachionus* sp.) in three batches per day at approximately 8h intervals. Feeding was done during the light cycle, the last feeding 1h before initiation of darkness. Additional *Artemia* was fed from day 29 posthatch. Green water was applied by one daily addition of 30ml *Nannochloropsis* algal paste. The experiment was ended at day 33 posthatch. Larval samples were collected and incubated for immuno-cytochemical staining of cell division at day 11 and 32 posthatch. After staining, the larvae were preserved in

4% paraformaldehyde with 0.1M phosphate buffer. The visualization of newly divided cells was based on protocols successfully applied for immunocytochemical staining of neurogenesis in various crustaceans (Beltz and Sandeman, 2003), and was based on BromodeoxyUridine (BrdU), that switches for the thymidine nucleotide during DNA duplication in the S-phase of the cell replication cycle. Newly born cells were easily visualized by BrdU incubation of live cod larvae, followed by fixation, a two-step antibody staining process, whole-mount embedding in Gel/Mount, and subsequent inspection by confocal microscopy. Special attention was given to the thin cell layers covering the eye disc, where cell division could be quantified. Cell proliferation rate was determined, together with eye size (average eye diameter and eye disc area calculated from vertical and horizontal eye diameters), standard length (SL), and wet weight (WW). Morphometrics were carried out both on the preserved and embedded larvae (day 11) and the preserved larvae only (day 32).

The second experiment was a repetition of the GBA experiment, but extending until the fish reached 10g for radiological evaluation of spinal deformities. The experiment was carried out in six $0.4m^3$ circular tanks of black polyethylene at Institute of Marine Research (IMR), Austevoll Research Station, Norway. Each light treatment (24h continuous light versus 16L/8D photoperiod) was set up in triplicates, but with one tank from each light treatment switching light regime after weaning at day 69 post-hatch. Standard rearing protocols were used (van der Meeren et al., 2007), with close resemblance to the GBA experiment (*Brachionus* sp. and *Artemia* as live feed, 3 daily batches of feed, and green water made by *Nannochloropsis*). No immunocytochemical techniques were used in this experiment. Cod larvae were sampled at days 11 and 32 post-hatch, preserved in 4% formaldehyde with 0.1M phosphate buffer, and photographed for morphometric measurements. Radiographs of 10g juveniles were taken using a portable X-ray apparatus (HI-Ray 100, Eickenmeyer Medizintechnik für Tierärzte e.K., Tuttlingen, Germany) and 30×40cm film (AGFA D4 DW). The vertebral column of each fish was thoroughly examined (Photoshop version 6.0), and the number of the affected vertebrae and type of deformity recorded.

Results and discussion

The results in 11-day-old larvae from the GBA experiment showed that eye disc cell proliferation rate in the photoperiod group did not show a diurnal pattern, but was significantly lower than in the continuous light group (3547 and 4541 $cells.mm^{-2}$ in the photoperiod and continuous light treatment, respectively). Eye size at day 11 was not different between the treatments (average eye disc area for both treatments was $0.224mm^2$). However, at day 32 the photoperiod-exposed larvae have developed significantly smaller eyes at similar larval size when compared to 24h light-exposed larvae. At this age, the eye diameter to larval standard length ratio was 0.079 and 0.083 in the photoperiod and continuous

light group, respectively. Larval size was also slightly less in the photoperiod treatment, but only significant for SL (9.8 and 10.1mm for photoperiod and continuous light, respectively). The data suggest that instantaneous cell growth measurement is an excellent tool to assess immediate effects of extrinsic factors on growth, effects that normally would need to accumulate over a longer period (and hence would need chronic exposure) to be detected by standard body morphometrics.

Regarding the effect of photoperiod on eye and body morphometrics, similar results were observed in the IMR experiment as in the GBA experiment. The eye diameter to standard length ratio was significantly higher for the continuous light group at days 32 and 134 post-hatch. However, the difference was small but consistent. Furthermore, irrespective of light regime the eye size relative to body length increased to a maximum towards the late larval period, suggesting that eye growth is not isometric with body growth throughout larval development in cod. This may point to the importance of the visual system for successful larval function, feeding, and survival during late larval and early juvenile stages.

In the sampled material at day 134 post-hatch (50 juveniles from each of the two tank that did not change light regime), no clear effects on bone deformities were observed from the X-ray micrographs, although some differences in deformity type were indicated. Average fractions of the two replicate tanks of deformed vertebrae and the bent neck deformity was 15 and 19% respectively for the continuous light treatment, and 19 and 8% respectively for the photoperiod treatment. Lordosis was only observed in the continuous light group (6 and 2% in the two replicate tanks, respectively).

Acknowledgements

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DEVELOPMENT OF ZINC AND MANGANESE ENRICHMENT METHODS IN LIVE FOOD AND NUTRITIONAL IMPORTANCE IN MARINE FISH LARVAE

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Manipulation of live food nutrition is essential for current hatchery protocol. Numerous studies have demonstrated highly unsaturated fatty acids are deficient in rotifers and *Artemia* nauplii but essential for marine fish larvae. However, much less attention has been paid to other nutritional components such as trace elements. Here we develop new methodology to enrich Zn and Mn in rotifers and *Artemia* nauplii to the levels found in natural zooplankton. Since minerals are important for skeletal development of fish, it is expected from these studies that mineral supplementation to the level in natural zooplankton will benefit normal growth and skeletal development of the experimental fish. The results obtained demonstrated that enrichment of rotifer and *Artemia* with zinc and manganese was successfully performed using microalgae. We also found that direct inclusion of Zn in culture medium failed to enrich this mineral in rotifers. Using this enrichment method, we examined the effect of *Artemia* enriched with Zn and Mn on early growth, survival, proximate composition, and the occurrence of skeletal deformity in red sea bream (*Pagrus major*). Fish fed *Artemia* enriched with Zn and Mn showed significantly improved growth performance and reduced occurrence of skeletal deformity. We will discuss possible mechanism of Zn and Mn incorporation in live foods and future perspectives of mineral nutrition studies in marine fish larvae.

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EFFECT OF FEEDING REGIMES ON GROWTH AND SURVIVAL OF CLARIAS GARIEPINUS LARVAE: REPLACEMENT OF *ARTEMIA* BY ARTIFICIAL FOOD AND DETERMINATION OF A FEEDING MODEL

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Introduction

Larvae of African catfish (*Clarias gariepinus*) are generally weaned with *Artemia*. The first aim of the study was to evaluate the possibility of partially or fully replacing live-feed (*Artemia* nauplius) by artificial food. The second objective was to calculate a R_{max} feeding model for larvae from first feeding to a body weight of 1g.

Materials and methods

The larvae (initial weight: 3mg) were reared in aquaria (n = 500 per 50-l aquaria) in a recirculating system at 28°C, with constant aeration (O_2>5ppm) and renewal rate (0.5 $l.min^{-1}$). First experiment was conducted in two phases: during the first 13 days post-first feeding, larvae were fed with or without *Artemia* nauplii and with different feed (6 feeding regimes in duplicate, Table I). Two commercial larval feed were used: Gemma micro®, Skretting (protein content 55%, lipid content 15%), a high quality marine larval feed or Nippai, Lucky Star® (protein content 56%, lipid content 8.3%), a "classic" larval feed. In the second phase, from D13 to D32, weaned larvae were reared at 200 $fish.aquaria^{-1}$ and fed with the same aliment (Nippai) to evaluate the effect of first feeding regimes on growth and survival after the weaning period.

In a second experiment, to calculate a R_{max} feeding model, larvae were reared at different feeding levels (from 5% to 40% of the total biomass) from D1 to D8 (mean weight at D8 = 35mg), from D10 to D18 (mean weight at D18 = 300mg), and from D20 to D28 (mean weight at D28 = 900mg).

In each experiment, specific growth rate, food conversion ratio and survival rate were calculated. Relationships between feeding level and growth were con-

structed to determine R_{max} in the 3 body weight classes, and then the relationship between body weight and R_{max}.

Table I. Feeding regimes for *C. gariepinus* larvae from D1 to D32 post-first feeding.

Feeding days	1	2	3	4	5	6	7	8	9	10	11	12	13	13→32
Regime 1	*Artemia*			co-feeding *Artemia* + "Nippaï"										"Nippaï"
Regime 2	*Artemia*			co-feeding *Artemia* + "Gemma micro"										"Nippaï"
Regime 3	*Artemia*			co-feeding *Artemia* + "Nippaï"			"Nippaï"							"Nippaï"
Regime 4	*Artemia*			co-feeding *Artemia* + "Gemma micro"			"Gemma micro"							"Nippaï"
Regime 5	"Nippaï"													"Nippaï"
Regime 6	"Gemma micro"													"Nippaï"

Results were analysed by one-way ANOVA. Significant ANOVAs were followed by a LSD multiple comparison test to identify differences among treatments. Mortality data were compared with Chi-square (χ^2) test. Level of significance was accepted at $P < 0.05$.

