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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 ± 0.5 °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 α-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 μm 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

with MS-222, washed with distilled water and immediately frozen at −20 °C. Total lipids were extracted in chloroform:methanol (2:1, v:v) using the method described by Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow, followed by vacuum desiccation overnight. Total lipids were stored in chloroform:methanol (2:1, 20 mg mL^{-1}) containing 0.01% butylated hydroxytoluene (BHT) at −20 °C prior to analysis. Methyl esters were extracted twice using isohexane:diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas–liquid chromatography on a Thermo Electron-TraceGC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m × 0.25 mm id; SGE, UK). A two-stage thermal gradient was used, from 50 °C (injection temperature) to 150 °C, after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}. Helium (1.2 mL min^{-1} constant flow rate) was used as the carrier gas and on-column injection and flame ionization detection was performed at 250 °C. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well characterized fish oil, and quantified using an internal standard, 21:0 fatty acid, added prior to transmethylation using a Chrom-card for Windows (TraceGC, Thermo Finnigan, Italy).

The fatty acid composition of the *Artemia* metanauplii varied in relation to the enrichment with the four emulsions. *Artemia* enriched with the cod emulsion presented the highest proportions of total saturated fatty acids (SFA), mainly 18:0. The amount of total monounsaturated fatty acids (MUFA), mainly oleic acid (OA, 18:1$n-9$), was the highest in the *Artemia* enriched with the olive emulsion and intermediary in those enriched with the cod emulsion. *Artemia* enriched with the soybean emulsion had the most elevated contents of total $n-6$ polyunsaturated fatty acids ($n-6$ PUFA), mainly linoleic acid (LA, 18:2$n-6$). Arachidonic acid (ARA) levels were relatively stable among the dietary treatments, and ranged from 1.2 (olive diet) to 1.5 (cod and soybean diets). The content of $n-3$ PUFA was the highest in the *Artemia* enriched with the linseed emulsion, mainly due to linolenic acid (LNA, 18:3$n-3$) contents. Finally, levels of $n-3$ LC-PUFA, EPA and DHA, were highest in *Artemia* enriched with the cod emulsion.

The fatty acid composition of the sole larvae fed with the four different dietary treatments reflected the fatty acid pattern observed in the enriched *Artemia* metanauplii (Table 1). Sole larvae fed with the cod emulsion presented the highest proportions of total saturated fatty acids (SFA), EPA and DHA. Respectively, EPA and DHA in larvae fed the cod diet was 4.9 and 7.2 compared to ranges of 0.9–1.2 and 2.3–2.7 in larvae fed with the other diets. The amount of total monounsaturated fatty acids (MUFA), mainly oleic acid (OA, 18:1$n-9$), was the highest (35.8) in the sole larvae fed with the olive emulsion and lower ranging from 21.6 to 22.6 in larvae fed with the other diets. Sole larvae fed with the soybean diet had the most elevated contents of total $n-6$ polyunsaturated fatty acids ($n-6$ PUFA), mainly linoleic acid (LA, 18:2$n-6$) with a level of 27.4 compared to 11.7–19.8 with other diets. ARA levels were relatively stable among the dietary treatments, and ranged from 2.7 (olive diet) to 3.9 (linseed diet). The content of $n-3$ PUFA was the highest in the sole fed with the linseed emulsion, mainly due to linolenic acid (LNA, 18:3$n-3$) contents.

