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Dietary novel oils modulate neural function and preserve locomotor response in gilthead sea bream (*Sparus aurata*) juveniles by regulating synthesis and contents of fatty acids in brain

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ABSTRACT

N-3 long-chain polyunsaturated fatty acids (LC-PUFA), particularly docosahexaenoic acid (22:6n-3, DHA), are the most important fatty acids with physiological significance in brain function of all vertebrates. Recently, novel lipid sources are available for the industry, including products obtained from microorganisms. Dietary fatty acid profiles may affect tissue composition and cell functioning. To determine the effect of novel lipid sources on behaviour and neural function in gilthead sea bream (*Sparus aurata*), juveniles were fed three isoproteic and isoenergetic diets containing either (FO), a microalgae oil combined with poultry oil (DD) or only poultry oil (PO). Behaviour response, brain fatty acid composition and relative expression of neurogenesis and neural activity related-genes in telencephalon were evaluated during 5 months of feeding with the experimental diets. Brain of sea bream fed diet PO showed the highest DHA content as well as increased desaturation and elongation products, contrary to the dietary pattern. This, together with an increased telencephalic *fads2* expression denoted the effective activation of LC-PUFA synthesis and retention as a compensatory mechanism for a dietary deficiency. Furthermore, *neurod6*, *bdnf* and *nos1* were proportionally upregulated in relation to the neural DHA content. Locomotor performance during cruising and escape responses was not affected by the experimental diets. However, there was a tendency for escape latency to be longest in fish fed PO and DD diets, and a relationship between escape latency and brain n-6 docosapentaenoic acid (DPA) contents was suggested. Indeed, the relation of n-6 DPA with behavioural deficits was shown previously in mammals and might deserve further attention and corroboration in fish as well in the future.

1. Introduction

N-3 long-chain polyunsaturated fatty acids (LC-PUFA) are involved in several metabolic pathways, such as energy production, membrane structure and function, control of lipid homeostasis and production of eicosanoids (Tocher, 2003). In particular, docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) have significant biological roles and are essential fatty acids (EFA) for marine species, due to their limited desaturases (FADS) and elongases (ELOVL) activities, necessary for their endogenous synthesis (Izquierdo and Koven, 2011; Tocher, 2015). Despite the well-known positive effects of DHA and EPA on vertebrate growth, reproduction, and disease prevention, these FA are also main components of polar lipids, which play critical roles in the regulation of membrane integrity and function. Moreover, they strongly participate in neural development and performance (Sargent et al., 1995; Izquierdo and Koven, 2011). Indeed, DHA is the most

Abbreviations: ARA, arachidonic acid; Bdnf, brain-derived neurotrofic factor; DB, double bend; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; egr1, early growth response gene 1; ef1α, elongation factor 1 alpha; EFA, essential fatty acids; ELOVL, fatty acid elongase; EPA, eicosapentaenic acid; FADS, fatty acid desaturase; FO, fish oil; LC-PUFA, long-chain polyunsaturated fatty acids; neurod6, neurogenic differentiation factor 6; nos1, nitric oxid synthase 1; PO, poultry oil; SB, single bend.