Results and discussion

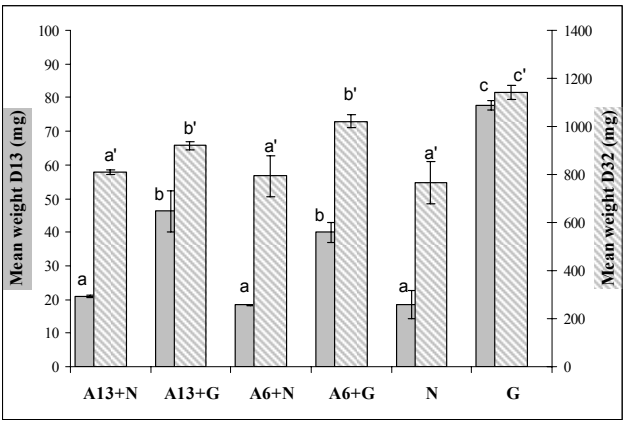

Fig. 1. Body weight of *C. gariepinus* after 13 days (feeding regimes test) and 32 days (follow-up of growth) experiment. A = *Artemia* (during 6 or 13 days); N = Nippai; G = Gemma micro.

In the first phase of the first experiment (D1 to D13), growth (final body weight: 77.8 ± 1.4mg) and survival (91.5 ± 3.8%) of larvae fed with the "Gemma micro only" regime was significantly higher ($P<0.05$) than the other regimes.

In the second phase (follow-up from D14 to D32, after weaning period), growth (final body weight: 1142.0 ± 30.0mg) was significantly higher ($P<0.05$) than the other regimes. Survival (86.3 ± 1.1%) of larvae fed with the "Gemma micro only" regime was higher than the other regimes, but only significantly with the "*Artemia* 13 days + Nippai" and "Nippai only" regimes. (Figs. 1 and 2).

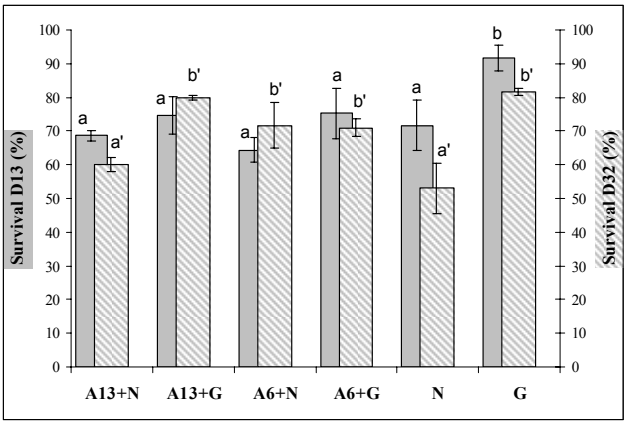

Fig. 2. Survival of *C. gariepinus* after 13 days (feeding regimes test) and 32 days (follow-up of growth) experiment. A = *Artemia* (during 6 or 13 days); N = Nippai; G = Gemma micro.

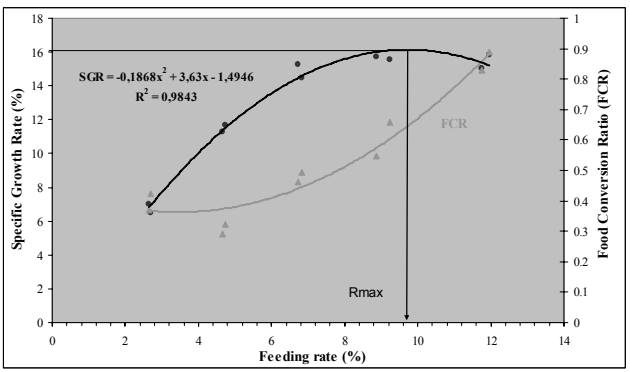

Fig. 3. Relationship between specific growth rate, food conversion ratio and feeding rate of *C. gariepinus* larvae from D20 to D28 (mean body weigh at D28: 909 ± 147mg) at 28°C.

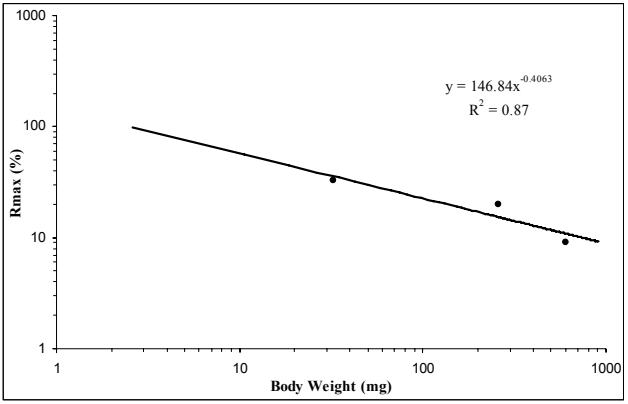

Fig. 4. Relationship between body weight (from 3 to 1000 mg) of *C. gariepinus* and maximal ration (with Gemma micro) at 28°C.

Results of the second experiment showed that the relationship between R_{max} (% of the body weight) and body weight (mg) was expressed by a power function: $R_{max} = 146.84$ (body weight)$^{-0.4063}$; $R^2 = 0.87$ ($P<0.05$) (Figs. 3 and 4).

Conclusions

Only feeding with high quality feed (Gemma micro) and without *Artemia* during weaning period showed best results in terms of growth and survival.

Higher energy content of this artificial food combined with a good supply in micro-nutriment (amino acids, phospholipids, vitamins, carotenoids) probably could explain this result.

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EFFECTS OF FEEDING *CHLORELLA VULGARIS* CULTURED ON DIFFERENT CONCENTRATIONS OF NITROGEN AND PHOSPHOROUS ON THE POPULATION GROWTH AND PROXIMATE COMPOSITION OF THE ROTIFER *BRACHIONUS CALYCIFLORUS*

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Introduction

Rotifers are widely used as a live food for the early stages of fish of commercial importance and must meet their nutritional requirements for optimal development and growth (Sarma, 1991). Long-term enrichment of rotifers with microalgae is well know for marine species, but knowledge in freshwater species is lacking and thus, the aim of this research was to evaluate population growth and proximate composition (protein, lipid, and moisture contents) of the rotifer *Brachionus calyciflorus* fed on *Chlorella vulgaris*, cultivated on different concentrations of phosphorus (P) and nitrogen (N).

Materials and methods

Chlorella vulgaris was batch-cultured on 2-l plastic reactors using Bold basal medium (Nichols, 1979) with different initial concentrations of N and P (Table I). The cultures were maintained under continuous illumination (57µmol.m^{-1}.s) and temperature of 25±1°C. After 10 days of culture, microalgae biomass was collected by centrifugation, washed, and kept at 4°C prior to use.

Table I. Initial concentrations of the treatments with phosphorus (P) and nitrogen (N) of the culture media of *Chlorella vulgaris.*

Treatment	Concentration (mg.l^{-1})	
	P	N
Control	2.5	2.5
P1.25	1.25	2.5
P0.625	0.625	2.5
N1.25	2.5	1.25
N0.625	2.5	0.625

The culture of *Brachionus calyciflorus* was done in 100-ml jars with 90ml of EPA medium (Pavón-Meza et al., 2007), with an initial concentration of 1 organism per ml. The rotifers were fed with the respective microalgae cultured on the different concentrations of P and N; and each treatment had four replicates. Daily, *C. vulgaris* was added at 1×10^6cells.ml^{-1} (Pavón-Meza et al., 2007) and conditions during the culture were as mentioned for microalgae culture. To obtain the population growth, rotifers were counted on daily basis. Counting was followed up to 3 days after the populations reach the maximum density. To obtain enough biomass for the proximate composition analysis, separated cultures were set up under the condition already mentioned. By day 15, rotifers were harvested with a mesh of 50μm, washed, and kept at -4°C until analysis.

The proximate composition analysis of the rotifer biomass was performed according to AOAC (1990) for moisture and Bligh and Dyer (1959) for total lipids. Protein content was determined with the Peterson's modification of micro-Lowry method (reagent kit from Sigma-Aldrich Co. St. Louis, MO, USA).

The population growth rate (PGR) was calculated with the formula $r = (\ln N_t - \ln N_o).t^{-1}$, where r was the PGR; N_t, final number of individuals; N_o, initial number of individuals and t, the time (days). Data of PGR, contents of protein, lipids and moisture of rotifer biomass were analyzed with a Kruskal-Wallis test ($P < 0.05$).

Results and discussion

The PGR and maximum density (MD) of *B. calyciflorus* obtained are shown in Table II. The PGRs of rotifers that consumed *C. vulgaris* cultured on low P concentrations showed significantly higher values than observed in the control group. Actually, the growth was faster on the groups with low P than the control (Fig. 1, top). On other hand, low N cultured *C. vulgaris* affected the growth of *B. calyciflorus*, as the PGRs were significantly lower than the control. The control group has a faster growth than N1.25 and N0.625 groups (Fig. 1, bottom). Table III shows the proximate composition of rotifer biomass. There was tendency for lower values of crude protein in the treatments than observed in the control group. In contrast, lipid contents tended to be higher in the treatments than observed in the control.

The PGRs of *B. calyciflorus* were higher than reported by other authors (Peredo-Álvarez et al., 2003; Sarma et al., 2001). This indicates that even *C. vulgaris* cultured on low N allows population growth. However, the possibility of using the rotifers fed microalgae with low N is not an option because of the low biomass protein content, which is necessary for growth and development of fish larvae (Sarma, 1991). The low P cultured microalgae, on other hand, does not have a

negative effect on *B. calyciflorus* and their population growth and proximate composition are suitable to be used as food for fish larvae.