2.5. Proactive–reactive behavioural characterization

2.5.1. Individual-based test

Forty-two Senegalese sole larvae (aged 40 dph) from each of the four replicate tanks per treatment were tested. Larvae were captured in groups of six from the experimental tank and placed in a 1 L beaker. After a standardized 1 min in the beaker to ensure all larvae had a similar pre-test treatment each larva was individually introduced into a separate plastic transparent Petri plates (six dishes of 9 cm of diameter, see Fig. 1A) used to perform the individual test. Larvae were always placed in the same position (close to the edge) in the Petri plate, which was gridded (squares equal to 1 cm^2, Fig. 1A). Petri plates were filled with 15 mL of sea water from the holding tanks and renewed for each sampled larvae. A digital camera (Casio-Exilim, model EX-ZS100, Japan) was fixed 40 cm above the Petri plates (Fig. 1B) to video record larval activity for 5 min. The observer stood 1 m away from the working area, in order to cause minimal disturbance to larvae, following the criteria described by Øverli et al. (2002). Once video recording were achieved, three behavioural responses were registered for each larvae, being: (i) the latency time to move defined as the time (in seconds) of first forward movement since the beginning of the test (adapted from Bell, 2005; Archard et al., 2012); (ii) the total activity time considered as the total swimming activity time (in seconds) of the larvae in the new environment (adapted from Millot et al., 2009; Benhaïm et al., 2012); and (iii) the Total Distance Moved determined by counting the total number of squares crossed by the larvae during the experimental time (adapted from Millot et al., 2009; Benhaïm et al., 2012). Larvae total length was measured as well, with a Vernier scale (0.001 error; model CD-20PK; Mitutoyo, Japan Corp).

2.5.2. Group-based risk test

In the group-based risk test, a different pool of larvae than the one used in the individual-based test was screened in a risk taking experience. Experimental methodology was adapted from previous studies performed in stickleback, *Gasterosteus aculeatus* (Bell, 2005; Ruiz-Gomez and Huntingford, 2012), gilthead sea bream (Castanheira et al., 2013), common carp, *Cyprinus carpio* (Huntingford et al., 2010) and bluegill sunfish, *Lepomis macrochirus* (Wilson and Godin, 2009). Four group risk tests were made per treatment, one test per treatment replica. The test consisted in placing 30 individuals in a rectangular tank (80 cm length × 10.5 cm depth × 13 cm width) divided into two areas, in order to determine the capacity of larvae to cross from a known area defined as a "comfort zone" to an unknown area defined as a "risk zone" (Fig. 1C). The experimental tank was divided in two equal volumes with a black plastic barrier, including a 1.5 cm width–length window at the base of the barrier, through which fish were able to pass (Fig. 1C). During the acclimation period, each group of fish ($n = 30$) was retained for 1 h in the comfort zone, by keeping the window closed until the beginning of the test. The comfort zone was completely isolated from light by a black plastic cover, while the risk area was illuminated by a fluorescent white light (OSRAM DULUX, 48 W, 450 lx in surface) placed 25 cm above the water surface. A gentle water flow (2 L h^{-1}) and aeration were provided during the period of the test and similar water parameters than those in the housing tanks (temperature of 16.5 ± 0.5 °C, salinity of 35 ppm and dissolved oxygen of 7.5 mg L^{-1}) were maintained. A digital camera (Casio-Exilim, model EX-ZS100, Japan) was fixed on the risk zone to video record the larvae that successfully crossed from comfort zone to the risk zone.

After the 1 h acclimation the door was opened and the behaviour registered during 1 h with the door open. Larval behaviour was established following the criteria of exploration activity, defining proactive larvae as those that crossed from the comfort to the risk zone in the first 20 min, intermediate larvae as those that took to cross between 21 and 40 min and reactive larvae as those that crossed more than 41 min after the beginning of the test or as those that did not cross (Wilson and Godin, 2009; Huntingford et al., 2010; Ruiz-Gomez and Huntingford, 2012; Castanheira et al., 2013). The time larvae crossed from the comfort zone to the risk zone was registered from the video recording. No larvae were observed to return to the comfort zone during the short experimental period.

Table 1
Fatty acid composition of sole post-larvae fed with *Artemia* metanauplii enriched with different experimental emulsions.