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important fatty acid with physiological significance for brain function in vertebrates, including in fish (Bourre, 2004; Marszalek and Lodish, 2005). This fatty acid is especially relevant during very early life stages, accounting for 30–40% of fatty acids contained in neural cell membranes (Lauritzen et al., 2001). Despite being less studied, EPA may also play important roles in neural function, by enhancing brain development in fish (Furuita et al., 1998), whereas in mammals, EPA has been shown to have a neuro-protective role (Joffre et al., 2019). The high demand for n-3 LC-PUFA from the brain in early stages of life is one of the most significant reasons behind the considerable higher DHA and EPA requirements in larvae than in juveniles or sub-adult fish (Izquierdo, 2005). However, despite these important functions in fish neuromodulation, there is still little information regarding the effects of dietary n-3 LC-PUFA on brain function of teleosts. Indeed, composition of neural cells can directly influence brain activity that, in turn, modulates fish behaviour (Benítez-Santana et al., 2012, 2014). Behaviour is a consequence of the connection between the perception of the environment by the animal and its nervous system that translate the sensory perception into a specific reaction (Lund et al., 2014). Therefore, normal fish behaviour is associated with a normal neural function and consequently, closely related with fish welfare and health status. Like in the natural environment, that fish faces several stressors, for instance, predators, in captivity, fish also experience stressful situations, including confinement due to high density, chasing due to fish netting, etc. These types of external stimuli are usually translated into typical behavioural response patterns that need to be monitored for fish welfare. For instance, previous studies with gilthead sea bream larvae (*Sparus aurata*) provided the first evidence regarding the effects of n-3 LC-PUFA on Mauthner neurons and escape behaviour (Benítez-Santana et al., 2012, 2014). DHA-deficient diets were shown to greatly reduce DHA incorporation in larval brain tissue and, consequently, delaying escape response to a sound stimulus (Benítez-Santana et al., 2012, 2014). Additionally, EFA-deficient diets also have short (28 days) and long-term (112 days) negative consequences in behaviour of pikeperch (*Sander lucioperca*) larvae (Lund et al., 2014). These unsuccessful or altered behavioural responses could be derived from a failure of neuronal activity or a low perception of the stimulus. Escape responses are natural mechanisms for avoiding stressors. They consist of both non-locomotor, for instance escape latency, as well as locomotor variables (e.g. turning rate, escape velocity and distance) (Domenici et al., 2007), that are activated by fish neural circuits, particularly Mauthner neurons, and allow fish to avoid threats (Domenici and Hale, 2019). In juveniles, recent studies with rainbow trout (*Oncorhynchus mykiss*) also linked dietary n-3 LC-PUFA and feeding behaviour, as well as inflammatory, oxidant and stress status of fish brain (Roy et al., 2020). Although some work has been carried out on larvae of farmed fish species, very few studies have been conducted to understand the impact of n-3 LC-PUFA in brain and behaviour responses of fish in later stages of development and, to our knowledge, no studies were carried out in gilthead sea bream (*S. aurata*) juveniles. This topic deserves further attention on more species for a number of reasons: (1) With the increasing replacement of fish oil (FO) in aquafeeds, mostly with terrestrial plant ingredients, n-3 LC-PUFA dietary contents could be significantly reduced. Dietary fatty acid composition is reflected in fish tissues and could affect fish cell responses (Turchini et al., 2009). Therefore, fish nutrition research has been recently focusing in finding new n-3 LC-PUFA sources for fish feeds, in which microalgae are some of the most promising alternatives (Shah et al., 2018). (2) Fish behaviour and neural function are good indicators of fish health and development (Martins et al., 2012), thus being of pivotal importance their study and monitoring for an effective production of fish (Benítez-Santana et al., 2012, 2014; Lund et al., 2014). (3) Fish models are gaining interest in biomedical and behavioural research as alternatives to mammals, in which DHA and EPA were shown to ameliorate neuropathological disorders, such as depression, schizophrenia, Alzheimer's disease or autism (Sanchez-Villegas et al., 2018; Shahidi and Ambigaipalan, 2018). Thus, studying the effect of novel oils as lipid sources for aquafeeds and alternative to FO, on fish neural function could contribute to increasing our knowledge in these fields.

Therefore, using a long-term feeding protocol for sea bream juveniles, the aim of the present study was to investigate the effect of three different dietary formulations with conventional or novel lipid sources with distinct dietary fatty acid composition on (1) brain fatty acid incorporation, (2) fish behavioural response to an external mechanosensory stimulus and (3) expression of neurogenesis and neural activity-related markers in the telencephalon.

