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DETERMINISM AND CAUSATIVE FACTORS FOR MORPHOLOGICAL ANOMALIES IN REARED EUROPEAN FISHES

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The presence of sublethal morphological deformities represents one of the main bottleneck of the industrial finfish hatchery production, resulting in major economic loss due to reduced growth and marketing ability of the final product, that has to be transformed (filets) or sold for fish flour. Furthermore, the elimination of deformed fishes from the productive cycle needs for periodic selections at present carried out by manual sorting. This represents an additional economic cost, and a stress for fishes. Some ethical issues (fish welfare) are also involved for experienced and future life: malformed fish are often unable to properly nourish, swim, escape from cannibal fishes and fall more easily victim of pathogens. They grow slowly and sick. Finally, commercialized fish with different morphology from the wild animal induces diffidence in consumers regarding aquaculture products.

Among morphological anomalies, deformed bones and cartilages, anomalous meristic counts, malformed scales or lateral line, anomalous shape, abnormalities in the olfactory organ, eye cataract and inflammation are quite widely reported in reared Teleosts.

As far as causes are concerned, one statement shared among all the scientists involved in morphological anomalies is that many epigenetic different causes can induce the same morphological anomalies in different species and that the same causes can induce different malformation in diverse fish species.

A quite exhaustive list of putative causative factors is achievable from scientific literature but the causative factors are only partially understood. Basically, there are as many causative factors as there are biological fields: genetic, nutritional, physiological, physical, chemical, biomolecular and environmental causes are blamed by the different authors for inducing pigmentation or skeletal anomalies in fish. In some cases, sibling juveniles show a different typology and incidence of skeletal anomalies if reared in different conditions, from which it may be inferred that an epigenetic control is exerted over skeletal development and growth. In other cases, genetic factors have undoubtedly a causative effect.

The main causes individuated as inducers of morphological anomalies are epigenetic and genetic ones. Among epigenetic causes, the following are the most investigated:

- epigenetic conditions: broodstock and egg quality, stock density, fast growth conditions (all the conditions facilitating growth, such as light treatment, high water temperature, high feed level), handling stress, hydrodynamism/water turbulence/water supply rate, intensive vs semi-intensive hatchery conditions, light, mechanical factors, oil films on water surface, levels of O_2/CO_2, pH, physical trauma/mechanical stress, pathogens, parasites, radiation, salinity variation, typology of substratum (mainly for flatfish), tank (volume, shape, color, material) characteristics; variation of temperature, toxins;
- xenobiotics with teratologic effects on fish: antibiotics, hormones, heavy metals, herbicides, metals, non-ionised ammonia, PCBs, pesticides, pollutants;
- nutrition: indirect nutritional deficiencies, underfeeding, inert diet, aminoacids, mineral deficiencies, oxidized lipids, peptides (hydrolysates), phospholipids, PUFAs, and lipid and water soluble vitamins, *e.g*. Vit A, Vit. C, Vit. D, Vit. K, manganese, copper and zinc;
- physiology: distress, inflammation, immunological responses, thyroid hormone growth regulation, effects of vaccinations;
- association with other anomalies; mechanical factors; pathogens, parasites; toxins.

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The genetic causes mainly deal with: ascertained heritability for some teratologies, genetic drift, generic genetic factors, genetic modifications, inbreeding, mutation, selective breeding, triploidy. However, some idiopathic causes are still individuated.

Up to now, available data seem to indicate that anomalies are the consequence of so many influential factors acting and interacting among themselves that interdisciplinary studies combining anatomic, genetic and biomolecular with physiologic data on larvae welfare conditions will probably be necessary.

Vertebral malformations may now be the norm in hatchery lots: it is therefore clear that all reared fish should be considered as 'distressed' fish, in which epigenetic and genetic factors strive to buffer the environmental noise effects but not always successfully. Intensive alimentation and the lack of predators allow the deformed individuals to live and that is the problem for the farmers.

Acknowledgments

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CHARACTERIZATION OF LORDOSIS-KYPHOSIS VERTEBRAL DEFORMITIES IN JUVENILE SENEGALESE SOLE (*Solea senegalensis*, KAUP 1858) BY IMMUNO-HISTOCHEMICAL TECHNIQUES