	LSO[b]	CLO[a]	SBO[c]	OO[d]
Formulation (mg g^{-1})				
Cod liver oil[a]	0	528	0	0
Linseed oil[b]	528	0	0	0
Soybean oil[c]	0	0	528	0
Olive oil[d]	0	0	0	528
Supplements[e]	52	52	52	52
Distilled water	420	420	420	420
Fatty acid composition (%TFA)				
Total saturated	16.5 ± 2.2	19.7 ± 1.2	17.9 ± 0.7	15.6 ± 0.7
18:1*n* – 9 (OA)	22.3 ± 0.7	22.6 ± 0.4	21.2 ± 0.5	35.8 ± 0.8
Total monounsaturated (MUFA)	30.0 ± 1.0	35.4 ± 0.6	29.0 ± 0.7	45.6 ± 1.3
18:2*n* – 6 (LA)	14.9 ± 0.2	7.8 ± 0.7	22.9 ± 0.5	10.1 ± 0.3
20:4*n* – 6 (ARA)	3.9 ± 0.3	3.0 ± 0.1	3.2 ± 0.1	2.7 ± 0.4
Total *n* – 6 PUFA	19.8 ± 0.7	11.7 ± 1.0	27.4 ± 0.3	14.2 ± 0.7
18:3*n* – 3 (LNA)	25.2 ± 3.6	13.1 ± 1.1	15.1 ± 1.5	14.3 ± 0.8
20:5*n* – 3 (EPA)	0.9 ± 0.2	4.9 ± 0.2	1.0 ± 0.2	1.2 ± 0.2
22:6*n* – 3 (DHA)	2.3 ± 0.6	7.2 ± 0.2	2.7 ± 0.5	3.1 ± 1.2
Total *n* – 3 PUFA	31.6 ± 3.5	30.7 ± 1.2	22.4 ± 0.7	22.4 ± 0.9
Total PUFA	51.4 ± 3.5	42.4 ± 1.9	49.8 ± 1.0	36.6 ± 1.2
MUFA/PUFA	0.6	0.8	0.6	1.2
n – 3/*n* – 6	1.6	2.6	0.8	1.6
DHA/EPA	2.6	1.5	2.7	2.6
ARA/DHA	1.7	0.4	1.2	0.9
ARA/EPA	4.3	0.6	3.2	2.3

[a] CLO: cod liver oil.
[b] LSO: linseed oil.
[c] SBO: soybean oil.
[d] OO: olive oil.
[e] Supplements: soy lecithin, 4 g; vitamin E 1,2 g.

The video analysis was corroborated with real time counts that were made every 10 min of the risk zones.

2.6. Statistics

Results were expressed as means ± standard error S.E. (n = 168 for the individual tests and TL). All data were checked for normality (Shapiro–Wilk test) and homogeneity of variance (Bartlett's test). Individual-based test means (n = 42) from each replica within each treatment were compared with a one-way Analysis of Variance (ANOVA) and as no differences were observed the data from replicas was combined. Two Multivariate Analysis of Variance (MANOVA) were performed, first among treatments on larval activity parameters ($n = 42 \times 4 = 168$) of the individual-based test and the *post hoc* Tukey HSD test was performed when significant differences were found and second to detect possible interferences among the position of the Petri plates (Fig. 1A) on the individuals responses of sole larvae (length, latency to move, total activity time and total distance moved). No statistical differences were observed among the location of the Petri plates (Fig. 1B) for the larvae length (F_5 = 1.29, P = 0.30), the latency time to move (F_5 = 0.65, P = 0.65), total activity time (F_5 = 0.88, P = 0.49) and total distance moved (F_5 = 0.63, P = 0.67) to discount that there was an effect on behaviour of Petri dish position of the experimental setup. A Pearson correlation was performed in order to determine possible correlations between variables including total length. A rank analysis, with N-tiles frequencies subcommand, was performed to examine the possible effect of size of larvae on the latency time to move, the total activity and the total distance moved. Individuals from all dietary treatments taken together were separated into size ranges: small from 0.5 to 0.8 mm, medium from 0.9 to 1.0 mm and large from 1.1 to 1.9 mm. The numbers of individuals in the medium size range were 49, 64, 67 and 76 respectively for cod, linseed, olive and soybean dietary treatments. A one-way ANOVA was performed to compare the behavioural parameters amongst the treatments for the selected larvae that were all in the same medium size range. Groups of 30 larvae (n = 30) from each replica (4 replicas) in each treatment (4 treatments) were used in each group test (total $4 \times 4 = 16$ group tests). A Chi square (χ^2) test was performed on data from the grouped-based tests, in order to determine the differences in the proportions of proactive, intermediate and reactive larvae. First, a Chi squared test was made to compare the data from