2. Materials and methods

2.1. Experimental diets and nutritional trial

Three experimental diets were formulated containing similar amounts of digestible protein, lipid and energy (Table 1) but differing in their dietary fatty acid composition (Table 2). A control diet was formulated based on FO (FO diet), whereas the two other dies were based on either a combination of poultry oil and a DHA and n-6 docosapentaenoic acid (DPA, 22:5n-6)-rich microalgae oil (DD diet), or only poultry oil (PO diet) (Table 1). All diets had similar contents in saturated fatty acids (Table 2). The FO and DD diets were similar in MUFA, n-9, n-3, n-3 PUFA and EPA + DHA. However, FO diet was the highest in EPA, EPA/ARA and EPA/DHA, whereas the DD diet was the highest on DHA, DPA and n-6 LC-PUFA, containing 5 times more DHA than EPA (Table 2). On the contrary, the PO diet contained low levels of n-3, n-3 PUFA, EPA and DHA. Thus, the n-3 LC-PUFA contents in PO diet were below the requirements estimated for gilthead sea bream juveniles (1.3–1.6% of dry weight of the diet; Izquierdo, 2005) and could cause a marginal EFA-deficiency. The diets were manufactured by Skretting ARC at their Feed Technology Plant (Stavanger, Norway).

A nutritional trial of 2.5 months was conducted in triplicated groups of 55 gilthead sea bream (*S. aurata*) fingerlings (initial body weight of 2.50 ± 0.01 g), whose results concerning fish growth performance and hepatic lipid metabolism are described elsewhere (Carvalho et al., 2020a, 2020b) and were not the aim of the present study. After this

Table 1
Ingredients and proximate composition of the experimental diets.

Ingredients (%)	FO	DD	PO
Fish meal[a]	15.0	15.0	15.0
Wheat[a]	12.3	12.1	11.4
Corn gluten[a]	6.6	6.2	10.0
Hi-pro soya[a]	5.0	5.0	5.0
Wheat gluten[a]	17.7	17.9	15.4
Soya protein concentrate[a]	25.0	25.0	25.0
Faba beans[a]	5.0	5.0	5.0
Fish oil[a]	5.3	0.0	0.0
Rapseed oil[a]	7.9	5.7	7.3
DHA Natur oil[b]	0.00	3.7	0.0
Poultry oil[c]	0.0	3.9	5.7
Vitamin premix[d]	0.1	0.1	0.1
Mineral premix[e]	0.1	0.1	0.1
Proximate composition (%)			
Crude protein	49.31	48.06	50.18
Crude lipids	18.19	18.23	17.50
Neutral lipids	89.32	90.15	91.96
Polar lipids	10.68	9.85	8.04
Moisture	6.60	7.85	8.28
Ash	4.66	4.70	4.64
Starch (theorical value)	10.03	10.18	9.87
Fiber (theorical value)	2.53	2.53	2.54

[d,e]Nclude vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC,
[a] Skretting AS (Norway).
[b] DHA Natur oil (ADM Animal Nutrition, USA).
[c] Poultry oil: Sonac. B.V. The Netherlands.

Table 2
Fatty acid (% of total fatty acids) composition of the experimental diets.