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Introduction

Senegalese sole (*Solea senegalensis*) is an important species for the aquaculture industry in Southern Europe, particularly in Portugal and Spain (Dinis et al., 1999), reaching high commercial values. As in other produced species, Senegalese sole exhibits a high incidence of vertebral deformities (Gavaia et al., 2009). In sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) some histological studies focusing on deformed vertebrae have already been developed (Kranenbarg et al., 2005b) showing that in an adaptive response to increased strain, the affected vertebrae presented an increase in bone formation as well as the development of chondroidal ossification at the articular surfaces (Kranenbarg et al., 2005a; Roberto et al., 2007). Unlike this actively swimming species, Senegalese sole has a typical benthic lifestyle through juvenile and adulthood so differences in histological and physiological adaptations to vertebral deformities are expected. However, little is known about the histological/histochemical/physiological alterations resulting from spine curvature vertebral deformities in Senegalese sole. In this scope, we have carried out a study in order to characterize vertebral deformities in Senegalese sole using histological, histochemical and immunochemical approaches to verify the response at the tissue and cellular level, concerning bone mineral deposition and resorption and effects in associated tissues.

Materials and methods

S. senegalensis eggs were obtained from a spawning stock at A. Coelho & Castro, Lda. and then incubated at IPIMAR facilities where larval rearing was also conducted. Fish were maintained under standard conditions with the following feeding schedule: rotifers from 3 to 14 days post hatching (dph), fresh *Artemia* nauplii EG from 8 to 22 dph, frozen *Artemia* nauplii EG from 15 to 56 dph and inert food beginning at 29 dph. For the analyses we have used juvenile Senegalese sole showing clear lordotic/kyphotic lesions. Normally developed specimens were used as reference. Vertebral tissues were embedded in methacrylate and 5 µm sections were performed. To determine what happens to deformed vertebrae and associated tissues and alterations in bone forming and resorption processes, we have characterized the deformities at histological, histochemical and immunohistochemical levels. Sections were submitted to toluidine blue and azan trichrome for general tissue staining and later on von Kossa's staining was performed to reveal mineralized structures. Determination of alkaline phosphatase activity sites was carried out to infer about bone formation sites, as well as the immunolocalization of bone markers accumulation, such as osteocalcin (Oc) and matrix gla protein (Mgp). Sections were then analyzed and photographed in an Olympus IX-81 microscope equipped with an Altra20 camera.

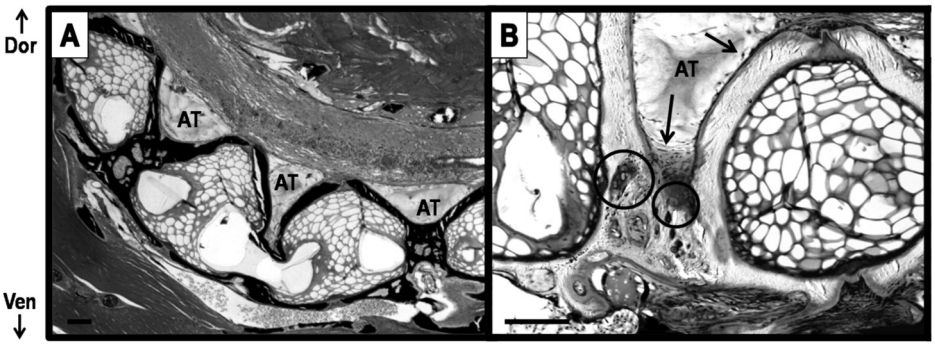

Figure 1: Lordotic deformity in *S. senegalensis*, showing the formation of atypical adaptive tissue (AT). von Kossa's staining showed the absence of mineralization within this tissue (A) and Toluidine blue staining revealed the presence of fibroblast-like cells (arrows, B) and chondrocyte-like cells within the deformed vertebral centra (circles, B). Scale bars represent 100 µm.

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Results

The analyses showed that, compared to non-deformed vertebrae, deformed ones developed what appeared to be a colloid tissue within the areas submitted to higher mechanical strains, located in the articular inner surfaces between vertebral centra involved in lordotic/kyphotic vertebral deformity. Toluidine blue and azan trichrome staining procedures allowed the detection of a few fibroblast-like cells sparsely distributed within this matter, revealing the formation of an ectopic tissue in response to the altered strains exerted on deformed vertebra. von Kossa's staining excluded the hypothesis that this tissue might be under mineralization since, unlike vertebra and arches, none of the surrounding tissues presented any kind of staining. Furthermore, the ectopic presence of chondrocyte-like cells within the inner-most part of the deformity cohesive to bone in vertebral centra was detected by general staining procedures.