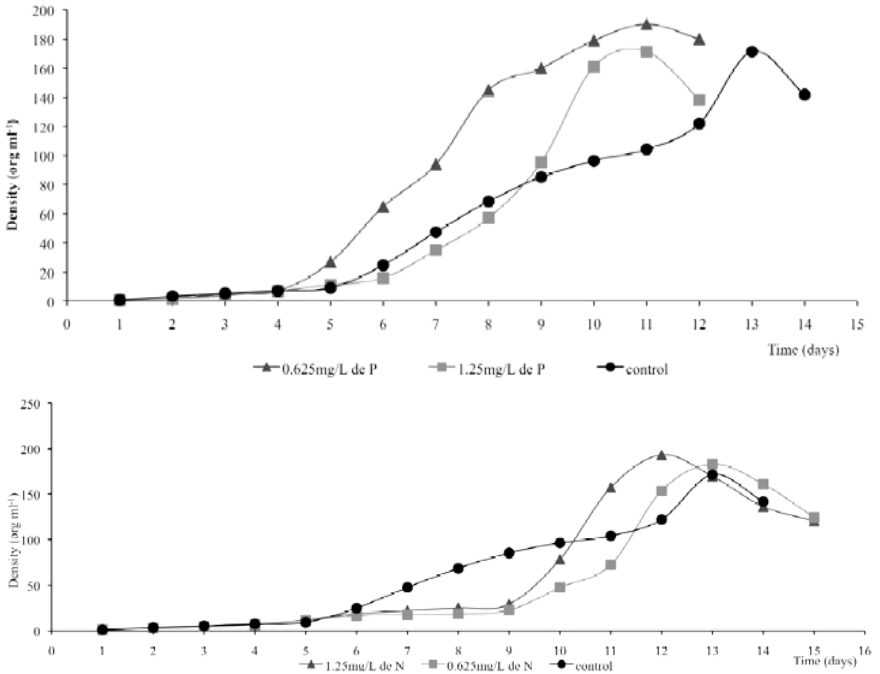

Fig. 1. Population growth of *Brachionus calyciflorus* fed on *Chlorella vulgaris* cultured on different concentrations of phosphorus (top) and nitrogen (bottom).

Table II. PGR and maximum density MD of *Brachionus calyciflorus* fed on *Chlorella vulgaris* cultured different concentrations of phosphorus and nitrogen. Values are the means of four replicates ± SD. Means with different letters in the same column differ significantly ($P < 0.05$).

Treatment	PGR	MD (org.ml^{-1})
Control	0.40 ± 0.01a	171 ± 4a
P1.25	0.50 ± 0.005b	171 ± 4a
P0.625	0.49 ± 0.005b	190 ± 5b
N1.25	0.35 ± 0.005 c	192 ± 5b
N0.625	0.34 ± 0.006c	182 ± 3c

Table III.Proximate composition of *Brachionus calyciflorus* biomass fed on *Chlorella vulgaris* cultured different concentrations of phosphorus and nitrogen. Values are the means of four replicates ± SD. Means with different letters in the same column differ significantly ($P < 0.05$).

Treatment	Crude protein[1]	Crude lipid[1]	Moisture (%)
Control	33.9 ± 0.2a	11.4 ± 0.03a	94.5 ± 0.1 a
P1.25	26.3 ± 2.2b	15.1 ± 0.03b	91.2 ± 0.1
P0.625	27.2 ± 2.1b	14.1 ± 0.04b	93.5 ± 0.1
N1.25	30.5 ± 0.7a	17.6 ± 0.02c	94.3 ± 0.02
N0.625	20.9 ± 6.2c	18.5 ± 0.1c	94.5 ± 0.1

[1]% in dry weight basis

Conclusions

The present research shows that *B. calyciflorus* fed on microalgae cultured on low P might be used on freshwater fish larvae cultures and help to reduce costs without affecting the production and nutritional quality of rotifers.

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BROODSTOCK MANAGEMENT AND INDUCED SPAWNING OF PERUVIAN ANCHOVY (*ENGRAULIS RINGENS*) UNDER A CLOSED RECIRCULATION SYSTEM

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Introduction

One the aims of our laboratory is to understand the limits of environmental tolerance in marine species of economic importance (fisheries and aquaculture), using the methodological tools of experimental biology. Laboratory tests under closed recirculation system were conducted in order to ascertain the degree to which variation of environmental conditions affects the reproductive process and spawning of the Peruvian anchovy.

Materials and methods

Peruvian anchovy adults (n=1298, with average total length of 13.7 ± 1.1cm) were captured off Callao, Peru, using a system of lights and a liftnet, designed according to the methodology used in the Fiji Islands (Nedelec, 1975). Fish were acclimated in 10-m^3 circular fibreglass tanks and kept under recirculation seawater system (RSS) with a photoperiod of 10L:14D, 16±1ºC, 35ppt of salinity, and pH 7.5-8.5. During acclimation, the fish were first fed on LARVAL AP100®, which was gradually replaced with 2×3mm (4.61kcal.g^{-1} fish per day) extruded feed for rainbow trout NICOVITA®.

The first experiment was conduced in 2-m^3 tanks under RSS (two replicates) for 72 days and samples were taken at 0, 30, 45, and 72 days, respectively. The daily rations of food were 46 and 138cal.g^{-1} per fish, equivalent to 1% (ration R1) and 3% (ration R2) of the biomass, respectively. A total of 20 individuals per treatment were sacrificed in each sampling (anaesthetized with tricaine 80ppm and sacrificed by decapitation) and assessed for sex, total length (TL), total weight (TW), and gonad weight (GW). These data were used to calculate gonadosomatic index (GSI = GW*100 (TW-GW) $^{-1}$). The percentage of total body fat (%F) was determined using a Soxhlet SER 148/3 automatic fat extractor. The size of yolked oocytes was compared from histological samples using a Nikon DS-Li digital image processor.

A second experiment was conduced in 2-m^3 tanks under RSS for 90 days with density of 73.3 fish.m^3. Three temperatures were tested: 12, 16, and 24ºC (three replicates). All fish were fed ad libitum. A total of 90 individuals were sacrificed in the same method as in the first experiment and data collected for GSI.

The induction of spawning done via intraperitoneal injection of Conceptal (busereline acetate, GnRHa). The effects of two hormonal induction treatments were examined on spawning of Peruvian anchovy broodstock. T1: 0.005µg GnRHa.g^{-1} body weight (bw) in single injection both male and female. T2: 0.004µg GnRHa.g^{-1} bw in single injection for male and 0.010µg GnRHa.g^{-1} bw in two doses for female (40% in first injection and second injection 13 hours later). Determination of sex was done following Leong (1971).

A significance level of 0.05 was considered for all the statistical tests, using SYSTAT 8.0 for Windows. To determine significant differences of oocyte size between treatments, a t-student with Bonferroni adjustment was used. To determine significant differences of GSI and F% were used analysis of variance (ANOVA) and Tukey test for comparisons between days of the same treatment; whereas the t-student test with Bonferroni adjustment for comparisons between treatments of the same day.

Results and discussion

In the second and third month of the experiment, the IGS of the males and the F% of both sexes in the experiment were higher than those reported by the FPM (Table I), indicating an efficient lipogenic mechanism in this species. Schülein et al. (1995) mention that clupeiforms are known as efficient anabolizers and catabolizers of lipids reserves which are required for growth, movement, and reproduction.

Table I. Values (means ± SEM) of gonadosomatic index (GSI) and percentage of total body fat (F %) of individuals of Peruvian anchovy. FMP: Fishing Monitoring 2003 Program from Callao. R1: Fish in captivity fed with a ration of 46cal.g^{-1} fish per day. R2: Fish in captivity fed with ration of 138cal.g^{-1} fish per day. Different superscripts are significantly different ($p < 0.05$).

			October	November	December
GSI (%)	Female	FPM	5.23 ± 0.14	4.69 ± 0.31^a	5.70 ± 0.22^b
		R1	4.41 ± 1.16	4.79 ± 0.60^a	6.40 ± 0.73^b
		R2	3.61 ± 2.29	6.13 ± 0.58^b	4.43 ± 0.46^a
	Male	FPM	6.28 ± 0.22^a	4.78 ± 0.30^a	6.42 ± 0.26^a
		R1	9.73 ± 1.29^b	6.67 ± 0.97^a	9.04 ± 1.50^b
		R2	6.02 ± 1.16^a	9.38 ± 0.75^b	8.60 ± 1.10^b
F%		FPM	5.81 ± 1.03	5.24 ± 0.56^a	3.39 ± 0.00^a
		R1	4.94 ± 0.68	10.33 ± 1.33^b	15.44 ± 1.57^b
		R2	4.67 ± 1.01	12.19 ± 0.84^b	15.97 ± 0.48^b

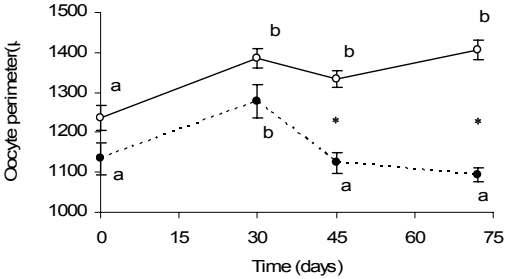

Fig. 1. Size of yolked oocytes (μm) in Peruvian anchovy held in captivity. Black circles: corresponds to individuals fed 46cal.g^{-1} fish per day (R1) and white circles to individuals fed 138cal.g^{-1} fish per day (R2). Bars represent standard error. Different letters indicate statistically significant differences between days of the same treatment and asterisks indicate differences between treatments ($p < 0.05$).