Fatty acid	FO	DD	PO
14:0	2.05	1.33	0.72
14:1n-7	0.40	0.34	0.08
14:1n-5	0.37	0.35	0.03
15:0	0.45	0.35	0.10
15:1n-5	0.29	0.36	0.03
16:0ISO	0.37	0.40	0.03
16:0	9.72	10.51	11.59
16:1n-7	2.51	1.42	1.90
16:1n-5	0.31	0.38	0.06
16:2n-4	0.44	0.52	0.07
17:0	0.49	0.41	0.06
16:3n-4	0.28	0.35	0.11
16:3n-3	0.31	0.49	0.04
16:3n-1	0.28	0.46	0.05
16:4n-3	0.54	0.44	0.13
18:0	2.72	2.70	3.40
18:1n-9	30.01	29.26	39.78
18:1n-7	2.68	1.73	2.38
18:1n-5	0.37	0.47	0.07
18:2n-9	0.41	0.39	0.00
18:2n-6	15.74	16.36	23.16
18:2n-4	0.32	0.41	0.03
18:3n-6	0.23	0.52	0.08
18:3n-4	0.34	0.49	0.10
18:3n-3	5.02	3.61	5.29
18:3n-1	0.29	0.46	0.00
18:4n-3	0.94	0.53	0.39
20:0	0.68	0.50	0.52
20:1n-9	0.37	0.43	0.18
20:1n-7	2.33	1.91	1.96
20:1n-5	0.47	0.41	0.17
20:2n-9	0.30	0.35	0.05
20:2n-6	0.32	0.56	0.27
20:3n-9	0.28	0.00	0.18
20:3n-6	0.32	0.58	0.11
ARA (20:4n-6)	0.59	0.48	0.32
20:3n-3	0.29	0.46	0.10
20:4n-3	0.44	0.64	0.11
EPA (20:5n-3)	5.30	1.54	1.24
22:1n-11	2.37	1.56	1.94
22:1n-9	0.56	0.58	0.39
22:4n-6	0.36	1.32	0.15
DPA (22:5n-6)	0.47	3.05	0.13
22:5n-3	1.31	1.09	0.31
DHA (22:6n-3)	5.36	9.04	2.11
ΣSFA	16.11	15.80	16.39
ΣMUFA	43.02	39.20	48.96
Σn-9	31.93	31.01	40.58
Σn-6	17.79	22.88	24.29
Σn-6 LC-PUFA	2.06	5.99	0.98
Σn-3	19.51	17.84	9.72
Σn-3 LC-PUFA	12.70	12.77	3.87
EPA + DHA	10.66	10.58	3.35
EPA/ARA	8.91	3.22	3.88
EPA/DHA	0.99	0.17	0.59

ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acids.

feeding period, 12 fish per tank (36 per treatment) were transferred to 500 L tanks kept under natural photoperiod and continuously supplied with filtered sea water (salinity: 37 g/L, water temperature: 23.1 ± 0.3 °C) and aeration (dissolved oxygen: 6.4 ± 0.2 mg/L). Fish were fed with the corresponding experimental diet for another 2.5 months.

2.2. Behavioural response to an external mechano-sensory stimulus

After the long-term feeding period (5 months), 9 fish per treatment were randomly selected for testing behaviour response to an external mechano-sensory stimulus. Gilthead sea bream is a schooling species and presents social hierarchies between individuals (Goldan et al., 2003; Montero et al., 2009; Arechavala-Lopez et al., 2019, 2020). Previous observations noticed a freezing-like behaviour (no movement) in gilthead sea bream under isolation in a tank (author's own observations), as also observed in other fish species under fear/anxious situations (Midttun et al., 2020). Therefore, to avoid the stress of isolation, for the present trial fish were tested in three groups per treatment, of three fish each, using a white bottom-tank of 100 L, indirectly illuminated, and covered by a dark plastic canvas to avoid disturbance during acclimation of 1 h and testing. Each fish was gently tagged with a colour pearl attached to the base of the first ray of the dorsal fin, under anaesthesia with clove oil (4 ml/100 L). Preliminary observations showed that tagging does not compromise fish normal behaviour regarding swimming or response to the stimulus, and this type of tagging was previously used in fish behaviour trials (Alfonso et al., 2019; Cerqueira et al., 2020). For that, prior to tagging fish, preliminary test trials were conducted to observe if the typical pattern of escape response and swimming activity were influenced by tagging and acclimation time. Although these trials were only observational, non-tagged fish showed the regular swimming activity, similar to tagged fish after 1 h of acclimation. Furthermore, all tagged fish responded to the stimulus as well as non-tagged fish, indicating that tagging and acclimation protocol did not affect the responsiveness and type of response of the fish to the stimulus. Even if clove oil and tagging may have had an effect on fish behaviour, the protocol was strictly maintained in all fish in order to accurately compare behavioural parameters among the different experimental groups. Then, fish were let to recovered and acclimated in the testing tank for 1 h, according the acclimation time used in most of locomotor activity- related studies in several fish species (Melvin et al., 2017). After this acclimation period, fish were exposed to a mechano-sensorial vibrational stimulus, which consisted in an iron pendulum of 600 g that was dropped without any additional force against the tank wall from a distance of 71 cm. Fish behaviour was monitored by a video-camera (Xiaomi Mijia 4 k, China) placed above the tank and recording at 200 fps. The first 60 s of recording prior to stimulation were used to measure fish basal activity level (cruise speed), while the first 300 ms immediately after applying the stimulus were used for measuring the activity level (measured through swimming speed) in fish after stimulation. Furthermore, fish motion during the escape response (Domenici and Hale, 2019) was analysed at the moment of applying the stimulus using the Kinovea® software (Kinovea Project, 2016; da Silva Souza et al., 2020) software for measuring specific points on the fish, i.e. the centre of mass of the fish (at 0.35 lengths, based on other fish species with similar body shape) (Webb, 1978; Dadda et al., 2010) and the tip of the head, as well as turning angles. The following variables were analysed:

