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The effects of dietary arachidonic acid on Senegalese sole morphogenesis: A synthesis of recent findings

Anaïs Boglino [a], Asher Wishkerman [a,b], Maria J. Darias [c], Pablo de la Iglesia [a], Alicia Estévez [a], Karl B. Andree [a], Enric Gisbert [a,*]

[a] IRTA-SCR, Unitat de Cultius Aqüicoles, Ctra. Poble Nou km. 5.5, 43540 Sant Carles de la Rápita, Tarragona, Spain
[b] School of Marine Sciences, Ruppin Academic Center, Michmoret, 4029700 Israel
[c] IRD, UMR 226 ISE-M, Place Eugène Bataillon Bât. 24– CC063, 34095 Montpellier, France

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ABSTRACT

In this study we evaluated the effects of high dietary arachidonic acid (ARA) levels on prostaglandin E_2 (PGE_2) and E_3 (PGE_3) production and their effect on two morphogenetic processes occurring during metamorphosis: the establishment of the juvenile pigmentation pattern and eye migration and remodeling of cranial bones. In this sense, Senegalese sole larvae were fed from 2 to 50 days post-hatch (dph) with live prey enriched with an experimental emulsion containing high levels of ARA (ARA-H; 10.2 and 7.1% TFA in enriched rotifer and *Artemia*, respectively) versus a reference commercial enriching product (Algamac 3050®, AGM; 1.0 and 1.4% TFA in enriched rotifer and *Artemia*, respectively). High dietary ARA levels did not affect larval growth performance at 50 dph, but significantly induced malpigmentation (81.4 ± 7.5%, versus 0.9 ± 0.3% in larvae fed the AGM diet). This malpigmentation was linked to the higher prostaglandin E2 (PGE_2) levels observed in pseudo-albino fish as compared to normally pigmented individuals. The PGE_2 levels were higher in normally pigmented specimens fed the ARA-H diet than in those fed the AGM diet. The effects of ARA on normally pigmented fish fed the AGM diet and pseudo-albino specimens fed the ARA-H diet were evaluated by means of the density of melanophores and the texture and image segmentation analyses in the dorsal skin of post-metamorphic fish. The skin of pseudo-albino specimens had a more uniform and homogeneous melanophore pattern than normally pigmented fish. Melanophores in pseudo-albino specimens were less abundant and not so aggregated in patches as they were in normally pigmented ones, whereas their shape differed (round vs. dendritic) suggesting their inability to disperse melanin. In addition, fish fed the ARA-H diet presented a higher percentage of cranial deformities (95.1 ± 1.5%) than those fed the control diet (1.9 ± 1.9%) that was significantly and negatively correlated with the incidence of normally-pigmented animals ($R^2 = -0.88$, $P < 0.001$). Cranial deformities in pseudo-albino fish were associated with an impaired migration of the eye from the ocular side (the right eye), whereas the left eye migrated from the blind side into the ocular side almost normally. The effects of high dietary ARA levels in the eye migration and cranial bone remodeling processes in post-metamorphic larvae were evaluated by means of the staining of cranial skeletal elements. Pseudo-albino fish showed higher interocular distance and head height than normally pigmented individuals, a different disposition of the eyes with regard to the vertebral column and mouth axes, and a distinct osteological development of some skeletal structures from the neuro- and splanchnocranium, in relation to high dietary ARA contents and high PGE_2 production. These results brought new information about possible nutritional forcing factors and physiological mechanisms of pigmentary disorders and impaired eye migration, which are current major bottlenecks in flatfish aquaculture.

1. Introduction

One of the most important events during the early stages of development of fish is metamorphosis.

This process is characterized by major morphological and physiological changes that are accompanied by a drastic shift in habitat and behavior (Rousseau and Dufour, 2012). In flatfishes (order Pleuronectiformes), this process involves the acquisition of body asymmetry with the migration of one of the eyes across the top of the head to the other side, as well as the maturation of different organs and systems (i.e. digestive tract, trunk musculature, erythropoietic tissue, nervous system and gills) in order to adapt the post-metamorphic specimen to its new benthic habitat (Inui and Miwa, 2012; Schreiber, 2013). From an aquaculture point of view, the development of an asymmetric and juvenile-like pigmentation pattern, as well as the migration

* Corresponding author. Tel.: +34 977745427; fax: +34 977443138.
E-mail address: enric.gisbert@irta.cat (E. Gisbert).