Discussion

Like other reared teleosts, *S. senegalensis* seems to undergo adaptations leading to the formation of ectopic tissue at the articular surfaces of vertebrae subjected to increased strains due to lordotic or kyphotic vertebral deformities. This response will apparently reduce strain effects within vertebrae since, as proposed by Kranenbarg (2005b), an increased articular surface area will reduce the pressure on the dorsal zygapophyses. However, in Senegalese sole, the formed tissue does not appear to be chondroid as in *D. labrax* (Kranenbarg et al., 2005a, b) or in *S. aurata* (Roberto et al., 2007), but seems to be a fibrous colloid-rich tissue. Possibly because of *S. senegalensis* less active and benthic lifestyle, the production of a tissue such as chondroid is not as essential as in actively swimming teleosts. However, the presence of chondrocyte-like cells within the articular surfaces with increased strain of deformed vertebrae suggests that there are similar response mechanisms leading to the adaptive processes on vertebral curvature deformities. This also suggests that, like observed in other species, the abnormal presence of chondrocyte-like cells might have an important adaptive role within vertebral deformities in response to increased strains in affected areas (Hall, 2005).

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EFFECTS OF DIETARY LIPID SOURCES ON BONE COMPOSITION AND METABOLISM IN GILTHEAD SEABREAM (*Sparus aurata*) JUVENILES

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Introduction

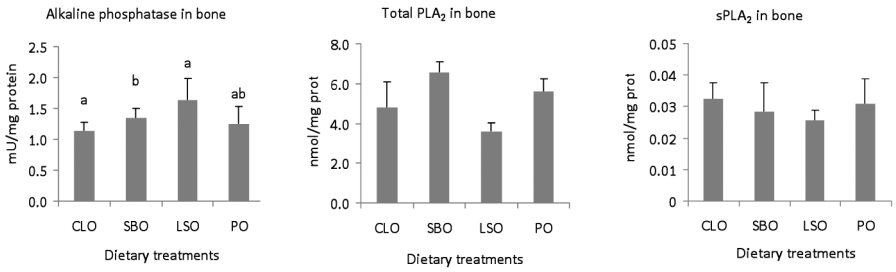
Presently, the replacement of significant amounts of marine fish oil by vegetable oils is a major trend in the aquaculture feed industry. Most studies involving fish oil replacement by vegetable oil sources show clear changes in the pattern and ratios of fatty acids circulating in plasma and deposited in various tissues. However, the metabolic effects of such changes are still under evaluation in fish. Emerging evidence from human and terrestrial vertebrate studies supports the hypothesis that dietary lipids play an important role in skeletal biology and bone health. Several studies showed that dietary lipids alter the fatty acid composition of bone compartments, which would impact the local production of factors influencing bone modeling in animals. In humans, the mechanism has been largely attributed to reduced prostaglandin E2 synthesis in bone. Knowledge on the mechanisms underlying the nutritional regulation of bone metabolism is extremely scarce in fish.

We speculate that changes in the dietary ratio of n-6:n-3 fatty acids may modulate tissue eicosanoids production and affect bone formation in fast-growing gilthead seabream, an important fish species for aquaculture in the Mediterranean region. Therefore, a trial was undertaken to evaluate the effect of dietary lipid sources as modulators of bone fatty acid and mineral composition, mineralization pattern and skeletal metabolism in seabream juveniles.

Material & Methods

Four isoproteic (crude protein, 46% DM) and isolipidic (15% DM) experimental diets were formulated to fulfill the nutritional requirements of juvenile seabream. Diets were formulated with purified ingredients to allow a detailed control over its composition and guarantee the target dietary changes. The dietary lipid sources under testing were: a) cod liver oil (CLO, a source of n-3 HUFA); b) soybean oil (SBO, a source of n-6 fatty acids); c) linseed oil (LSO, a source of n-3 fatty acids, non HUFA); and d) palm oil (PO, a source of saturated fatty acids).

Twelve homogenous groups of 25 seabream juveniles each, with a mean initial body weight of 39.3 g were stocked in circular plastic tanks (volume: 90L; water-flow rate: 3.5L·min^{-1}), supplied with flow-through seawater (temperature: 24±1°C; salinity: 33-34g·L^{-1}, dissolved oxygen above 6mg·L^{-1}). A 12/12 fluorescent light/dark cycle was adopted. Each dietary treatment was tested in triplicate tanks over 46 days. Fish were fed to apparent satiety, by hand, four times a day and feed intake was recorded on a weekly basis. Utmost care was taken to avoid feed losses. All fish were individually weighed at the beginning, bulk weighed every three weeks and at the end of the trial, following one day of feed deprivation. At the beginning and immediately before the end of the trial, fish from each tank were be immersed for 5 min in a 0.1% tetracycline bath to mark the mineral deposition of fish and allow the identification of the mineralization patterns associated to experimental feeding. Samples of the vertebral column (freed of soft tissue) were collected in both initial and final fish and frozen at -80°C until subsequent analysis in bone of fatty acid profile, calcium and phosphate content, activities of alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), Ca-dependent (cPLA2) and secretory (sPLA2)



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phospholipase A2 activity in bone homogenate. Vertebrae from tetracycline marked fish were fixed in formalin and processed for methacrylate embedding and sectioning. Differences in mineral deposition were determined by measuring the distance between the two tetracycline fluorescent bands under microscope observation. Points of active bone resorption/remodelling were identified by observing gaps in the marked bands.