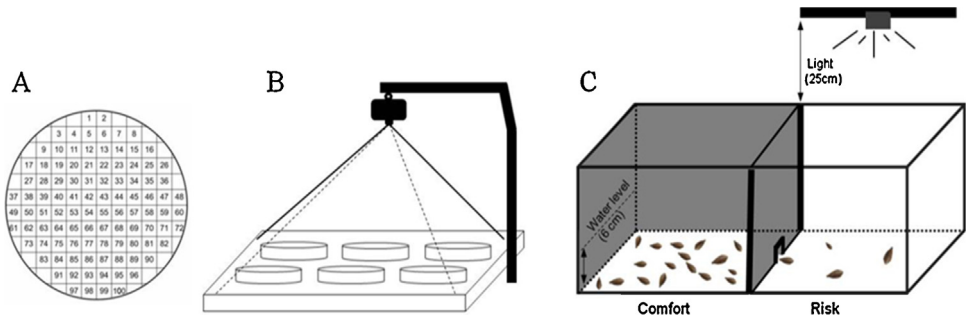

Fig. 1. Equipment for determining performance and behavioural personality of Senegalese sole (*Solea senegalensis*) larvae. (A) Petri plate gridded (1 cm^2) to measure larvae performance; (B) platform and camera installed to analyze individual performance; and (C) tank for grouping test (comfort zone = covered; risk zone = illuminated).

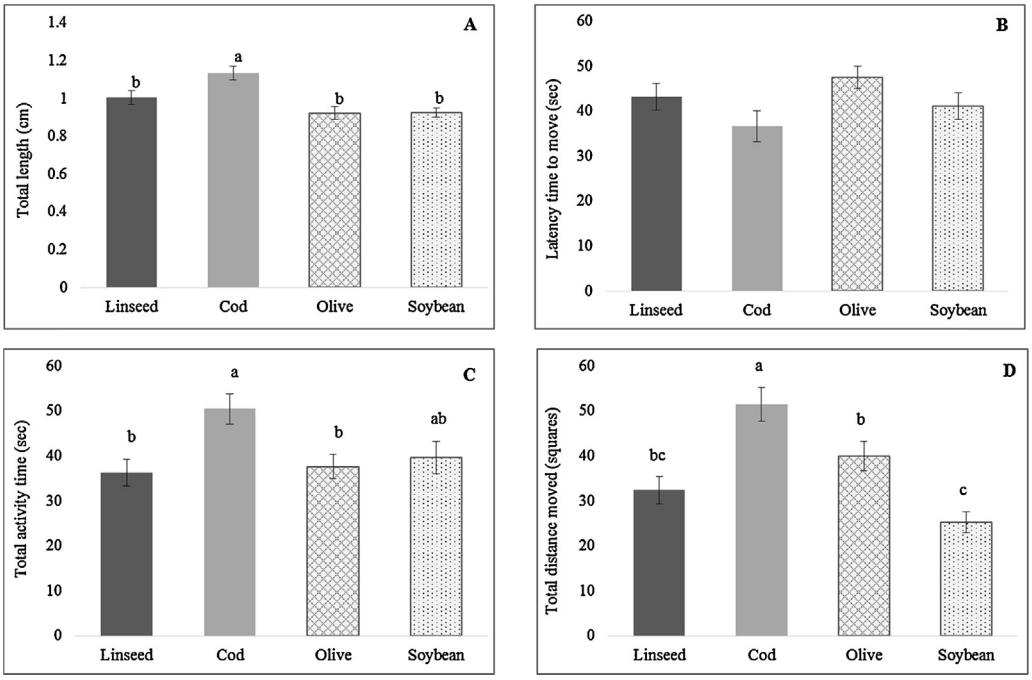

Fig. 2. Size and behavioural performance of Senegalese sole (*Solea senegalensis*) larvae fed with *Artemia* metanauplii enriched with one of four different oil emulsions: linseed oil; cod liver oil; olive oil and soybean oil. (A) Total length; (B) latency time to move; (C) total activity time and (D) total distance moved. $n = 168$ per treatment. Different letters indicate significant differences between treatments.