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of the eye and subsequent cranial remodeling might be considered as two of the major developmental processes occurring during flatfish metamorphosis with a great impact on larval quality (Bolker and Hill, 2000; Darias et al., 2013a; Power et al., 2008; Schreiber, 2013). In this sense, pigmentation abnormalities are considered as one of the most serious defects in cultured flatfish, since fish which manifest albinism and pseudo-albinism are either culled during production or rejected in the market by consumers, ultimately reducing profits and the sustainability of the sector. Additionally, juveniles with pigmentary disorders lack cryptic coloration and are therefore easily seen by predators in natural environments (Bolker and Hill, 2000; Inui and Miwa, 2012), which contributes to poor survival rates when hatchery-raised fish are used for stock enhancement programs (Miller et al., 2010; Yamashita and Aritaki, 2010). The development of symmetrical juvenile flatfish due to abnormal metamorphic craniofacial remodeling ("arrested development" or failure of the eye to migrate) and vertebral column deformities (Fernández and Gisbert, 2011; Power et al., 2008) is also a common phenomenon in mass cultured commercial flatfish species. Regardless of the anomaly considered, both developmental disorders generally take place during pre- and prometamorphosis (Inui and Miwa, 2012).

Metamorphosis in flatfishes is highly sensitive to intrinsic and extrinsic signals, leading to a tightly regulated climax event required for the successful achievement of the juvenile phenotype (Hamre et al., 2007; Power et al., 2008; Schreiber, 2013). This process is primarily controlled by the pituitary–thyroid axis and many biotic and abiotic factors have been described to disrupt metamorphosis (Inui and Miwa, 2012). Among these causative factors, larval nutrition at first feeding has been recognized by many studies as one of the key parameters that affect skeletogenesis and pigmentation (Boglione et al., 2013; Hamre et al., 2005; Lall and Lewis-McCrea, 2007). Two groups of nutrients, essential fatty acids and retinoids, have been shown to be the major dietary factors influencing flatfish pigmentation and skeletal disorders in hatchery reared flatfish (Fernández and Gisbert, 2011; Hamre et al., 2005; Inui and Miwa, 2012). Lipid and fatty acid nutrition are known to be one of the key factors involved in proper larval development and pigmentation in marine fish (Izquierdo and Koven, 2011) and particularly, proper levels of docosahexanoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA), as well as their ratio have been considered important for the proper development of skin pigmentation (Estévez and Kanazawa, 1995; Hamre et al., 2005) and bone formation (Boglino et al., 2012a; Dâmaso-Rodrigues et al., 2010; Roo et al., 2009). In contrast, less attention has been paid to arachidonic acid (20:4n-6, ARA) with regard to the above-mentioned essential fatty acids. However, ARA is the major precursor for eicosanoid synthesis, enhancing the immune system and resistance to stress, among other important physiological processes and it directly competes with EPA for the enzymes involved in prostaglandin (PG) biosynthesis (Bell and Sargent, 2003). High amounts of dietary ARA have been reported to result in imbalances in the relative content of EPA and DHA (Moren et al., 2011) and therefore, in the relative proportions of PGE_2 and PGE_3 (Bell and Sargent, 2003). This has been suggested to cause biochemical stress and developmental disorders related to pigmentation patterns (e.g. pseudo-albinism) and disrupted eye migration in several flatfish species (Copeman et al., 2002; Estévez et al., 1999, 2001; Hamre and Harboe, 2008; Hamre et al., 2007; Lund et al., 2008; McEvoy et al., 1998; Villalta et al., 2005, 2008).