Results

Overall growth performance criteria were not significantly affected by the various dietary experimental treatments ($P>0.05$). Over a period of 46 days, fish reached a final body weight of ranging from 75 to 80g and DGI for varied between 1.80 and 1.99. Feed efficiency ranged from 0.58 to 0.66. Whole-body composition of fish was not affected by dietary treatments.

As expected, analysis of the fatty acid composition of the experimental diets reflected that of dietary oil sources, confirming that CLO diet had the highest level of ω-3 HUFA, SBO diet the highest level of ω-6 fatty acids, LSO diet a high level of ω-3 fatty acids but non HUFA and PO diet the highest level of saturated fatty acids. The proximate composition of bone, namely moisture, ash and fat level are significantly affected by dietary treatments ($P<0.05$). Preliminary data on the fatty acid profile of bone suggests that its composition is altered by the dietary fatty acid profile. However, the patterns associated to a dietary modulation also suggest the occurrence of conservative mechanisms for some specific fatty acids. Activities of ALP (a marker of bone turnover) and phospholipase A_2 (regulatory step in arachidonic acid degradation, a precursor of eicosanoids) clearly show that dietary lipids have significant effects on bone metabolism in seabream.

Microscopic observation of tetracycline-marked vertebrae from fish fed the various experimental diets allowed us to identify active points of bone resorption/remodelling. While the calcium content of bone was little affected by the dietary treatments, bone phosphate level in seabream fed the CLO diet was significantly higher than those found in fish fed the vegetable oil diets.

In the overall, our data suggests that dietary lipid sources are important modulators of bone metabolism in a marine teleost, such as gilthead seabream.

Acnowledgments

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EFFECT OF TWO CULTURE CONDITIONS ON GROWTH AND SKELETOGENESIS OF *Solea senegalensis* EARLY LIFE STAGES

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Introduction

Senegalese sole (Solea senegalensis Kaup, 1858) is one of the flatfish of interest for diversification of Mediterranean and Southern Atlantic waters aquaculture. Interest in aquaculture for this specie is encouraged by a high market price but abnormalities that affect the morphology/anatomy are considered to be a major significant biological problem (Dinis *et al*., 1999). Indeed, fish produced under intensive conditions are known to develop a higher number and diverse skeletal deformities compared to wild ones. Previous studies suggest that malformations are induced at early stages, and larval nutrition at first feeding is one of the key parameters that affect skeletogenesis during early development in flatfish (Lall and Lewis-McCrew, 2007).

The objective of the present study was to evaluate the skeletal deformities occurring during the early stages of development of Senegalese sole when reared under extensive mesoscosm and intensive rearing systems.

Methods

Larvae were collected from natural spawning of Senegalese sole broodstock at the IPIMAR aquaculture station. The newly hatched larvae were distributed and maintained either in mesocosms or intensive rearing tanks using the standard rearing techniques described for this species (Engola *et al*., 2009).

To identify and quantify the skeletal deformities of specimens from the different systems, 20 to 30 larvae per tank were sampled at 45 and 60 days after hatching (DAH) and stained for bone and cartilage in whole-mount preparations using a modification of the method described by Walker and Kimmel (2007). After staining, skeletal abnormalities were observed, focusing on the cranial, axial and appendicular regions.

Results and Discussion

In this study, 162 individuals reared in intensive system and 160 individuals reared in extensive system were stained for bone and cartilage, observed and photographed (Fig.1). In addition larvae were also sampled at HCMR (Greece) using similar intensive (69 larvae) and extensive (75 larvae) rearing systems and data compared (table I).