Significant differences were observed in sizes of yolked oocytes from both treatments on days 45 and 72 (Fig. 1). It is evident that the greater amount of food allowed fishes fed on R2 ration to mature their gonads faster than those fed on R1 ration. Also, it is possible that more or better reserves were stored inside the oocytes of fishes fed with R2 ration, allowing the oocytes a longer lifespan before starting atresia. We can suppose that in the case of fishes fed with R1 ration, the small perimeter of oocytes was because of the need to allocate food resources to other vital functions. However this doesn't seem to be the case because fat storage increased through all the test duration (Table I), indicating that food availability was sufficient.

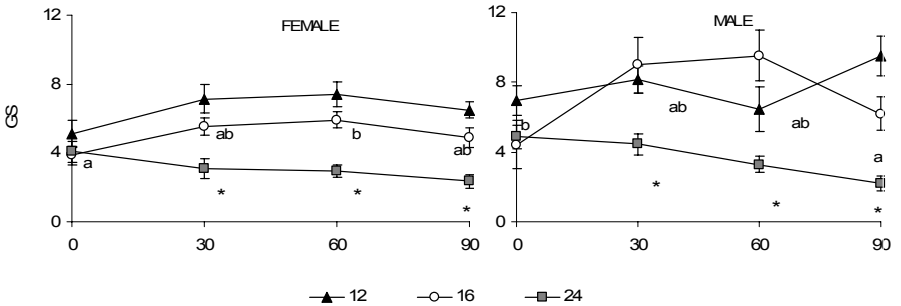

Fig. 2. Gonadosomatic index (GSI) of broodstock Peruvian anchovy held in captivity at three temperatures: 12, 16, 24ºC. Different letters indicate statistically significant differences between days of the same treatment ($p < 0.05$). Asterisk indicates statistically significant differences between treatments of the same day.

The gonadosomatic index can indicate an effect of temperature on the gonads; i.e., significant differences in the size of them, both in females as in males

($p<0.05$), mainly in treatment of 24ºC, and this effect was consistent for both females and males from the 30th to the end of the experiment. Nonetheless, the mortality (38%) at 24ºC was zero at day 69 until the end of the experiment and the percentage of total body fat increased from 9 to 18% in 90 days (data not shown). This suggests the physiological plasticity of this species.

Table II. Effect of two treatments the hormonal induction on spawning of broodstock of Peruvian anchovy. T1: 0.005µg GnRHa.g^{-1} body weight (bw) in single injection both male and female. T2: 0.004µg GnRHa.g^{-1} bw in single injection for male and 0.010µg GnRHa.g^{-1} bw in two doses for female (40% in first injection and second injection 13 hours later).

Inductions treatments	Female broodstock	Male broodstock	Total eggs	Eggs Fertilized (%)
T1	6	6	11 478	0
	9	6	8 563	0
	8	7	5 236	0
	10	5	0	0
T2	11	9	10 526	5.99
	10	20	12 603	4.17
	18	12	16 654	3.73

The broodstock spawned in both treatments fertilized eggs were not found in T1. The main problem is the asynchrony in the spawning in the Peruvian anchovy. Application of the two injections in females was more effective, but a low percentage of fertilized eggs was obtained. These results suggest that GnRHa (busereline acetate) injection has an inductor effect on final oocyte maturation in mature broodstock of *E. ringens*, ending with spawning between 24 and 48 hours post-injection.

Acknowledgements

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IMPACT OF LIVE VERSUS DEAD BACTERIA ON SURVIVAL, GROWTH, AND GENE EXPRESSION IN ATLANTIC COD (*GADUS MORHUA*) LARVAE

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Introduction

Probiotics are one candidate for exploring novel strategies to increase microbial control in cod farming and to reduce problems concerning the production of high quality larvae. The present work intends to explore the effects of live versus dead probiotic candidates on the survival, growth and gene expression in Atlantic cod larvae.

Materials and methods

The cod eggs were disinfected once before arrival and then washed in filtered (0.2µm Micropore filter) autoclaved seawater (FASW). The eggs were then disinfected once more, by cautious stirring for 10 minutes in FASW with glutaraldehyde (final concentration 400mg.l-1) (Salvesen and Vadstein, 1995; Salvesen et al., 1997). The eggs were washed in FASW, and transferred to large Petri dishes with FASW added 10ppm ampicillin and rifampicin. The temperature pre-hatching was 8°C.

After hatching the temperature was increased by 1°C.day-1 until it reached 12°C. The larva were reared in 2-litre bottles (30 larvae.l^{-1}) with aeration, and fed bacteria free rotifers, *Brachionus nevada*, from day 3 to day 17. Bacteria-free *Isochrysis* sp. algae was added to achieve green-water (Naas et al., 1992), and as feed for the rotifers. Dead larvae and algal waste was removed every third day. Three treatments were tested: bacteria free larvae (Control), live *Microbacterium* sp. ND2-7 (two replicates abbreviated LM1 and LM2) (Fjellheim, 2006), and dead *Microbacterium* sp. ND2-7 (abbreviated DM). The bacteria were added to a total concentration of 10^6CFU.ml^{-1}.

On day 17 post hatching, the experiment was terminated. Live larvae were collected from the rearing bottles, transferred to a FASW with metomidate (0.5g.l^{-1}, lethal dose), and their picture was taken to determine the length of the larvae.

They were then transferred to tubes containing RNA later. RNA was isolated from these larvae, and the gene expression of 8 different genes was quantified by means of real-time PCR. The results were normalized to the expression of the housekeeping gene *β-actin*.

We also conducted a short term experiment with 3 different probiotic candidates, (Fjellheim, 2006) various mixtures of these, and one known pathogen. The larvae in this experiment were reared in 50ml bottles (30 larvae.50ml^{-1}), without aeration and feeding. The experiment was terminated after 5 days, and sampled as the first feeding experiment.

The results were analyzed by a t-test (survival) and ANOVA using Tukey's Honestly-Significant-Difference Test for multiple comparison (growth and gene expression). This was done using statistic software package Systat.

Results and discussion

The survival was high during the first week of the experiment, and decreased rapidly after day 10, the day for transition to exclusively exogenous feeding (Kjørsvik et al., 2004). On day 17 post hatching the survival was higher in the control treatment (57%), than in the treatments with live *Microbacterium* sp. (35 and 33%). There were no significant difference between the control and the treatment with dead *Microbacterium* sp. (47%). It therefore seems as this strain of bacteria was not probiotic when it comes to cod. However, one should also have a challenge experiment with known pathogens, in order to see the effect of *Microbacterium* sp. when interacting with other bacteria. The fact that the control treatment had the highest survival rate was expected.

For the short term experiment the survival was higher for the treatments with dead probiotic candidates than for those with live probiotic candidates. Live and dead *Microbacterium* sp. was also used in this experiment, and this treatment showed a lower survival with live bacteria already on day 3. The difference in survival increased throughout the experiment, but the survival was still higher than for the treatment with the known pathogen.

At day 17 the larvae from the first feeding experiment given live *Microbacterium* sp. (LM1) were significantly longer than the larvae from the treatment with dead *Microbacterium* sp. (Fig. 1). There were no differences in larval length between the other treatments. This implies that the dead bacteria do not have a positive effect on larval growth compared to larvae given live bacteria. The reason for the difference between the two treatments with live bacteria is unclear, but it might be that the conditions in the two bottles were so different that the effect of the added bacteria was overruled.

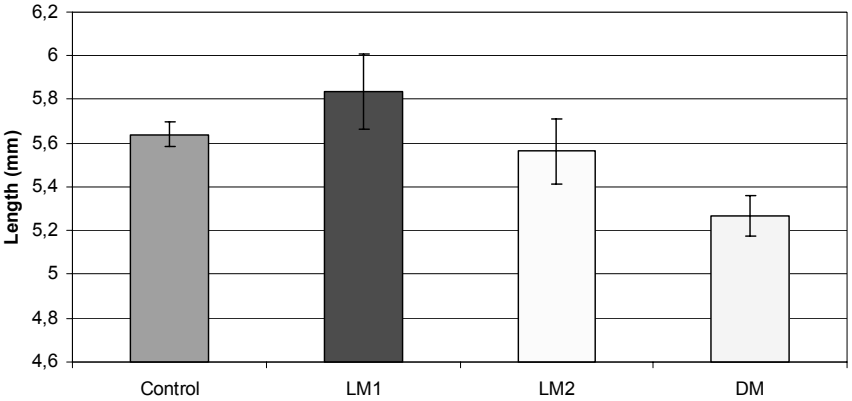

Fig. 1. The average length of the cod larvae in the different treatments of the first feed ing experiment. Between 19 and 32 fish were measured in the various treatments. Error bars are standard error.

