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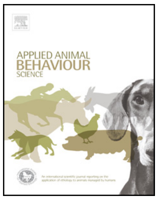

# Dietary fatty acid composition significantly influenced the proactive–reactive behaviour of Senegalese sole (*Solea senegalensis*) post-larvae

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ABSTRACT

Few studies have examined the influence of diet on larval proactive–reactive behavioural dimension of stress coping style responses. The present study evaluated the influence of using different vegetables oils (Linseed; Soybean; Olive) and fish oil (Cod liver) for *Artemia* metanauplii nutritional enrichment on the proactive–reactive behavioural responses of Senegalese sole (*Solea senegalensis*) post-larvae (40 days post hatch). Forty-two Senegalese sole larvae from each of the four replicate tanks per treatment were tested. Two tests were performed: a new environment individual-based test, which evaluate the larvae latency time to move, total activity time and total distance moved; and a risk group-based test, which consisted in evaluating the larval capacity to cross from a “comfort” zone to a “risk” zone. In the group-based test, proactive, intermediate and reactive individuals were identified depending on the time taken to cross between two zones. Larvae fed with *Artemia* metanauplii enriched with the cod liver oil emulsion were significantly ($P = 0.01$) larger and in the individual-based test presented significantly higher total activity time ($P = 0.08$) and total distance moved ($P = 0.01$) than larvae from the other dietary treatments. No significant correlations ($P > 0.05$) were observed between larvae total length and latency time to move, total activity time or total distance moved across all treatments or within any dietary treatment. In the group-based test, fish fed with *Artemia* enriched with the cod liver oil emulsion presented a significantly higher proportion of proactive larvae ($P = 0.02$) and the lower proportion of reactive larvae. The present study showed for the first time that (i) Senegalese sole presented a defined proactive–reactive behaviour from early ontogenesis and (ii) dietary fatty acid composition significantly influenced the proactive–reactive behavioural dimension of stress coping style of sole larvae. The current study has practical implications that open the possibility to produce organisms that have behavioural styles that could ultimately result in improved aquaculture productivity.

## 1. Introduction

Animals including fish when confronted with a threatening or stressful situation have been recognized to have two different behavioural responses, respectively named as proactive and reactive (Koolhaas et al., 1999; Øverli et al., 2007). So-called proactive fish have been characterized to have fight-flight behavioural response and were observed to explore unfamiliar environments and take risks (Bell, 2005; Brelin et al., 2005; Koolhaas et al., 2007; Castanheira et al., 2015). By contrast, reactive fish have been characterized to freeze or hide and generally have lower activity, avoid risk and tend to stay immobile when submitted to novel environments (Brelin et al., 2005; Koolhaas et al., 2007; Toms et al., 2010; Castanheira et al., 2015). Physiologically, proactive fish were characterized by a low hypothalamus–pituitary–adrenal/interrenal (HPA/HPI) axis activity, leading to low post-stress levels of glucocorticoids, in contrast to reactive fish, which were characterized by a higher HPA/HPI response and levels of glucocorticoids (Koolhaas et al., 2010; Conrad et al., 2011). Together the behavioural and physiological dimensions combined with a consistency in responses over time or contexts have been described as the stress coping style of an organism (Koolhaas et al., 1999; Conrad et al., 2011; Castanheira et al., 2015). A diverse range of behavioural tests have been used to identify the behavioural dimension of stress coping style responses in teleost fish, such as reaction to confinement (Brelin et al., 2005), feeding motivation after being transferred into a novel environment (Mota-Silva et al., 2010), inter-individual

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aggression (Ruiz-Gomez and Huntingford, 2012), predatory situations (Archard et al., 2012) and group based tests (Bell, 2005; Wilson and Godin, 2009; Castanheira et al., 2013).

The performance of fish with different stress coping styles has been reported for different fish species. In a captive or aquaculture type environment proactive organisms were generally found to present higher growth (Mas-Muñoz et al., 2011), lower disease susceptibility (MacKenzie et al., 2009), lower latency time to recover and feed after a disturbance (Øverli et al., 2007) and were more disposed to follow routines ignoring novel changes in the environment (Ruiz-Gomez et al., 2011), which are characteristics that would favour aquaculture production. Thus, studying the behaviour during larval stages of fish species reared in aquaculture might be of great interest, in order to establish the period of development of behavioural characteristics, factors that influence the development and the consequences of presenting different behavioural traits on growth, performance and development. Among various factors assumed to influence larval survival, growth and quality, the importance of early larval nutrition, especially dietary lipids and essential fatty acids (EFA), have been highlighted in many studies (Izquierdo et al., 2000; Sargent et al., 2002). Diverse studies have addressed the effect of diets on fish larvae behaviour (e.g. swimming speed, escape reaction, etc.) in several fish species such as gilthead seabream (*Sparus aurata*) (Benítez-Santana et al., 2007), black sea bass (*Centropristis striata*) (Rezeck et al., 2010) and pikeperch (*Sander lucioperca*) (Lund et al., 2013). Moreover, different authors have indicated that vegetable oils and/or fish oils, used to improve the nutrition of live preys, may influence the fish growth, fatty acid body composition, gene expression and neuronal activity (Montero et al., 2003; Sales and Glencross, 2010; Benítez-Dorta et al., 2012; Benítez-Santana et al., 2014). Studies on the effects of vegetable oils on all life stages (larval, juvenile: pre-ongrowing and adult: ongrowing and breeders) of aquaculture species are required as the replacement of fish oils with vegetable oils is increasing rapidly to increase the sustainability of the aquaculture industry (Sargent et al., 2002; Naylor et al., 2009). Nonetheless, the question of how these dietary nutrients and the sources might have an impact on larvae behavioural dimension of stress coping style has, to our knowledge, received little attention.