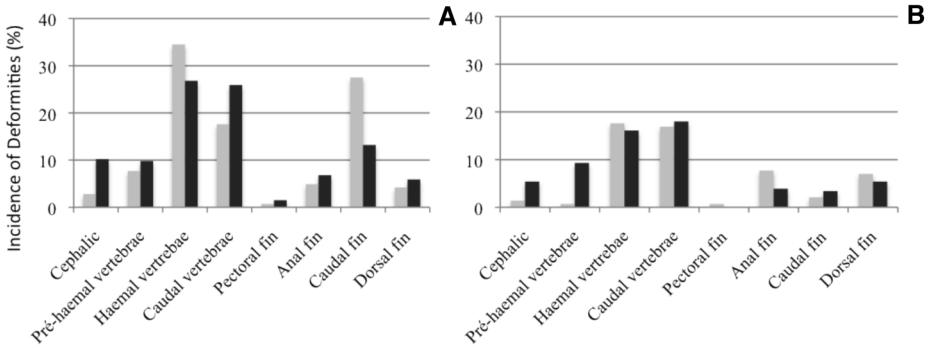

Figure 1. Distribution of the abnormalities detected according to the affected structures in 45 (gray) and 60 DAH (black) specimens cultured in intensive rearing system (A) and extensive (mesocosms) rearing system (B) at IPIMAR facilities.

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Sample	Deformed fish
Intensive IPIMAR	83,80%
Mesocosms IPIMAR	57,50%
Intensive Greece	60,80%
Mesocosms Greece	41%

Different studies have shown a high incidence of skeletal deformities in hatchery-reared early juveniles, ranging from 44% (Gavaia *et al.*, 2002) to 80% (Engrola *et al*., 2009).

In this study the incidence of malformations ranged from fish with only one anomaly to fish displaying multiple deformities with different degrees of severity. Overall, all the skeletal structures were affected by deformities, with special incidence in haemal and caudal vertebra and caudal fin in both rearing conditions. Among the abnormalities observed in Senegalese sole, the most common were vertebral fusions, malformed neural and haemal arches and spines, indicating that development of these structures may be the most susceptible to rearing conditions in captivity. However, the incidence of deformities affecting anal and dorsal fins did not differ between the two systems. At 45DAH (post-metamorphic larvae) the number of deformities was higher than at 60DAH. Intensive rearing system appeared to affect more the intramembranous ossification of vertebral centrums, which led to fused and compressed vertebra. In addition, this condition also affected those structures from vertebra and caudal fin formed by endochondral ossification, leading to morphological defects and fusions.

Larvae maintained in intensive system showed percentages of fish with one or more deformities between 60 and 83%, while fish maintained in mesocosms presented lower values (41 to 57,5%), although still higher than larval specimens captured in the wild in which levels of deformities did not go above 23%.

Conclusion

Under the present experimental conditions, and independently of the feeding treatment, Senegalese sole exhibited high levels of skeletal abnormalities, particularly in the vertebrae and caudal fin complex. Establishment of both nutritional and abiotic parameters involved in mechanisms leading to the appearance of these deformities must be determined in order to prevent a high incidence of malformations particularly under intensive culture conditions.

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EARLY SKELETAL DEVELOPMENT OF THE DUSKY GROUPER (*Epinephelus marginatus*, LOWE), PRODUCED UNDER EXTENSIVE MESOCOSMS CONDITIONS

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Introduction

The dusky grouper (*Epinephelus marginatus*) is an endemic species from the Atlantic and Mediterranean were it is becoming endangered. The first successful efforts for reproduction of this species were conducted in the 90's (Spedicato *et al.*, 1995) but the aquaculture production of larvae and juveniles for reintroduction still faces technical problems such as finding the correct feeding requirements. Furthermore there is a high incidence of skeletal deformities when the larvae are produced under intensive conditions (Boglione *et al.*, 2009). The production of high quality juveniles relies on the improvement of reproduction control, rearing conditions and feeding protocols, that will ensure wild-like individuals for reintroduction. This work is focusing on the ontogenic development of structures of the axial and appendicular skeleton, in particular the vertebral column and caudal fin complex since these structures are strongly affected by skeletal deformities.

Materials and Methods

Fertilized dusky grouper eggs were obtained from a captive broodstock maintained in a 10-m^3 indoor tank at the Aquaculture Research Station (EPPO) of the National Institute of Biological Resources (INRB-IPIMAR) with a mean density of 4.0 $kg.m^{-3}$ and fed a diet of fresh/frozen squid and sardine. Females were hormonally induced for spawning as described by Marino *et al*. (2003). Males were obtained from hormonally sex-reversed individuals according to Cabrita *et al.* (2009). Eggs were collected in sterile containers by abdominal massage, and *in vitro* fertilization was performed by the wet method (Marino *et al*., 2003). Fertilized eggs were incubated in 200-l cylinder-conical fiberglass tanks and newly hatched larvae were released into 3-m^3 mesocosms tanks.