The results of the real-time PCR for the first feeding experiment showed that for 6 out of 8 genes, there was a large difference in gene expression between the two parallel treatments with live *Microbacterium* sp. This includes the genes CLECT*, C3, Cyp1a1, fdps, gpx2*, and *Itgb1bp3*. This is consistent with the fact that the larvae in the two parallel treatments also showed a difference in growth. The genes *WARS* and *fiaf* showed similar expression patterns between treatments, with highest expression for the two parallel treatments with live *Microbacterium* sp., followed by the control treatment and the treatment with dead *Microbacterium* sp. The gene expression in the larvae from the treatment with dead *Microbacterium* sp. was the lowest one for all the genes except *Cyp1a1*. This suggest that the bacteria have to be alive and active in order to induce a response in the cod larvae.

For the short term experiment, so far expression of only the gene *Cyp1a1* has been quantified, but gene expression on other genes will be quantified later.

Conclusions

Our results suggest that *Microbacterium* sp. did not have a probiotic effect on the cod larvae in terms of survival and growth. Moreover, the probiotic candidates used in this experiment had to be live and active to induce a gene response in cod larvae.

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LIPIDS AND FATTY ACIDS OF *OCTOPUS VULGARIS* PARALARVAE REARED WITH ENRICHED ON-GROWN *ARTEMIA*

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Introduction

Up today, rearing of common octopus, *Octopus vulgaris*, paralarvae is severely limited by the lack of success during the paralarval stage, with generalised mortalities occurring before the settlement of the juveniles. The use of on-grown *Artemia* cultured with the microalgae *Isochrysis galbana* and further enriched with *Nannochloropsis* sp. has proven a certain degree of success during the first month of life (Hamazaki et al., 1991). The present works aims at studying the effects of this rearing protocol on the lipid and fatty acid composition of paralarvae, by comparison with a diet based on on-grown *Artemia* further enriched with a high polyunsaturated fatty acid (PUFA) oil emulsion.

Materials and methods

Octopus vulgaris paralarvae were fed during 28 days with on-grown *Artemia* (1.5-2mm) biomass cultured *Isochrysis galbana* and further enriched during 24h with either *Nannochloropsis* sp. (Nanno treatment) or a PUFA-rich oil emulsion based on a speciality oil (M70 treatment).

Cultures were carried out at 21-22°C in 1000-l black tanks at a larval density of 5 individuals.l^{-1}. Continuous illumination and gentle aeration were provided. A concentration of 1×10^6 cells of *Nannochloropsis*.ml^{-1} was kept in the culture medium. Enriched *Artemia* was added (3 to 4 times per day) at a density of 0.5 prey ml^{-1} until day 11, and at 0.2 prey.ml^{-1} onwards.

At the end of the trial, the lipids of diets and paralarvae were extracted, an aliquot was fractionated into polar and neutral classes by thin layer chromatography, and the fatty acids analysed by high resolution capillary gas chromatography. The fatty acid profiles thus obtained were subsequently analysed chemometrically by principal component analysis (PCA). The score plot resulting after

the generation of the two principal components was used to identify patterns of similarity among cases.

Results and discussion

After 28 days, paralarvae fed M70 weighed 1.88±0.22mg (n=10), whereas those fed Nanno, averaged 1.76±0.28mg (n=9). No significant differences (t-test $p>0.05$) were found between both groups. Survival at 28 days was roughly estimated at 3% for M70 and 22.5% for Nanno. Culture of this last group was followed until day 35, reaching an average paralarval weight of 1.83±0.28mg (n=10) and a 3% survival. These data indicate that after one month, the biometrical outcome of both dietary treatments was very similar. At this time the Nanno group showed better survival, but this hypothetical advantage was lost one week later.

Table I. Selected fatty acids (% of total fatty acids) of the total lipid of enriched on-grown *Artemia*, and of total, polar and neutral lipid of *Octopus vulgaris* paralarvae fed on them (mean, n=3).

	Artemia		**Paralarvae**					
Fatty acid	**Total Lipid**		**Total Lipid**		**Polar Lipid**		**Neutral Lipid**	
	M70	**Nanno**	**M70**	**Nanno**	**M70**	**Nanno**	**M70**	**Nanno**
14:0	1.43	1.13	0.74	1.62	0.58	1.09	2.56	5.19
16:0	10.54	15.71	21.09	21.23	18.53	18.25	20.21	18.22
16:1n-7	5.19	23.69	5.02	4.31	2.07	2.47	16.22	13.01
18:0	7.26	5.40	12.61	12.51	14.21	13.56	10.50	8.19
18:1n-9	16.22	12.31	6.93	8.32	3.94	5.33	12.36	17.25
18:1n-7	9.27	7.89	5.58	5.28	4.08	3.88	6.27	6.10
18:2n-6	12.15	2.34	1.50	2.95	1.11	2.08	2.52	7.06
18:3n-3	5.05	0.38	0.94	0.77	0.22	0.56	0.24	1.81
20:4n-6	2.75	2.88	6.35	5.50	7.69	6.31	1.19	0.49
20:5n-3	11.99	22.55	21.89	20.33	25.73	23.31	5.33	2.31
22:6n-3	8.10	nd	5.55	5.21	6.44	5.63	nd	nd
Sat	20.38	23.33	34.57	35.54	33.73	33.35	34.62	31.94
Mono	31.58	44.19	20.20	20.45	12.81	14.55	36.41	37.19
Poly	44.42	28.96	41.57	40.48	46.06	43.42	11.96	13.99
n-3	27.92	23.04	30.98	29.09	35.45	32.75	5.57	4.56
n-6	16.67	5.62	9.73	10.72	10.75	10.56	4.16	9.25
HUFAn-3	20.93	22.57	29.92	28.00	35.16	32.01	5.33	2.31
HUFAn-6	3.83	3.06	7.02	6.35	8.38	7.12	1.64	1.60

Sat: saturates; Mono: monoenes; Poly: polyunsaturated; HUFA: highly unsaturated fatty acids (>20C). nd: not detected. Standard deviations were bellow 10%

Lipid content of diets (% dry weight (DW)) was significantly different (t-test $p<0.05$): 21.01±0.84 for Nanno and 18.23±0.70 for M70. Analysis of the fatty acids (Table I) showed that main differences were due to Nanno treatment being higher in 16:1n-7 (which resulted in higher monoenes content), and 20:5n-3; whereas M70 showed higher 18:2n-6 (thus increasing total n-6 content) and 22:6n-3, which was absent in the other diet (Table I).

Differences in the lipid content of diets did not translate (P>0.05) into the lipid content of the paralarvae (Total lipid (% DW): M70=16.31±1.04; Nanno=17.5±0.23). Similarly, a first look into the fatty acid profiles of the total lipids of both dietary groups did not reveal streaking differences between them (Table I). Especially notable is the case of 16:1n-7 and 18:2n-6, which showed very different values in the diets. Besides, the paralarvae seemed to be able to cope with a 22:6n-3 deficient diet, although the poor performance of the two dietary groups could also point towards a dietary deficiency of both treatments.

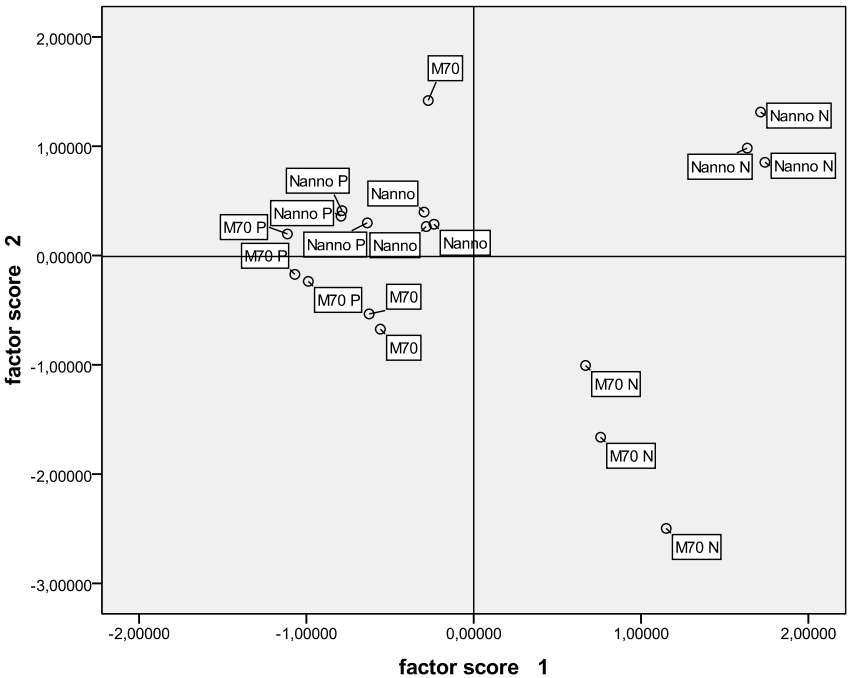

Fig. 1. Score plot generated after PCA of the fatty acid patterns of *Octopus vulgaris* paralarvae fed two enriched on-grown *Artemia* diets.