replicas within each treatment (3×4 matrix). No differences were observed amongst replicas within treatments. The replicate data was combined ($n = 30 \times 4 = 120$) and dietary treatments were compared (3×4 matrix). A value of $P < 0.05$ was accepted as statistically significant for all statistical tests realized on data from the individual and grouping tests. All the statistical analysis was conducted using SPSS statistics V.17 (IBM Inc., Chicago, Illinois, USA).

3. Results

3.1. Individual behaviour characteristics

Feeding Senegalese sole larvae with *Artemia* metanauplii enriched with the four different emulsions significantly affected growth and individual activity performance when introduced into a new environment (Fig. 2). Larvae fed with *Artemia* metanauplii enriched with the cod emulsion presented a significant higher total length (Fig. 2A; $F_{1,24} = 8.6$, $P = 0.01$; 1.13 ± 0.03 cm) than larvae fed *Artemia* enriched with linseed, olive and soybean emulsions (1.01 ± 0.03 cm; 0.92 ± 0.03 cm and 0.93 ± 0.02 cm, respectively). The activity of larvae in the new Petri dish environment in all treatments ranged from larvae that that did not move to larvae that moved immediately (low latency time) with a high level of activity (distance moved and time of activity) during the 5 min. No significant difference ($F_{1,24} = 2.3$, $P = 0.079$) was found in latency time to move among larvae fed the different dietary treatments (Fig. 2B), although larvae fed with *Artemia* enriched with the cod emulsion tended to have a lower latency time to move (36.6 ± 3.4 s) and those on the olive treatment had a slightly higher latency time (47.5 ± 2.4 s). Larvae from the cod group had significantly higher total activity time (Fig. 2C; $F_{1,24} = 4.1$, $P = 0.08$; 50.4 ± 3.7 s) than those from the linseed and olive groups (36.3 ± 3 s and 37.6 ± 2.7 s, respectively), while larvae fed the soybean diet displayed an intermediate and non-significantly different total activity time value (39.6 ± 3.6 s). Similarly, larvae fed with *Artemia* metanauplii enriched with the cod emulsion showed a significantly higher total distance moved (Fig. 2D; $F_{1,24} = 12.8$, $P = 0.01$; 51.47 ± 3.4 squares) than those fed the three other diets and larvae fed with *Artemia* enriched with the soybean emulsion presented a significantly lower total distance moved ($F_{1,24} = 4.4$, $P = 0.06$; 25.3 ± 2.3 squares) than the olive group (39.9 ± 3.2 squares), while those fed the linseed diet showed an intermediate value of total distance moved (32.4 ± 3 squares). When larvae were selected by medium size (0.9–1.0 mm, medium of all treatments) from each treatment group and compared the same pattern of differences was found amongst treatment groups. The 0.9–1.0 mm larvae from the Cod treatment presented significantly lower latency time to move ($F_{252} = 3.603$, $P = 0.014$), significantly higher total activity time ($F_{252} = 5.926$, $P = 0.001$) and significantly higher total distance moved ($F_{252} = 20.414$, $P < 0.001$) than 0.9–1.0 mm larvae from some or all of the other treatments. In addition, no significant correlations ($P > 0.05$) were observed between larval total length and latency time to move, total activity time or total distance moved across all treatments or within any dietary treatment. The correlation coefficients were low ranging from the highest of $R^2 = 0.27$, $P = 0.08$ observed between total length and total activity time in the cod treatment to the lowest between total length and total distance moved in the linseed treatment ($R^2 = 0.08$, $P = 0.62$).