Larval feeding was based on prey developed in the tanks, complemented after mouth opening by addition of enriched rotifers (*Brachionus plicatilis*) and later with *Artemia* sp. nauplii. Dry feed was gradually introduced after 18 DPH (days post hatching) (Cunha *et al*. 2009). Larval stages of *E. marginatus* (n=10) were sampled every day from hatching (0 DPH) until 10 DPH, every two days until 20 DPH and every 5 days until 50 DPH. The specimens were euthanized by excess 2-phenoxyethanol, weighted and measured for standard and total length and subsequently fixed in 4% paraformaldehyde. To visualize the skeleton the specimens were submitted to whole-mount double staining of cartilage and bone with alcian blue 8GX and alizarin red S, according to Gavaia *et al*. (2000). The cleared and stained specimens were observed under a Leica MZ6 stereomicroscope and images recorded with an Olympus C3030 camera.

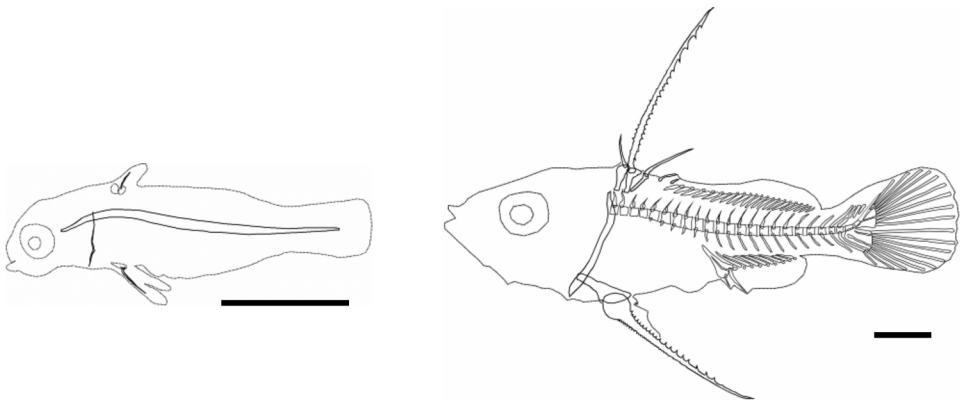

Figure 1. Early stages of development of the axial and a appendicular skeletal structures. At 8 DPH (left scheme), the pelvic fin spine and second dorsal fin spine appear. At 16 DPH (right scheme), all the vertebral structures are formed and undergoing calcification. The pelvic and dorsal spines have elongated distally and display serrated edges. The caudal complex is fully developed and the dorsal and anal fin pterigophores are under formation.

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Results and discussion

At hatching, the *E. marginatus* larval skeleton was formed only by the notochord, pectoral fin plates attached to the cleithrum and a primordial fin fold. At 8 DPH, the fast growing larvae showed the onset of the second dorsal fin and the first pelvic fin spiny rays (Fig. 1). The formation of first vertebral skeleton elements was visible at 10 DPH, with mineralization of the basioccipital process and the second cephalic vertebral centrum. The hypurals and caudal fin rays were first observed in 12-DPH larvae, which already displayed extensive calcification in cranial membranous bones, feeding and breeding skeletal elements. Final number of elements on the vertebral column was observed at 16 DPH (Fig. 1) with ossification still progressing on the posterior most vertebrae, arches and spines. The pelvic and dorsal spines have elongated distally and display serrated edges. All the caudal complex elements have developed and are calcifying. The dorsal and anal fin cartilaginous pterigophores are still under formation. At 35 DPH *E. marginatus* have completed the development of all axial elements with complete ossification of all the vertebral elements, caudal fin complex, dorsal and anal fins, but pelvic and pectoral fins are still undergoing differentiation and ossification of internal and external structures. The second dorsal and first pelvic spines have reduced the proportion to body length and are not conspicuous. The juvenile specimens at 50 DPH have completed the formation of all the axial and appendicular elements of the skeleton. We have not found any skeletal deformities in the specimens used for this study in contrast to what was observed in intensively produced fish, where an incidence of up to 78% was detected (Boglione *et al.,* 2009), indicating that *E. marginatus* produced under extensive conditions will be more similar to wild specimens, and more fit to be used in reintroduction attempts.

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THE *vasa* MATERNAL FACTOR IN SENEGALESE SOLE, *Solea senegalensis:* MOLECULAR CHARACTERIZATION AND GENE EXPRESSION PATTERN ANALYSIS DURING EARLY DEVELOPMENT AND ADULTHOOD.

