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# Senegalese sole (*Solea senegalensis*) metamorphic larvae are more sensitive to pseudo-albinism induced by high dietary arachidonic acid levels than post-metamorphic larvae

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ABSTRACT

High dietary levels of arachidonic acid (ARA) and its relative proportions with eicosapentaenoic acid (EPA), fed during early larval stages, have been associated with malpigmentation in various flatfish species. This study investigated whether the nutritional induction of pigmentary disorders at larval stages was related to a specific larval period of increased sensitivity to ARA in Senegalese sole (*Solea senegalensis* Kaup, 1858). Senegalese sole larvae were fed high dietary ARA levels during pre- and pro-metamorphosis (2–15 dph) and/or post-metamorphosis (15–50 dph). Larval tissues reflected the dietary fatty acid composition. Malpigmentations were significantly related to elevated dietary and larval ARA contents and ARA/EPA ratio. This study reports evidence for a "pigmentation window", with a higher larval sensitivity to dietary ARA during pre- and pro-metamorphosis than post-metamorphosis. High dietary ARA fed to larvae during pre-metamorphosis enhanced survival, but did not affect growth nor eye migration. The aspect and density of melanophores in the skin of the ocular side of ARA-induced pseudo-albinos were significantly reduced in comparison to normally pigmented individuals, even more in the pseudo-albino fish fed high dietary ARA levels during the pre-metamorphic stage. Pseudo-albino fish fed high dietary ARA levels during post-metamorphosis showed higher concentrations of 2- and 3-series prostaglandins ($PGE_2$ and $PGE_3$) than normally pigmented specimens fed the same diets. An increased sensitivity to ARA-induced malpigmentations has been identified at pre-metamorphosis and early metamorphosis in Senegalese sole. Supplying high dietary ARA amounts imbalanced the dietary ARA/EPA ratio and disrupted the relative concentrations of derived $PGE_2$ and $PGE_3$, resulting in 20 to 81.7% pseudo-albino individuals, depending on the dietary treatment. The administration of high levels of dietary ARA at different developmental stages did not only affect the incidence of animals with pigmentary disorders, but it also affect the melanophore density and skin aspect in normally pigmented and pseudoalbino fish as image segmentation and texture analyses indicated.



## 1. Introduction

Flatfish development and ontogenesis are characterized by a crucial event occurring at an early stage, the metamorphosis, a process associated with important morphological, physiological, hormonal, behavioral and ecological changes (Geffen et al., 2007). The functional modifications occurring during the transition of a larva to a juvenile in Pleuronectiformes include many critical processes, from the acquisition of asymmetry with the migration of one eye from one side of the body (blind side) to the other (ocular side), to the maturation of different organs and systems, bones and myomeres remodeling and the differentiation of pigment cells (Geffen et al., 2007; Power et al., 2008). The pigmentation of the ocular side of the fish, occurring during metamorphosis, makes this a critical period for the acquisition of the adult phenotype (Bolker and Hill, 2000; Darias et al., 2013a). Pigmentation development is highly sensitive to epigenetic factors, and their impact on the pigmentary process may vary depending on the stage of larval development (pre-, pro- and post-metamorphosis) at which they were exerted (Alves-Martins et al., 2011; Darias et al., 2013a; Power et al., 2008).

Lipid nutrition and fatty acid nutrition are known to be one of the key factors involved in proper larval development and pigmentation in marine fish (Bolker and Hill, 2000; Cahu et al., 2003; Hamre et al., 2007). High dietary contents of arachidonic acid (20:4n−6, ARA) have previously been associated to the incidence of pigmentary

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anomalies in various flatfish species (Darias et al., 2013a; Estévez et al., 1999; Hamre and Harboe, 2008; Lund et al., 2007; McEvoy et al., 1998; Villalta et al., 2005a). ARA is the major precursor for eicosanoid synthesis, enhancing the immune system and resistance to stress, among other important physiological processes (Bell and Sargent, 2003) and it directly competes with eicosapentaenoic acid (20:5n − 3, EPA) for the enzymes involved in prostaglandin biosynthesis; ARA gives rise to prostaglandins of the 2-series ($PGE_2$), while EPA is the substrate for the synthesis of prostaglandins of the 3-series ($PGE_3$) (Bell et al., 1995). High amounts of dietary ARA have been reported to result in imbalances in the relative content of other essential fatty acids 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. pseudoalbinism) and delayed eye migration in several flatfish species (Boglino et al., 2013; Copeman et al., 2002; Estévez et al., 2001; Hamre and Harboe, 2008; Lund et al., 2008, 2010; McEvoy et al., 1998; Villalta et al., 2008).

Senegalese sole (*Solea senegalensis* Kaup, 1858) is a high-value flatfish commonly reared under intensive aquaculture in Spain and Portugal, which larval quality is still hindered by the incidence of pigmentation disorders in some production batches. These pigmentary disorders in flatfishes generally affect fish survival, growth and development, downgrading the market value of the final product. The high incidence of malpigmented fish represents a current major bottleneck in flatfish farming and limits the development of the production sector for this species (Hamre and Harboe, 2008; Power et al., 2008). Pigmentary anomalies in flatfish have previously been described and qualitatively classified (Seikai, 1985). However, the study of pigmentation is complex and requires objective and quantitative tools to characterize the different types of pigmentation pattern, such as the skin texture image analysis (Bharati et al., 2004) that we used in this study.