(1) Responsiveness: responsiveness to the stimulation was defined as the number of fish that responded to the sensory-mechanical stimulus with an escape response (Domenici and Hale, 2019); non-responders were considered individuals that did not display any movement at the moment of the stimulation;
(2) Escape type: Escape responses consists of a fast body muscular contraction, usually in C-shape form (stage 1), where a second contralateral contraction (stage 2) may or may not following (Domenici and Blake, 1997). Thus, escape type was defined as either single if escape response consisted of stage 1 alone (SB) or double bend (DB) if stage 2 followed stage 1 (Domenici and Blake, 1997);
(3) Escape latency: Escape latency was measured as the time (in ms) from the frame F0 in which the stimulus started disappearing below the upper edge of the tank seen from the camera above until the first detectable movement by the fish. Because F0 presumably occurred a few milliseconds before the stimulus hit the side of the tank, these latency values are likely to be longer than those typically found in teleosts. They are, however, a proxy of the readiness to react, since the stimulus was delivered to the side of the tank in a consistent manner;

(4) Turning rate: Turning rate was measured as the ratio between the turning angle observed in stage 1 and the duration of the stage 1 (degrees /s) (Domenici et al., 2008). The duration of this stage was measured between the frame 0 (prior to the first visible reaction) and the moment when a change in the direction of rotation of the head occurred (Domenici and Blake, 1997). Stage 1 turning angle was measured (in degrees) as the rotation of the line passing through the center of mass and the tip of the head of each individual, during the whole duration of stage 1;
(5) Distance covered: Distance covered was defined as the distance (in cm) between the center of mass of the fish at the frame before the first visible response and 70 ms later. The 70 ms time-period was chosen as a shortest fixed time through which all fish had completed stage 2 (if DB) or stage 1 (if SB) (Meager et al., 2006; Turesson et al., 2009). This fixed time was chosen because stage 1 and 2 correspond to the period considered crucial for survival when avoiding stressors, for instance ambush predator attacks (Walker et al., 2005);
(6) Basal activity level: Basal activity level (in cm/s) was defined as the cruise speed (distance/ time) during the first 60 s prior to stimulation;
(7) Post-stimulation activity level: Post-stimulation activity level (in cm/s) was defined as the swimming speed (distance/ time) during the 300 ms immediately after the stimulation.