A closer look into the polar and neutral lipid fatty acid composition showed the polar lipids being much richer in 20:5n-3 and 22:6n-3 (absent in the neutrals), which was reflected in a higher polyunsaturated, n-3 and HUFA n-3 content. Neutrals on their turn were richer in monounsaturated fatty acids. Within the polar lipids, M70 treatment generally increased the polyunsaturated, n-3 and HUFA n-3 content with respect to Nanno. Within the neutral lipids, Nanno treatment could be associated to higher n-6 content, whereas M70 seemed to increase HUFA n-3.

These trends were endorsed by the results of the PCA. The first component explained 76% of variance and was associated to variables 14:0, 18:1n-9, 18:2n-6, and 16:1n-7 on the positive side and to variables 20:5n-3, 20:4n-6, 22:6n-3, and 18:0 on the negative side. Second component explained only 11% of variance. The results of the score plot (Fig. 1) showed two main clouds of scores corresponding to polar (associated to variables on the negative side) and total lipids on one side, clearly distinct from neutral lipids (associated to variables on the positive side). Within the polar and neutral lipids, the scores corresponding to the different dietary treatments were distinguishable, whereas those corresponding to the neutral lipids were spread among the polars. The scores of the polar lipids were more grouped than those of the neutral lipids indicating a higher similarity in the fatty acid patterns, which speaks about the structural role of this lipid class (Gurr and Hardwood, 1991).

Navarro and Villanueva (2003) found that main dietary changes were very evident in the total lipids of *O. vulgaris* paralarvae, although they were also clear in the polar lipids. However these authors did not report on neutral lipid composition. The extent of the dietary changes may be dependent on the composition of the diets. The results reported here show that although the fatty acid pattern of paralarvae is influenced to more or less extent by the composition of diets, it tends to be more conservative in the structural (polar) lipids, with the neutral lipids reflecting the dietary differences in essential fatty acids.

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EFFECT OF LIGHT SPECTRUM AND PHOTOPERIOD ON THE GROWTH, DEVELOPMENT, AND SURVIVAL OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) AND ATLANTIC COD (*GADUS MORHUA*) LARVAE

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The lighting regime during the early development of cultured teleost fish is established in order to improve important elements like growth and survival, however, unsuitable illumination is linked with a number of functional and skeletal abnormalities. This study investigates how the characteristics (spectrum and photoperiod) of artificial lights affect European sea bass and Atlantic cod eggs and larvae from -1 day to 40-60 days post-hatching (dph).

From -1 to 40dph, five lighting regimes (with three replicates) were applied to sea bass eggs and larvae using LED lamps: 12L:12D cycle with red (LDR, half-peak bandwidth 641-718nm), blue (LDB, half-peak bandwidth 435-500nm) and white (LDW, range of 367-1057nm), 24L:0D white (LL), and 0L:24D (DD) (FieldSpec®, ASD, Colorado, USA). Larvae were arbitrarily chosen (n=30) for total length, yolk sac and oil globule measurements (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK.), first feeding, development of fins, swim bladder, teeth and malformations. Wet weight and survival were observed at the end of the experiment. In Atlantic cod, the effects of light intensity and spectrum on larvae performances were tested using either narrow bandwith cold cathode lighting systems (CCL) or Light Emitting Diodes (LED). Cod larvae (from hatching, 5000.tank^{-1}) were exposed to continuous light regimes (LL) at either 1.39W.m^{-2} (LED), 0.5W.m^{-2} (CCL), or 0.25.W.m^{-2} (CCL) intensities at four different spectral bandwidths: blue (455nm), green (535nm), red (640nm) and white (two peaks at 460 and 560nm). Larvae were sampled randomly (30 per sampling point per tank) every 10 days until 60dph to measure dry weight, standard length, myotome height, eye diameter, condition index, swim bladder inflation, and others. Survival was determined at the end of each the trial.

The results showed that total length, wet weight, and development of different body structures were best in larvae reared under blue light in both species. In

contrast, growth and development of larvae reared under red light was lower. Sea bass larvae reared under red light did not feed on live prey (rotifers) until the addition of *Artemia* on 16dph while cod larvae under similar conditions were up to 4× smaller (by 60dph) than their siblings reared under shorter wavelengths. Light intensity also appeared to have significant effects on cod larvae performances with best growth achieved under higher intensities. This confirms previous findings in the same species. Furthermore, work in sea bass identified that in addition to spectral content the photoperiod can influence larval performance with the best survival being obtained under LL treatment, however significantly higher malformations were also identified.

These results are supported by a previous experiment that showed haddock larvae performed optimally under the spectral conditions most frequently encountered in their particular ecological niche. Overall this work demonstrates that the photic environment in which larvae are reared can play a significant role in development and survival. This work builds on previous findings of the importance of light intensity by demonstrating how shorter wavelengths also stimulate development in both sea bass and cod. Perhaps more importantly, they also demonstrate that at least in sea bass, photoperiod is essential for natural development. LDB treatment was close to the natural environmental characteristics that sea bass larvae encounter in the wild. Light and dark cycles are also required for the normal appearance of rhythmic circadian clock outputs, which, in fish, mature extremely early during larval development and are thought to regulate the temporal co-ordination of many physiological processes.

In conclusion, natural light and dark cycles with a blue spectrum (LDB) provided the best rearing conditions for sea bass larvae. “Unnatural” lighting conditions (LL, DD, and LDR) at this early stage resulted in a delay in development that compromised fish performance and welfare. However, in cod, LL regime clearly appears to stimulate growth and improve survival as previously reported. Differential light sensitivities may therefore exist among teleosts which support the need for the development of species specific husbandry protocols within the industry.

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THE EFFECT OF DHA LEVELS ON GROWTH, SURVIVAL, WEANING, AND PIGMENTATION OF CALIFORNIA HALIBUT (*PARALICHTHYS CALIFORNICUS*) LARVAE

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Introduction

The California halibut *(Paralichthys californicus*) is a good candidate for aquaculture due to its good growth, survival, and high commercial value. Several farms in the Western coast of North America are currently evaluating the potential of this species under commercial conditions. However, one of the main problems in the production of juveniles for commercial purposes is the high percentage of malpigmented fish obtained after metamorphosis (up to 80%). This problem seems to be related, among other things, to nutritional deficiencies during the larval period, in particular to the quantities and proportions of highly unsaturated fatty acids (HUFAs) in the diet.

As a first approach to reduce malpigmentation, improve growth, and determine the requirement of DHA in California halibut late larvae, we evaluated the effect of four levels of dietary DHA (0, 5, 10, and 15% of total of fatty acids in the diet) on growth, survival, weaning success and pigmentation.

Materials and methods

DHA was administered through enriched *Artemia* nauplii. Four experimental enrichment emulsions were prepared with increasing DHA levels: 0%, 5%, 10, and 15% DHA of the total fatty acids (TFA). A commercial enrichment product used to enrich live feed was used: spray-dried gold fat, Aqua Grow®, Advanced BioNutrition containing 42.8% DHA of TFA (15% DHA by weight).

Larvae standard length and wet weight were taken during the initial stage of *Artemia* nauplii supplementation (18 days post hatch, dph); at the beginning of the weaning period (50dph); and at the end of the experiment (75dph). Weaning success was defined here as the treatment producing the highest survival and the juveniles with the highest weight at the end of the experiment. Total fatty acids in 18- and 50-dph larvae and *Artemia* were analyzed by gas chromatography

(Hewlett Packard 5890II) using a flame ionization detector (260°C), a capillary column (Omegawax 320; 30m × 0.32mm, film thickness 0.25µm, Supelco Inc., Park City PA, USA), 1-µL sample injection, and helium as the carrier gas.

The fish were classified according to their degree of pigmentation into three categories: A) pigmented, when the ocular side was fully pigmented. B) pseudoalbino or hypomelanism, fish with completely white ocular side, C) patchy, with stains in the ocular side.

Results and discussion

The live prey enrichment protocol was successful resulting in increasing levels of DHA in *Artemia* nauplii from 0.16 ± 0.23 to 3.25 ± 0.37% of the total fatty acids (Table I).

Table I. Selected fatty acid profile of enriched *Artemia* nauplii with four DHA enrichment levels (% of total fatty acids), mean ± standard deviation, n = 2.

Fatty acids	0 % DHA	5% DHA	10% DHA	15% DHA
Total *n*-6 PUFA	18.60 ± 2.62	17.62 ± 2.47	16.83 ± 1.41	14.68 ± 2.13
C18:3 *n*-3	18.34 ± 5.11	19.90 ± 2.27	18.40 ± 2.93	18.13 ± 1.28
C20:5 *n*-3	4.41 ± 0.90	4.70 ± 0.11	4.77 ± 0.12	5.06 ± 0.08
C22:6 *n*-3	**0.16 ± 0.23^{a}**	**0.85 ± 0.05^{ab}**	**1.61 ± 0.12^{bc}**	**3.25 ± 0.37^{c}**
Total *n*-3 PUFA	22.92 ± 3.98	25.45 ± 2.21	24.78 ± 3.17	26.43 ± 0.83
Total PUFA	41.51 ± 1.37	43.07 ± 0.26	41.61 ± 1.75	41.12 ± 1.29
n-3/ *n*-6	1.23 ± 0.39	1.44 ± 0.33	1.47 ± 0.31	1.80 ± 0.32
DHA/EPA	0.04 ± 0.05^{a}	0.18 ± 0.02^{ab}	0.34 ± 0.02^{b}	0.64 ± 0.06^{c}
EPA/ARA	0.92 ± 0.20^{a}	0.84 ± 0.00^{a}	1.20 ± 0.05^{ab}	1.39 ± 0.07^{b}

a, b, and c superscripts denote significant differences among diets (P > 0.05)

No significant differences on growth, survival and pigmentation as a result of dietary DHA levels were found in 50-dph juveniles. However, larvae fed the highest DHA level resulted in the highest growth and survival at the end of the experiment (75dph), Table II. Additionally, highest weaning success was achieved with this treatment. Significantly higher numbers of normally pigmented fish (~33%) were obtained with the highest DHA level (15% DHA TFA) at 75dph compared to the low DHA levels (0 and 5% DHA TFA), Table II. However, since this treatment resulted in the highest survival a relatively high number of fish had abnormal pigmentation (~30% of the population).