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Introduction

Primordial Germ Cells (PGCs) identification and manipulation present considerable potential for hatchery practice and surrogate broodstocks. Donor PGCs from species whose hatchery practice is problematic can be transplanted to completely domesticated closely related species differentiating in donor species functional gametes. The development of such technologies requires the previous characterization of PGCs (Nagasawa et al., 2009). The gene *vasa* is a maternal factor and a molecular marker for PGCs that encodes a DEAD box family protein, which is a putative RNA helicase that was observed in both granules at the posterior end of the oocyte and the nuage structure in the germ cells in *Drosophila* (Kobayashi et al., 2000). *vasa* homologs have been cloned in several vertebrates, and the expression patterns in PGCs examined. Moreover, in tilapia (Kobayashi et al., 2000) and zebrafish (Krøvel and Olsen, 2004) a sexual dimorphic expression pattern of splice variants of *vasa* was described. The aim of this study was to clone the *vasa* gene from *S. senegalensis* and to establish its expression patterns during developmental stages and in adult organs, as a first step towards establishing germ cell transplantation techniques.

Materials and methods

Solea senegalensis embryos at different development stages were sampled at the Ramalhete Experimental Station (Faro, Portugal) while larval culture from 0DAH to 120DAH was carried out at IPIMAR (Olhao, Portugal). The adults used in this study were captured in the Gulf of Cadiz and maintained at the ICMAN-CSIC (Puerto Real, Spain) facilities. Samples were stored at -80ºC to perform total RNA extraction or fixed in PFA then stored at -20ºC in methanol to perform *in situ* hybridization. To amplify the *vasa* gene, total RNA from the gonad of an adult female was used as template and amplification was performed with *SMART™ RACE cDNA Amplification Kit* (Clontech). The RACE-PCR product was cloned into the plasmid pCR®2.1-TOPO® (Invitrogen). Quantitative real time PCR (QPCR) was performed to analyze *vasa* expression levels during embryonic and larval development and in tissues from adult fishes (kidney, spleen, brain, liver, testis and ovary were divided in three parts: anterior, medial part, posterior end). *AffinityScript QPCR cDNA Kit* (Stratagene), *Brillant II SYBER GREEN QPCR Master Mix* (Stratagene) and the *Miniopticon System* (Biorad) were used. Primers for the eEF1A1 (AB326302.1) housekeeping gene were chosen according to Infante et al., (2008). Efficiency standard curves were generated for each primer pair. QPCR results were analyzed using the Kruskal-Wallis non-parametric one-way analysis of variance for comparing more than two groups (tissues from adults fish or embryonic and larval stages) and Mann-Whitney *U* test for pairwise comparisons. Samples that resulted positive by QPCR analysis were hybridized with a (DIG)-labeled 3'UTR *vasa* riboprobe and *in situ* localization was performed according to Gavaia et al., (2006) with some modification.

Results

The Senegalese sole *vasa* cDNA (*ssvasa* cDNA) was approximately 2.0kb and showed high homology with other teleost *vasa* cDNAs (>80% identity). The resulting amino acid sequence contained the consensus sequences for the DEAD box protein family and a marked homology with VASA proteins of several teleosts (88%). QPCR results showed that in adult individuals the *ssvasa* was predominantly expressed in gonads rather than kidney, spleen, brain and liver. The testis expressed the highest level of *ssvasa* although it was not significantly different from the values observed for the posterior end of the ovary. Significant differences were obtained between *ssvasa* expression levels found in the three parts of the ovary. During embryonic development *ssvasa* expression was detected at the stage of 2-cells, 32-cells and early blastula. At mid-blastula stage the *ssvasa* expression level decreased significantly and disappeared at somitogenesis. *Ssvasa* expression was not detectable in larvae and young individuals before sexual differentiation age. *In situ* hybridization results confirmed the localization of *ssvasa* observed in QPCR positive samples.

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Discussion and conclusions

The complete *ssvasa* cDNA sequence was cloned and characterized. The nucleotide sequence and its deduced amino acids sequence showed high similarity with others *vasa* cDNA and Vasa proteins from several teleosts. The expression pattern observed by QPCR and *in situ* hybridization in gonads, embryos, larvae and fish before sexual differentiation suggested that the *ssvasa* mRNA characterized in this work is maternally provided as described in others organisms by several authors (Kobayashi et al., 2000). Nevertheless, zebrafish (Krøvel and Olsen, 2004) and tilapia (Kobayashi et al., 2000) display two isoforms of *vasa* homologues during germ cell differentiation and gonadal development. The *ssvasa* mRNA expression pattern described in this work showed some similarity with the observed during embryonic and larval development in zebrafish for its *vasa* isoform "*vas-l"* (AB005147) and in tilapia for its *vasa* isoform "*vas"* (AB032467). These results suggest the need for further studies to identify possible *vasa* splice variants in *Solea senegalensis*.