2.3. Sample collection

For sampling procedures, fish were fasted 24 h and euthanasia was carried out by an overdose of clove oil (6 ml/10 L). Fish brains were carefully removed from skull and collected at short (2.5 months of feeding) from 15 fish per treatment ($n = 5$ per triplicate) that were chosen randomly, for analysing their fatty acid composition. Brains were also weighed to calculate cephalic index (%) as following: Brain weight (g)/ Body weight (g) *100. Additionally, telencephalons of 9 fish per treatment were micro-dissected and conserved in RNA later (Sigma-Aldrich, Madrid, Spain) for molecular analysis. The remaining brain parts were used for analysing brain fatty acid composition after long-term feeding (5 months). All samples were frozen at −80 °C until analysis.

2.4. Diets and brain biochemical composition

All samples were homogenized prior to analysis. Dietary protein, ash and moisture contents were additionally analysed according A.O.A.C. (2000). Lipid content of diets and brains was determined by extraction with chloroform/methanol (2:1 *v*/v) (Folch et al., 1957) and then transmethylated to obtain fatty acid methyl esters (FAMES) (Christie, 1989). FAMES were then separated by gas liquid chromatography (Izquierdo et al., 1990), quantified by a flame ionization detector (Finnigan Focus SG, Thermo electron Corporation, Milan, Italy) and identified comparing with previously characterized standards.

2.5. Brain gene expression

2.5.1. RNA extraction, quantification and complementary DNA synthesis

Total RNA was obtained from fish telencephalons, using the RNeasy Mini Kit extraction (Qiagen, Hilden, Germany). Briefly, samples were homogenized using TissueLyzer-II (Qiagen) and adding TRI Reagent (Sigma-Aldrich, Sant Louis, MO, USA). Then, samples were centrifuged with chloroform at 12000 *g* for 15 min, at 4 °C, and RNA phase was mixed with 75% ethanol and transferred into a RNeasy spin column, using RW1 and RPE buffers (Qiagen) to purify RNA. RNA was posteriorly eluted with RNase-free water. The NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Gel Red™ staining (Biotium Inc., Hayward, CA) on 1.4% agarose electrophoresis gel were used to determine the quantity and integrity of RNA, respectively.

From the extracted RNA, complementary DNA (*cDNA*) was synthetized using iScriptcDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad).

2.5.2. RT-qPCR analysis

Brain (telencephalon) *mRNA* levels of the following genes were analysed from each individual: *neurogenic differentiation factor 6* (*neurod6*) and *early growth response gene 1* (egr1), related with neurogenesis (Alfonso et al., 2019); and *cerebral nitric oxid synthase 1* (*nos1*), *c-fos* and *brain-derived neurotrofic factor* (*bdnf*), markers of neural activity and plasticity (Alfonso et al., 2019), and *fatty acyl desaturase 2* (*fads2*), involved in PUFA biosynthesis (Izquierdo, 2005). Primer sequences were designed from gilthead sea bream genome using Primer3 software (Rozen and Skaletsky, 2000) (Table 3) and respective relative gene expressions were determined by RT-PCR in an iQ5 Multicolour Real-Time PCR detection system (Bio-Rad). *ef1α* and tubulin were used as reference gene (RG) and best RG to normalize the data was chosen by comparison of the two RGs with BestKeeper software. According to BestKeeper, both genes showed a very small variation (SD = 0.45 and 0.50 for *ef1a* and *tubulin*, respectively, below the maximum SD acceptable in the literature (Zhou et al., 2018), as well as r values close to 1 ($r = 0.95$ and 0.93, respectively). Therefore, although both genes were suitable for be used as RG, *ef1a* was chosen for presenting a minor slight lower SD and higher r value than tubulin. RT-PCR conditions used were the following: a first step of 3 min 30 s at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at annealing temperature for each primer (see Table 3), 30 s at 72 °C, 1 min at 95 °C, and a final denaturing step from 58 °C to 95 °C for 10 s. All PCR reactions were carried out in in a final volume of 15 μl, with 7.5 μl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 μl of each primer (10 mM), 5 μl of cDNA (1:10 dilution) and 1.3 μl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions. In each run, an analysis of melting curve leading to a melting peak specific for the amplified target DNA was carried out.