In a recent study, the morphological and molecular ontogeny of skin pigmentation was characterized in the ocular side of Senegalese sole; this work provided the knowledge to elucidate the formation mechanisms of the adult pigmentation pattern and to understand when and how the pseudo-albino phenotype appears (Darias et al., 2013b). This study revealed different stages of skin pigmentation and development that coincided with the progress of metamorphosis and patterns of gene expression. Subsequently, we investigated the morphological development of pseudo-albinism in Senegalese sole larvae induced by high dietary ARA levels supplied at pre-, pro- and post-metamorphosis and the molecular signaling accounting for such a pigmentation impairment (Darias et al., 2013b). This study demonstrated that although larval pigmentation was genetically determined, the establishment of the adult pigmentation phenotype could be modified by nutrition (Darias et al., 2013b), which differently affect the pigmentation whether they intervene before, during or after the metamorphosis process (Power et al., 2008). In a study on common sole (*Solea solea*), Lund et al. (2008) established the concept of the "pigmentation window", revealing that ARA-induced malpigmentation and sensitivity to dietary ARA and EPA relative proportions were higher during pre-metamorphosis than at later stages of development. In order to deepen the understanding of the sensitivity of larvae to high dietary ARA levels, this study aims to investigate whether the induction of pigmentary disorders during ontogeny is related to a specific larval period of increased sensitivity to ARA in Senegalese sole, as well as investigate the effects of high dietary ARA levels on the melanophore density and skin aspect in normally pigmented fish and in those displaying pigmentary disorders.

## 2. Material and methods

This study was carried out in accordance with the recommendations made by Kilkenny et al. (2010). Animal experimental procedures were conducted in compliance with the experimental research protocol (reference number 4978-T9900002) approved by the Committee of Ethic and Animal Experimentation of the IRTA and the Departament de Medi Ambient i Habitatge (DMAH, Generalitat de Catalunya, Spain) in accordance with EU regulation (EC Directive 86/609/EEC).

### 2.1. Larval rearing and feeding protocol

One-day-old Senegalese sole larvae were obtained from Stolt Sea Farm SA (Carnota, La Coruña, Spain). Larvae (110 larvae $l^{-1}$) were reared in 16 cylindro-conical tank (volume = 100 l) connected to a water recirculation unit IRTA mar™ at IRTA-SCR facilities. Water conditions were as follows: temperature 16.7 ± 0.4 °C, salinity 35‰, pH 8.0 ± 0.2, dissolved oxygen 7.5 ± 1.3 mg $l^{-1}$, gentle aeration in each tank and 50% daily water renewal in the recirculation system. Photoperiod was 16L:8D, and light intensity was 500 lx at the water surface. The following feeding protocol was used: larvae were fed twice a day, from 2 days post-hatching (dph) to 10 dph, with enriched rotifers (*Brachionus plicatilis*), at a density of 10 rotifers/ml from 2 to 8 dph and 5 rotifers/ml from 9 to 10 dph. Enriched *Artemia* metanauplii were supplied twice a day to larvae from 8 to 50 dph, at increasing densities ranging from 0.5 to 12 metanauplii/ml, adjusted based upon the increase of larval weight. The daily food ration was calculated as described by Cañavate et al. (2006). From 30 dph onwards, when larvae settled to the bottom of the tank, enriched *Artemia* metanauplii were supplied frozen as previously described (Villalta et al., 2008).

### 2.2. Experimental design, diets and live prey enrichment

The effect of high dietary ARA levels on Senegalese sole larval development was evaluated during two different developmental stages, pre-metamorphosis and pro-metamorphosis (2–15 dph at 17 °C) and post-metamorphosis (15–50 dph at 17 °C), in order to determine the differential sensitivity of the larvae to these high levels. Rotifer and *Artemia* metanauplii were enriched with a commercial enrichment (AGM, Algamac 3050™, Aquafauna Biomarine Inc., USA). 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). In addition, both live prey were enriched with an experimental emulsion containing high levels of ARA (ARA-H) that was prepared using a mixture of commercially available oils rich in ARA (Vevodar®, DSM Food Specialties, Netherlands; 43% ARA, 0% DHA, 0.2% EPA) and DHA (cod liver oil, Fluka©, Sigma-Aldrich, Chemie GmbH, Steinheim, Norway) in order to meet the larval nutritional requirements in n − 3 PUFA (Boglino et al., 2012a). Olive oil was added to the cod liver oil and the Vevodar® mixture to dilute and adjust n − 3 PUFA concentration in enriched live prey whereas α-tocopherol was included in the emulsion as an antioxidant. Oil mixture was emulsified with soy lecithin and distilled water with an Ultra-turrax T25 at high speed for 60 s. The ingredients used in the formulation of the ARA-H emulsion and the fatty acid composition of both emulsions are shown in Table 1.

Four experimental groups (four replicates each) were constituted in order to evaluate the sensitivity of Senegalese sole to high dietary ARA levels: a control group fed live prey enriched with AGM during the whole experiment (C; 2–50 dph); a second group of fish fed live prey enriched with ARA-H during the whole trial (ARA-H +/+; 2–50 dph); a third group fed live prey enriched with AGM (control diet) during pre- and pro-metamorphic stages (2–15 dph) and then with *Artemia* enriched with the ARA-H emulsion during post-metamorphic stage (15–50 dph) (ARA-H −/+); and a fourth group fed live prey enriched with the ARA-H emulsion during pre- and pro-metamorphic stages (2–15 dph) and then with *Artemia* enriched with AGM (control diet) during post-metamorphic stage (15–50 dph) (ARA-H +/−) (Fig. 1).

Rotifers were enriched in 20 l containers at 500 rotifer/ml at 26 °C with 0.6 g $l^{-1}$ of each emulsion. Half of the rotifers were supplied to the larvae after 2 h of enrichment and the other half after 6 h post-enrichment. No significant differences with regard to the biochemical