Table II. Length, weight and survival of 75 days post-hatch (DPH) California halibut, *Paralichthys californicus,* feed with enriched *Artemia* nauplii.

	0% DHA	5% DHA	10% DHA	15% DHA
Weight (mg)	106.3 ± 14.3^{a}	186.8 ± 19.0^{b}	275.3 ± 111.2^{c}	240.1 ± 57.9^{bc}
Lenght (mm)	19.2 ± 0.6^{a}	23.3 ± 0.1^{a}	26.3 ± 4.5^{bc}	26.6 ± 2.4^{c}
Survival (%)	9.6 ± 9.9	20.0 ± 11.1	23.3 ± 25.4	39.8 ± 6.7

Larval DHA content was affected by dietary treatments with the lowest DHA level found in the 0% DHA treatment (0.85 ± 0.78% of TFA) and the highest DHA content in the 10% DHA level (2.47 ± 0.78% TFA); however due to the high variability of the data no significant differences were found (Table III).

Table III.Selected fatty acid profile of *Paralichthys californicus* whole body of 1 and 50dph larvae fed with *Artemia* nauplii enriched DHA levels (% of total fatty acids), mean ± standard deviation, n = 2.

Fatty acids	Initial	0% DHA	5% DHA	10% DHA	15% DHA
Total *n*-6 PUFA	14.69	17.93 ± 1.45	17.32 ± 5.02	16.69 ± 1.22	11.47 ± 2.63
C18:3 *n*-3	0.00	7.16 ± 4.55	7.70 ± 3.30	6.86 ± 1.94	2.85 ± 1.63
C20:5 *n*-3	2.56	1.64 ± 1.74	1.56 ± 1.55	3.46 ± 1.00	4.27 ± 2.92
C22:5 *n*-3	1.94	0.28 ± 0.48	0.44 ± 0.76	0.55 ± 0.16	0.00
C22:6 *n*-3	5.31	0.85 ± 0.78	2.29 ± 2.15	2.47 ± 0.78	1.75 ± 2.89
Total *n*-3 PUFA	9.81	9.93 ± 7.35	11.99 ± 5.75	13.34 ± 3.81	8.87 ± 5.49
Total PUFA	24.50	27.86 ± 8.75	29.31 ± 10.61	30.03 ± 4.95	20.34 ± 8.11
n-3/ *n*-6	0.36	0.55 ± 0.35	0.69 ± 0.20	0.80 ± 0.17	0.77 ± 0.32
DHA/EPA	2.07	0.52 ± 0.10	1.46 ± 7.12	0.71 ± 0.13	0.41 ± 0.61
EPA/ARA	1.19	0.35 ± 0.35	0.24 ± 0.27	0.69 ± 0.07	1.73 ± 2.52

a, b, and c superscripts denote significant differences among diets (P > 0.05)

Based on a second-order polynomial regression model, the recommended DHA level in the diet to achieve the highest number of normally pigmented juveniles as estimated here for California halibut at 75dph, is 2.40% DHA of the TFA in the diet and 11.61% DHA of the enrichment emulsion. Similarly, for maximum growth and weaning success we recommend 2.49% DHA of the TFA on the diet and 14.08% DHA of the TFA of the emulsion. Additional research needs to be performed to evaluate the effect of EFA ratios (i.e., DHA:EPA:ARA) in California halibut larvae culture.

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FUNCTIONAL DEVELOPMENT OF THE DIGESTIVE SYSTEM IN ATLANTIC COD (*GADUS MORHUA* L.) LARVAE

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Atlantic cod (*Gadus morhua* L.) is one of the most important species in North Atlantic commercial fisheries. However, a predictive production of high quality juveniles is still a bottleneck.

Most marine fish species that are potential candidates for aquaculture have small pelagic and altricial larvae. Allometric growth is common during the larval development, and the most important features develop first, followed by those with lower priority for survival. In addition to the head with its sensory organs, the digestive organs are examples of organ systems which are prioritized during the early development.

In contrast to production of fry of salmonids, the production of many marine fish larvae relies on fairly complex feeding protocols involving micro algae and several live feed organisms, such as rotifers and *Artemia.* In order to reduce the reliance on live prey it is of interest to introduce formulated diets as early as possible to ensure a stable supply of nutrients. Experimental weaning of cod larvae before metamorphosis is often accompanied by reduced survival and growth rates compared with larvae fed cultivated live feed.

Cod larvae are capable of digesting proteins and lipids from the time of mouth opening, but digestive capacity does not depend only on digestive enzyme activity. Functional development of the gut and the liver are important parameters to be studied.

In this study, the functional development in relation to growth and nutrition was studied in cod larvae. Morphological development of liver and intestine were studied using stereology. Stereological techniques are powerful tools to quantify the growth and development of living tissue, such as organ volume and surface area, cell and organelle size, and numbers of organelles per cell.

The cod larval midgut brush border surface area increased 840 times in 18 days, demonstrating the rapid changes cod larvae go through during early development. During the same period midgut volume increased 40 times while larval dry weight increased 28 times. The liver developed allometrically, increasing its size 160 times while the larval dry weight increased 50 times in 37 days. Regardless of larval growth rates, energy was put into growth and cod larvae did not store larvae amounts of energy in the liver, making larvae vulnerable to starvation.

The midgut and the liver had similar developmental patterns, with hypertrophy, hyperplasia and increased density and number of mitochondria per cell. The development of these organs was closely related to larvae size rather than larval age and occurred rapidly during the larval exponential growth phase.

Liver morphology demonstrated the most sensitive parameters for cod larval growth rates and nutritional status. Hepatocyte nucleus size was reduced during weaning to formulated diets when growth rates were lower. The same pattern was observed in larvae fed formulated diets differing in nutritional compositions. In larvae suffering from malnutrition/starvation, swollen hepatocyte mitochondria were observed. Even in larvae in the exponential growth phase suffering from nutritional stress, mitochondria were enlarged. Lack of pathological effects in the midgut indicated that the gut tissue is flexible and can withstand periods of suboptimal food supply.

This study showed that cod larvae go through extensive and rapid development during the beginning of the exponential growth phase and before the development of a functional stomach. The results showed a positive correlation between larval growth and the functional development, demonstrating that dietary treatment may delay larval development on early stages of development. Therefore, for further success in cod rearing the larval phase should be carefully considered.

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EARLY DEVELOPMENT OF PRELEPTOCEPHALUS LARVAE OF THE JAPANESE EEL IN CAPTIVITY

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The Japanese eel *Anguilla japonica* is naturally found in Far East Asia and is a commercially important species. Intensive commercial culture of eel was initiated in Japan more than one hundred years ago, and nowadays the eel culture industry is large and important not only in Japan but also in Taiwan and China. In spite of its large farming production, the culture of eel still depends totally upon field-collected wild glass eels as seedlings, but the catch of glass eels is reported to be fluctuating annually with a continuous decreasing trend due to various reasons. Therefore the establishment of production techniques of glass eel is highly desired among fish farmers.

The first captive-bred glass eel was produced in the National Research Institute of Aquaculture (NRIA) in Japan using a kind of slurry-type diet made from spiny dogfish eggs as an initial feed, but the rearing techniques for mass production have not yet been established. The survival rate of eel larvae during its early developmental stage in captivity is still very low. The main constraint preventing development of Japanese eel larvae production is related to the initial feed used for eel larviculture. Preleptocephalus larvae of eel cannot be produced using *Brachionus* rotifers as done in many other marine teleost hatcheries.

In this research, the early development of preleptocephalus larvae of Japanese eel, with special reference to the ontogenetic development of larval feeding organs and their functions, were studied to clarify why rotifers were not suitable for the rearing of eel larvae. The rearing of the Japanese eel larvae was conducted using the facilities of NRIA in 2004-2007, under a research project sponsored by the Agriculture, Forestry and Fisheries Research Council of Japan.

Fertilized eggs were obtained by hand-stripping mature adults induced by hormonal treatments using NRIA protocol. The eggs were incubated and the newly-hatched larvae were reared in two different temperatures (21°C and 25°C) in 32-34psu sand-filtered seawater. The larvae were reared in the dark until the completion of yolk-absorption (10dph at 21°C, and 8dph at 25°C). Embryonic and early development until the completion of yolk was observed under microscopes

using live specimens and based on body measurements. Preserved and/or haematoxylin-eosin-stained specimens were used for the observation of larval morphology and histological studies of the digestive organs of eel larvae.