3.2. Risk taking by group

All treatments had larvae that passed through the door to the risk zone soon after opening (proactive) and larvae that did not cross from the comfort zone to the risk zone (reactive). Larvae from the cod treatment presented a significant higher proportion ($\chi^2 = 24.27$, df = 6, $P = 0.02$) of proactive and intermediate organisms (14 and 7, respectively) and the lowest number of reactive larvae (9) (Fig. 3). On the contrary, most of the larvae fed the soybean and olive diets did not cross from the comfort to the risk zone (21 and 25, respectively), suggesting predominantly reactive behaviour. Larvae fed with *Artemia* metanauplii enriched with the linseed emulsion presented intermediary values of proactive (9), intermediate (3) and reactive larvae (18) (Fig. 3). Larvae crossed only once from comfort zone to risk zone.

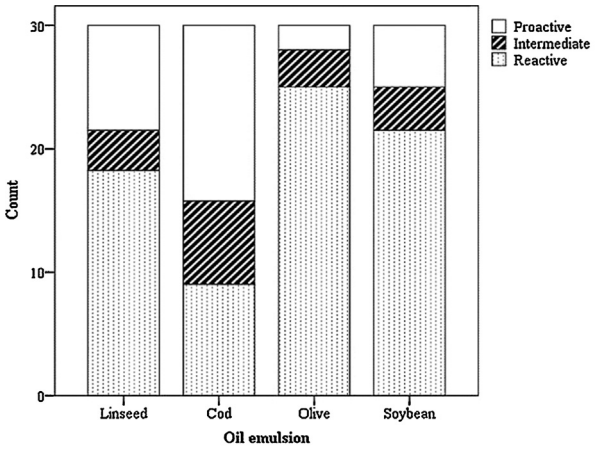

Fig. 3. Number of proactive, intermediate and reactive Senegalese sole (*Solea senegalensis*) larvae fed with *Artemia* metanauplii enriched with one of four different oil emulsions: linseed oil; cod liver oil; olive oil and soybean oil (χ^2, $P = 0.001$). $n = 120$ per treatment. *Indicates proportions were significantly different from expected values.

4. Discussion

This is one of the first studies that investigated the effect of diets on the proactive–reactive behavioural dimension of stress coping style during the larval development period. The tests employed in the present study enabled the characterization, for the first time, of proactive–reactive behaviour of Senegalese sole post-larvae at 40 dph. The activity of sole post-larvae in the new environment individual-based tests and latency to enter an unknown risk area in the risk group-based test enabled the identification of proactive and reactive behavioural traits. In agreement with the classification used in other studies, proactive larvae had low latency to move into a risk zone or in a new environment and were more active in a new environment, whilst reactive larvae had higher latency to move into a risk zone or in a new environment and were less active in a new environment. All dietary treatments had this range of behaviours, although the levels of activity in a new environment and proportions of proactive, intermediate and reactive larvae significantly changed depending on diet. The identified proactive–reactive behaviours as determined by activity in the individual-based tests did not appear to be related to the size of the larvae as the same pattern of differences were found in the behaviours of similarly sized (0.9–1.0 mm) larvae that were selected from each treatment group and there was no correlation between larvae length and individual-based test activity variables latency time to move, total activity or total distance moved. The activity in new environment and the risk taking tests used in the present study to differentiate the proactive and reactive behaviours have been both previously reported as relevant measurements to determine proactive and reactive traits in a wide range of fish species (Toms et al., 2010; Castanheira et al., 2013) including Senegalese sole (Mota-Silva et al., 2010; Martins et al., 2011). This behavioural dimension of stress coping styles were described in the damselfly *Lestes congener* (Brodin, 2008), common carp (Huntingford et al., 2010), rainbow trout *Oncorhynchus mykiss* (Ruiz-Gomez et al., 2011) and three-spined stickleback (Ruiz-Gomez and Huntingford, 2012). Altogether, these studies, which are in line with the present results, have provided a solid base of evidence for the existence of proactive–reactive coping style responses in those fish species and demonstrated that proactive individuals, when in novel environments, resumed activity earlier, tended to take risks and spent more time in movement than reactive individuals.