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IMPACTS OF DIETARY VITAMIN K SUPPLEMENTATION ON SKELETON QUALITY AND PROTEOME EXPRESSION OF SENEGALESE SOLE LARVAE

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Introduction

Skeletogenesis is a critical event during larval development of fish. A high incidence of skeletal deformities is commonly observed in marine fish hatcheries, affecting growth, morphology and survival of fish, and leading to an increase in production costs and a reduction of the market value of the final products. Since vitamin K is known to play an important role in bone metabolism, the aim of this study was to investigate the effects of dietary vitamin K_1 supplementation on skeleton deformities and on proteome expression of Senegalese sole.

Materials & methods

For the purpose, two experiments were conducted at one year interval (trial 1 and trial 2). Triplicate (trial 1) or quadruplicate (trial 2) groups of Senegalese sole (*Solea senegalensis*) larvae were fed from first-feeding onwards with rotifers and *Artemia* enriched with different levels of vitamin K_1 (0 to 250mg of vitamin K_1/kg of Selco). Larvae were sampled at 40 days after hatching. The quality of larval skeleton was analyzed though a double staining technique, using Alcian Blue to stain cartilage and Alizarin Red to stain calcified structures (Gavaia et al. 2000) in both trials. A comparative proteome expression analysis was performed by two-dimensional electrophoresis on entire larvae of trial 1, after removing of the head and the gut. Larvae from the same experimental conditions were pooled and four replicate analyses were performed per pooled sample. Proteins were extracted in a classical extraction buffer (containing urea, thiourea, CHAPS, dithiothreitol and a protease inhibitor cocktail), were isoelectric-focused on linear gradient pH 4-7 IPG strips in the first dimension and were then separated in a second dimension by SDS-PAGE on 4-12% Bis-Tris gels. Qualitative and quantitative analyses of gels were done with PDQuest 2-D analysis software. After spot detection and matching, the intensity volume of each spot was normalized by dividing it by the total intensity volume of valid spots. Evaluation of the statistical significance of spot variation between the two groups of larvae was performed using the non-parametric Mann-Whitney U test. Protein spots that exhibited statistically significant differences in normalized volume greater than 1.5-fold or lower than 0.67-fold between the two experimental conditions ($p<0.05$) were considered to be significantly differentially expressed. Some of the spots differentially expressed among the groups were excised from the gels, digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry. Peptides mass lists generated were used for protein identification through the MASCOT search engine (Matrix Science).

Results and Discussion

Larval skeleton quality analyses revealed that the incidence of deformed larvae tended to decrease with dietary vitamin K_1 supplementation in both trials, although the differences were not statistically significant. Moreover, larvae fed the diet supplemented in vitamin K_1, presented a statistically significant decrease in the number of deformities per individual, mainly due to a reduction in the incidence of deformities affecting the caudal area, compared to the larvae fed the control diet. Compared to control diet, supplemented diet induced a statistically significant decrease in the occurrence of vertebral fusions in the caudal area, deformed haemal arches in the haemal area in both trials. In trial 2, dietary vitamin K1 supplementation also induced a statistically significant decrease in the incidence of deformed hypurals.

Two-dimensional analysis of larvae proteome allowed the detection and the comparative quantification of a total of 486 protein spots across all gels. Among these spots, 76 showed significant variations between the two experimental groups, 47 were over-expressed and 29 were under-expressed in vitamin K_1 supplemented larvae relative to control. Among over-expressed protein spots in larvae fed the vitamin K_1 supplemented diet, some were identified as being involved in clotting process (fibrinogen beta), in cellular contractile system (myosin heavy chain) and cytoskeleton proteins (cytokeratin type I). In the same experimental group of larvae, some of the under-expressed protein spots were identified as being involved in energy metabolism (enolase, creatine kinase, ATP synthase F1 complex beta polypeptide), protein folding (chaperonin containing TCP1), in cellular contractile system (myosin light chain, myosin binding protein). Vitamin K_1 supplementation also induced a down-regulation of type VI collagen, which has already been shown to be expressed in most tissues and to be involved in matrix structural integrity and to bind other collagen fibrils and glycosaminoglycans.

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Conclusions

The results of this study showed that dietary vitamin K_1 supplementation in sole larvae leads to an improvement in larval quality, decreasing the incidence of skeletal deformities. The proteome analysis of the larvae revealed that this supplementation affected significantly the expression of 76 proteins. Among others, the vitamin K_1 supplementation affected specially clotting process and energetic metabolism, as well as the expression of type VI collagen, which is known to have a role in skeletal metabolism (Hall, 2005). Proteome analysis of the larvae from the trial 2 is underway and should extent the present results, contributing to a better understanding of mechanisms involved in skeleton development and the appearance of deformities in fish.

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