Senegalese sole (*Solea senegalensis*) is a flatfish species that has great interest and potential for aquaculture diversification in Europe. One of the advantages of this species for aquaculture is that compared to other marine species larval rearing is not complicated and larvae possess high growth rate and survival (Morais et al., 2014). Furthermore, sole have been found to be particularly resilient to handling stress during the larval and early post-larval stages compared to other cultured species (Rønnestad et al., 2001). However, this species present high size variation and high mortality rates have been observed at the weaning period (Morais et al., 2014). Therefore, Senegalese sole larvae are a particularly interesting fish model in which to study the behavioural dimension of stress coping styles in relation to fatty acid nutrition since it has been demonstrated that an inappropriate fatty acid profile affected growth and muscle formation (Benítez-Dorta et al., 2012), digestive system maturation (Boglino et al., 2012) and glucocorticoids regulation (Martins et al., 2013). In view of these arguments, the present study aimed to determine whether (i) Senegalese sole larvae exhibit proactive–reactive behaviours in standardized novel environment and risk tests and (ii) whether dietary fatty acid composition from vegetable oils and fish oils, used as rotifers and *Artemia* enrichments, can influence the behaviour of sole larvae. Results will provide novel information related to Senegalese sole larvae behaviour at early life stages and how diets could influence physical fitness and behaviour. In addition, results may be valuable for the aquaculture industry in order to produce larvae with a specific behavioural characteristic.

## 2. Materials and methods

### 2.1. Ethic statement

All the experimentation on fish that formed part of this study were in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA) and approved by the Animal Ethics Committee of IRTA.

### 2.2. Experimental animals and housing

Senegalese sole larvae, supplied by a commercial farm (Stolt Sea Farm S.A., Galicia, Spain), were housed in sixteen 100 L tanks at a density of 100 larvae $L^{-1}$, in four replicate tanks per treatment. Tanks were connected through a recirculation system (IRTAmar®) and 50% of total water was renewed daily. Water parameters, such as temperature, salinity and dissolved oxygen were maintained at $16.5 \pm 0.5\,^{\circ}C$, 35 ppm and 7.5 mg $L^{-1}$, respectively. Photoperiod was adjusted to follow a light–dark cycle of 16 L:8 D h.

### 2.3. Experimental emulsions, live feed enrichment and feeding protocol

Four experimental emulsions were prepared with different oils following the methodology described by Villalta et al. (2005) and used to enrich rotifers and *Artemia* metanauplii. The four oils used were: cod liver oil from *Gadus morhua*, cod (Sigma–Aldrich Co., St Louis, MO, Germany); linseed oil, linseed (Biolasi Products Naturals, S.L., Guipùzcoa, Spain); soybean oil, soybean (Huilerie Emile Noël, S.A.S., Pont Saint Esprit, France); and olive oil, olive (Borges Pont, Lleida, Spain). Oils were emulsified in warm distilled water (50 °C) with soy lecithin (Dietetica Rosa, S.A., Barcelona, Spain) and $\alpha$-tocopherol (Sigma–Aldrich Co., St Louis, MO, Germany), using an Ultra-turrax T25 homogenizer at high speed for 60–90 s. Emulsions were kept refrigerated at 4 °C in the absence of air until used for enriching the rotifers and *Artemia* metanauplii.

Senegalese sole larvae were fed twice a day, from 2 to 8 days post-hatching (dph) with rotifers (*Brachionus plicatilis*) enriched with the oil emulsions at a density of 10 rotifers $mL^{-1}$. Freshly enriched *Artemia* metanauplii (EG type; INVE, Belgium) were introduced in tanks at 6 dph until 24 dph, in quantities ranging from 0.5 to 6 metanauplii $mL^{-1}$, adjusted based upon the increase of weight of the larvae, with the daily food ration being calculated as described by Cañavate et al. (2006). As larvae metamorphosed and became benthonic, live *Artemia* metanauplii were gradually substituted with frozen *Artemia* metanauplii. From 19 to 24 dph and from 25 dph larvae were fed exclusively with frozen *Artemia* at a density of 6 metanauplii $mL^{-1}$ until 30 dph and then 12 metanauplii $mL^{-1}$ until the end of the experiment (40 dph).

Rotifers (*Brachionus plicatilis*) were cultured as described in Boglino et al. (2012). *Artemia* metanauplii were hatched in standard conditions and metanauplii enrichment was performed in 20 L conical containers at 150 metanauplii $mL^{-1}$ for 16 h at 28 °C with oxygen ($\geq$5 mg $L^{-1}$), and using 0.6 g of each emulsion $L^{-1}$. Subsequently, enriched *Artemia* metanauplii were washed with UV-treated filtered sea water and disinfected with hydrogen peroxide (8000 ppm) for 5 min. Then washed for 15 min period in 150 $\mu$m plankton nets with UV-treated filtered sea water. A batch of *Artemia* was frozen and kept at −20 °C until being given to post-metamorphosed larvae.

### 2.4. Lipid and fatty acid analysis

For biochemical analysis of the larvae, pools of 50 post-larvae were taken per tank at 37 dph. Sampled larvae were euthanized