The results from the present study have clearly shown that diets differing in fatty acid profile significantly influenced the proactive–reactive behavioural dimension of stress coping style of Senegalese sole larvae. To our knowledge, there is no information on the early interactions between dietary fatty acid composition and the stress coping style of Senegalese sole larvae, or any other fish larvae. Sole larvae fed with *Artemia* metanauplii enriched with the fish oil emulsion showed significantly higher activity time and swam longer distances in the new environment individual-based test and a significantly higher proportion of larvae took the risk to cross to and explore an unknown environment in the risk group-based test compared to larvae fed with *Artemia* enriched with the vegetable oil emulsions (linseed oil, olive oil and soybean oil). In contrast, larvae from the latter groups were less active in the individual-based test, more cautious in the reaction to novel situations and mostly stayed sheltered in the safe zone with a significantly lower proportion crossing to an unknown environment (risk group-based test). Therefore, the fish oil enrichment (cod liver oil) appeared to promote a proactive explorative behaviour, while vegetable oils (linseed oil, olive oil and soybean oil) resulted in a reactive behaviour in Senegalese sole larvae. Possible explanations for these significant dietary effects on proactive–reactive behaviour could be a direct effect of nutritional status on physiology, metabolic rate and/or development that resulted in retarded growth or differences in physical condition that affect activity and personality of the larvae.

Cod liver oil diet contained higher amounts of SFA and of the EFAs, EPA and DHA (15.2; 4.7 and 2.6% TFA, respectively) than the other diets. In addition, it had the second highest level of MUFA, after the olive treatment. Therefore, overall, this diet possibly supplied higher levels of important energy substrates, as well as of critical structural components of bio-membranes and precursors of essential metabolites, resulting in a more balanced diet for fish larval and metamorphosing stages with fast growth associated with high requirements for development and organogenesis (Conceição, 1997; Morais et al., 2005). This difference in nutrition may also explain the higher growth of sole fed the cod diet. Other studies have shown that red sea bream *Pagrus major* (Nakayama et al., 2003), gilthead seabream (Benítez-Santana et al., 2007) and pikeperch (Lund et al., 2012) fed with higher inclusions of PUFA, particularly DHA, in diets improved fish growth, activity, swimming speed, escape ability and reduced mortality after a stress confinement situation. Similarly, Liu et al. (2002) and Atalah et al. (2011) suggested that increasing the dietary amount of EPA and ARA supplied to gilthead seabream and European sea bass improved resistance to handling stress in comparison to larvae fed with diets containing poor levels of these ingredients. However, in the present study, the poor correlation between larval length and activity and the same pattern of behavioural differences amongst fish of the same size from the different treatments provides compelling evidence that the diet effect on the behavioural dimension of stress coping style was more profound than just increasing energy reserves and growth to increase activity and hence the proportion of proactive larvae.

Possibly one of the most relevant roles of DHA and EPA related to this study is its crucial importance for neurogenesis, being that DHA and EPA a major components of cell membranes in the eyes and brain of fish (Bell and Dick, 1991; Bell et al., 1996). Studies have demonstrated that providing an adequate DHA and EPA proportions during early development of fish might improve the brain development, the central nervous system, the neuromasts formation (i.e. sensory cells associated to lateral line), the activation of mauthner cells (i.e. neurons responsible for escape responses) optical tectum, cerebellum, vision, the antipredator behaviour, as others (Bell et al., 1995; Ishizaki et al., 2001; Nakayama et al., 2003; Benítez-Santana et al., 2007, 2014). Moreover, the same authors