In this study we present recent data on the effects of dietary ARA levels on several morphogenetic processes occurring during Senegalese sole (*Solea senegalensis*) metamorphosis in order to identify the effect of this essential fatty acid on the eye migration and cranial bone remodeling processes occurring during the transition from a pelagic to a benthic way of life, as well as the differentiation of pigment cells and the acquisition of the juvenile pigmentation type. In this sense, Senegalese sole was chosen as a model species since this is a high-value flatfish species whose intensive culture is still hindered by a high incidence of pigmentary and skeletal disorders (Fernández and Gisbert, 2011).

2. Materials and methods

2.1. Larval rearing and experimental feeding regimes

Fish larvae (1 day post-hatch, dph) used in this study were obtained from Stolt Sea Farm SA (La Carnota, A Coruna, Spain) and reared in eight, 100 l cylindro-conical tanks (initial density: 110 larvae·l^{-1}). Each tank was connected to a water recirculation unit (IRTAmar®) at IRTA-SCR facilities. Larval rearing conditions were as follows: temperature 16.7 ± 0.4 °C, salinity 35, pH 8.0 ± 0.2, dissolved oxygen 7.5 ± 1.3 ppm and photoperiod 16L:8D (500 lx at water surface). Feeding protocol was as follows: larvae were fed twice a day, from 2 to 10 dph, with enriched rotifers *Brachionus plicatilis* (10 rotifers·ml^{-1} from 2 to 8 dph and 5 rotifers·ml^{-1} from 9 to 10 dph). Enriched *Artemia* sp. metanauplii were supplied twice a day from 8 to 50 dph, at increasing densities ranging from 0.5 to 12.0 metanauplii ml^{-1}. From 30 dph onwards, when larvae settled to the bottom of the tank, enriched *Artemia* sp. metanauplii were supplied frozen as described by Villalta et al. (2007). Both types of live prey were enriched according to Boglino et al. (2013).

In order to evaluate the effects of dietary ARA on Senegalese sole morphogenesis, larvae were fed with live prey enriched with an experimental emulsion containing high levels of ARA (ARA-H; 10.2 and 7.1% TFA in enriched rotifer and *Artemia*, respectively) versus a reference commercial enriching product (Algamac 3050® Aquafauna, Biomarine Inc., AGM; 1.0 and 1.4% TFA in enriched rotifer and *Artemia*, respectively). The choice of using Algamac 3050® as a reference product for live prey enrichment was based on recent data from Boglino et al. (2012a,b). The experimental emulsion containing high levels of ARA was prepared from a mixture of two oils rich in DHA (cod liver oil, Fluka, Sigma-Aldrich) and ARA (Vevodar, DSM Food Specialties). Olive oil was added to the mixture in order to dilute and adjust n-3 polyunsaturated fatty acid (PUFA) concentration and α-tocopherol was included for preserving the emulsion from oxidation (Boglino et al., 2012c). Lipid and FA composition of enriching products, enriched live prey and early juveniles aged 50 dph was calculated as previously described in Boglino et al. (2012a) and values are shown in Table 1.

2.2. Larval survival and growth

Final survival was evaluated considering the number of sampled individuals during the experiment and the final number of fish in the rearing tanks. At the end of the trial (50 dph), 50 fish were randomly sampled from each tank and euthanized with an overdose of tricaine methane sulfonate (MS-222, Sigma) in accordance with EU regulations (EC Directive 86/609/EEC). Once euthanized, each fish was photographed (300 dpi image; Olympus DP25©, Olympus Corporation connected to a stereomicroscope Nikon SMZ 800) and the standard length (SL, mm) was measured to the nearest 0.1 mm using an image analysis software (AnalySIS, Soft Imaging Systems, GmbH, Olympus). Dry weight (DW, mg) was determined by rinsing larvae with distilled water to remove salt and then drying them at 60 °C for 24 h. Samples were weighed with an analytic microbalance (Sartorius BP211D).

2.3. Pigmentation analysis

The pigmentation success defined as the percentage of specimens displaying different levels of hypomelanosis (Bolker and Hill, 2000) and a pseudo-albino phenotype (Darias et al., 2013b) was determined in post-metamorphic fish at 50 dph. Pigmentation analyses – image segmentation and texture analysis procedures (Matlab; Mathworks Inc., Natick, Massachusetts, USA) – were conducted in order to evaluate