The eggs of the Japanese eel are transparent, non-adhesive, pelagic, and spherical in shape with a characteristic wide perivitelline space obtained after hydration (max. egg diameter: 1.6-1.7mm). Initial separated oil globules gradually gathered and eventually formed a single large globule (about 20% of egg diameter). Embryonic development was almost identical to that of other marine teleosts with pelagic eggs. Hatching of larvae began 45h after the fertilization at 21°C and 31.5h at 25°C and terminated within 3.5-4h after the onset of hatching. The mean total length of newly hatched eel larvae was 2.8-3.0mm. One of the biggest morphological characteristics of eel larvae is the appearance of four pairs of sharp pointed teeth at about one week after hatching. On 10dph at 21°C (211.7day-degrees) and 8dph at 25°C (201.5day-degrees), larvae of about 6.8-7.1mm TL completed yolk absorption and their larval teeth were fully developed and became functional under captive condition. At the present moment only the soft and slurry liquid-form diet made from shark-egg is applicable for larval rearing of eel in captivity. If larvae are fed on solid feeds including rotifers, eels larvae cannot swallow them even if particle size is small enough, probably due to the very thick oesophageal walls in eel lacking the mucous cells that facilitate swallowing feed particles.

The natural foods that eel larvae ingest in their natural nursery areas in the Pacific Ocean are still not completely understood, but the results of the present research would become an important clue to develop novel rearing feeds for eel larvae in the future.

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AMINO ACID PROFILES IN *SPARUS AURATA* LARVAE UNDER DIFFERENT FEEDING CONDITIONS – TESTING VEGETABLE PROTEIN SOURCES IN MICRODIETS

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Introduction

Proteins of vegetable origin are products offering a certain variety in the amino acid profile depending on the species and may be an interesting ingredient in larval diet formulation. Having this in mind, variations in total amino acid (AA) profiles related to different feeding conditions (live prey, microdiets and starved) were measured in early *Sparus aurata* larvae obtained from different spawns collected during two years. A first objective of the present work was to assess to what extent changes in feed quantity would be reflected in the amino acid profile of larvae. A second objective was to use such information as a reference for a further nutritional evaluation of microdiets which included a certain proportion of vegetable protein hydrolysates. Results were compared to previously published amino acid profiles in this species, in order to assess their potential variability.

Material and methods

Larvae were reared in 300-l tanks at 19.5ºC and 35g.l^{-1} and fed rotifers (*Brachionus rotunduformis* and *B. plicatilis*) and *Artemia* nauplii according to standard protocols. Determinations were carried out at different developmental stages on larvae either fed on live food or experimental microdiets, as well as on starved individuals. Whole-body amino acid content on larval pools were analysed by reversed-phase HPLC using D,L-α aminobutyric as internal standards (Alaiz et al., 2004). Microdiets were prepared according to Yúfera et al. (2005). The microdiets differed in the source of fish and vegetable hydrolysates (CPSP-90, rapeseed, sunflower and chickpea) used in their formulation. All diets were prepared with 27.5% fish meal, 30% of cuttlefish meal and 10% of protein hy-

drolysate on weight basis. Only particles with a diameter ranging between 80 and 200µm were considered in the present study.

Results and discussion

Changes in the AA profiles in rotifer fed larvae are shown in Figure 1. The general trends observed during embryo and growing phases were different. Threonine, isoleucine, leucine, serine, and alanine decreased during the endogenous feeding period, while glycine, asparagine + aspartic acid, and glutamine + glutamic acid increased. Lysine and cysteine showed small variations during the whole considered period. The day of first feeding is clearly discernible with the noticeable change in the content of some AA (methionine, valine, tyrosine, and asparagine + aspartic acid).

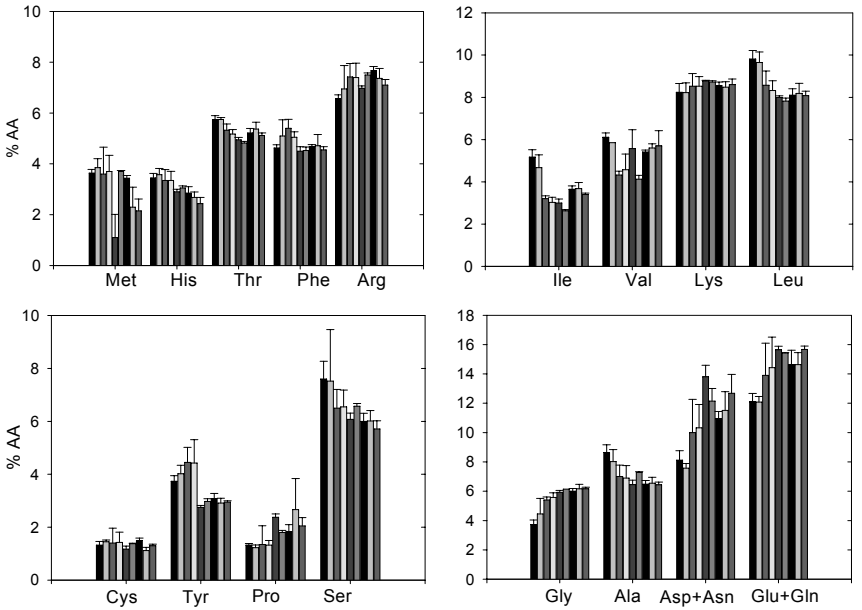

Fig. 1. Total amino acid profiles during embryo and larval development of *Sparus aurata* fed on live prey. Each bar group indicates the values at 0, 1, 2, 3, 4, 6, 9, 11, and 15 DAH consecutively.

On the other hand, unfed larvae after the mouth opening showed significant increase in glycine and decrease in isoleucine, leucine, threonine, and valine. This result indicates that larvae need to use also essential AA as metabolic fuel during this short period from mouth opening up to 9DAH. No significant changes were observed in other AA. Significant differences between profiles in fed and unfed larvae (ANCOVA; $p<0.05$) were found only in four AA; unfed larvae had higher

arginine and tyrosine content, while fed larvae had higher glutamic and proline content.

The microdiets prepared with different protein hydrolysates showed small differences in their profiles, being methionine and proline the AA showing a greater variation among the microdiets. The comparison of the profiles of indispensable AA in each microdiet to that of a 9-day-old larva is shown in Figure 2. All diets showed deficiencies in some AA; while microdiets including fish (CPSP90) or chickpea hydrolysates were deficient in valine and metionine, (being this latter particularly noticeable in the chickpea diet), those including rapeseed or sunflower hydrolysates exhibited deficiencies in valine and arginine.

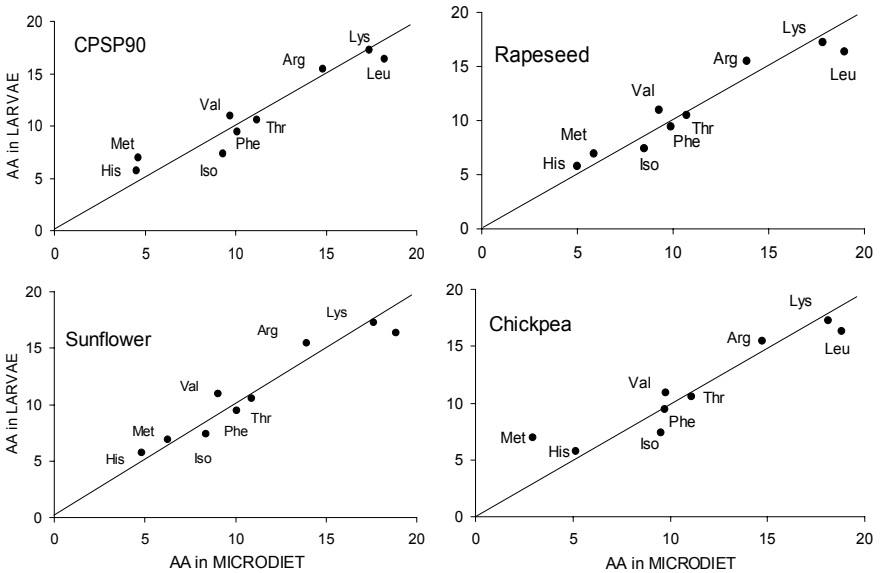

Fig. 2. Comparison of indispensable AA ratio of 9 DAH larvae with microdiets prepared with different hydrolysate sources.

It is interesting to remark that the present results are not exactly similar to those previously published for this species (Aragão et al., 2004). The main differences were observed in threonine, isoleucine, leucine, cysteine, tyrosine and proline. Such variations could be due to the use of totally different spawns or differences in larval rearing or analytical procedures, but in any case it will be necessary consider and examine the existence of such variability in future determinations.

Preliminary results on larvae fed on microdiets did not show significant differences in the body AA profile with larvae fed on live prey. Present results indicates that AA composition of microdiets including vegetable protein hydrolysates resembles to a great extent that of a microdiet prepared with a commercial

fish protein hydrolysate. Further experiments and analyses using vegetables protein isolates and hydrolysates, currently on course, will contribute to properly explore the potential of these ingredients in the formulation of larval microdiets, as well as to minimise dietary AA imbalances.

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