observed that larvae fed with higher inclusions of DHA and EPA showed higher ability to swim, formed schooling patterns and improved anti predator escape capacity. Therefore, higher dietary levels of LC-PUFA, particularly of DHA and EPA, as provided by the cod diet in the present study, could have had a positive effect on the ontogenic development of the neural system and sensorial organs which in turn influenced the fish brain development or neurogenesis, cognition, swimming activity, learning capacity and explorative behaviour of larvae's. These assumptions are in agreement with the studies performed by Øverli et al. (2007), Sørensen et al. (2007) and Koolhaas et al. (2010) whom confirmed that behavioural stress coping styles of fish at different ages are associated with neurogenesis and neural plasticity. In reference to the vegetable emulsions, these three diets led to similar coping style responses in sole larvae. A possible explanation of this is that vegetable oils presented lower DHA/EPA ratio, higher ARA/DHA and ARA/EPA ratios and unbalanced ratios of MUFA/PUFA and $n-3/n-6$ PUFAs. Therefore, it is possible to suggest that energy, physical fitness and enzymatic material of larvae was similar within the three vegetable emulsions, but lower in comparison with larvae fed fish oil emulsion (Sargent et al., 2002; Villalta et al., 2005; Benítez-Dorta et al., 2012; Boglino et al., 2012). However, further systematic studies should be performed in order to explore the relation between nutrition, neurogenesis and coping styles, since it is not entirely clear how these aspects can be involved in fish larval behaviour.

Lastly, it should be mentioned that stress coping style is defined as a coherent set of behavioural and physiological stress responses that is characteristic to a certain group of individual (Koolhaas et al., 1999). In the present study, diets influenced the behaviour of 40 DAH Senegalese sole larvae in a coherent way. The dietary treatments had a coherent influence on larval behaviour as no differences were found in the behavioural parameters amongst the four replicas within each dietary treatment indicating that the behavioural patterns in the replicas were highly consistent between individuals that had received the same treatment. Stress coping style has been shown to be determined or perhaps influenced by genetic and environmental factors (Dingemanse and Réale, 2005; Koolhaas et al., 2007). It has also been hypothesized that individual's behaviour may change in accordance with developmental stage or age (Groothuis and Trillmich, 2011). From an environmental point of view fish behaviour may depend on environmental stimuli (i.e. predators, density, etc.), food availability, social structure or motivational state and negative experiences (Koolhaas et al., 2007; Ruiz-Gomez et al., 2011; Frost et al., 2013). The present study highlighted that nutrition was an important environmental factor in determining or influencing behavioural development of the proactive–reactive behavioural dimension of stress coping style of sole larvae in different contexts.

The implication of understanding fish stress coping styles is of major importance, not only from an evolutionary perspective but also in practical disciplines, such as neurosciences (Benítez-Santana et al., 2014), disease susceptibility (MacKenzie et al., 2009) and especially aquaculture production (Øverli et al., 2002). In this context, the characterization of Senegalese sole larvae proactive–reactive behavioural dimension of stress coping style may have important practical applications for the aquaculture industry for the selection or production of larvae with specific behavioural characteristic that may have benefits for welfare protocols, selective genetic programmes, improved growth, stress resistance and/or survival. For instance, Øverli et al. (2002) and Pottinger (2006) found that selected lines of proactive rainbow trout juveniles presented higher levels of locomotor activity and growth rates. MacKenzie et al. (2009) indicated that coping style responses in common carp were related to susceptibility to diseases in inflammatory challenges. Hansen et al. (2009) showed that proactive Atlantic cod, *Gadus morhua*, individuals presented a higher learning ability compared to reactive fish. In the present study, proactive larvae (fed with cod oil) were more active, showed higher length and take more risk than reactive larvae (fed with vegetable oils).

5. Conclusion

The present study indicated that proactive–reactive behavioural dimension of stress coping style developed early during ontogenesis and, furthermore, that the proportion of the different proactive–reactive behaviour was modulated by diets. These findings are of great interest to certain sectors such the aquaculture, since it may offer the possibility to produce a higher proportion of organisms that have similar behavioural styles that may result in improved growth, welfare, stress resistance, fitness and thus increment aquaculture productivity.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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