2.6. Statistical analysis

All data are presented as mean ± standard deviation (SD). Shapiro–Wilk and Levene's tests were used to test data normality and homogeneity of variances, respectively. A one-way ANOVA was applied to growth, biochemical and gene expression, using diet as factor. When necessary, transformations to normalize data were applied (Sokal and Rohlf, 1981). If normalization was not possible, for instance in some minor FA, the data were analysed using the non-parametric Kruskall-Wallis test. Relative gene expression data were normalized according Livak method ($2^{-\Delta\Delta ct}$). Tukey (Tukey, 1949) was used as post-hoc test to compare means when significant differences were detected. When applicable, data were also subjected to the best fit regressions (linear, exponential or logarithmic), which were also checked for significance. Behaviour response such as responsiveness and type of escape response were analysed applying a Chi-2 square test (critical CHI values =5.99 for two degrees of freedom), while numeric variables including activity levels, latency, distance, and turning rates were analysed using a two-way ANOVA, where diet was used as fixed factor and group within each diet was used as random factor. Total length of each fish (cm) was used as co-variable for all numeric behavioural variables. A significance level of less than 5% was used for all statistical analysis ($P < 0.05$). Statistical treatment of the data was carried out using SPSS 21.0 for macOS 10.15 (IBM, 2020).

2.7. Ethical statement

All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010/63/EU) and Spanish legislation (RD 1201/2005) on the protection of animals for

Table 3
Primers sequences used in RT-PCR for measuring telencephalic neuronal markers relative expression in gilthead sea bream fed the different experimental diets.

Gene	Primer sequence	Temp (°C)	Access n°
neurod6	F: TCGGCAGGAAAAGAAAAAGA R: CACAATATCGGCTCCATGTG	61°	XM_030401584.1
egr1	F: GACGAGAGGAAGAGGCACAC R: ACGGGAGAGGGGTAAGAAGA	61°	XM_030396900.1
nos1	F: GGTCAACAAAGAGCCTCAGC R: ATTCCTCTGGCCTTCTCCAT	61°	XM_030416914.1
c-fos	F: TGACCTGTCCAACTCCCTCT R: GTTGCTGTTGCTTCCTCTCC	61°	XM_030405977.1
bdnf	F: ATCAGCAACCAAGTGCCTTT R: GCCGTCTTTTTATCCACAGC	61°	XM_030413189.1
fads2	F: GCAGGCGGAGAGCGACGGTCTGTTCC R:AGCAGGATGTGACCCAGGTGGAGGCAGAAG	60°	AY055749
ef1α	F: CATGGTTGTGGAGCCCTTCT R: TCCTGCACGACCATTCATTTC	60°	AF184170
tubulin	ATCACCAATGCCTGCTTCGA CTGTGGGAGGCTGGTAGTTG	60°	AY326430,1

neurod6, neurogenic differentiation factor 6; *egr1*, early growth response gene 1; *nos1*, nitric oxid synthase 1 (neuronal); *bdnf*, brain-derived neurotrophic factor; *ef1α*, elongation factor 1 alpha.

scientific purposes, at ECOAQUA-UI from University of Las Palmas de Gran Canaria (Canary Islands, Spain). The procedures used were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (Ref OEBA-ULPGC-21/2018 & OEBA-ULPGC-07/2019).

3. Results

3.1. Growth performance and cephalic indices

After 5 months of feeding the experimental diets, body weight of sea bream fed PO diet was significantly lower ($P < 0.05$) than those fed FO or DD diets (Fig. 1A). No significant differences were observed in cephalic index after 2.5 months of feeding (data not shown), whereas after 5 months of feeding, cephalic index was higher in fish fed DD and PO diets compared to those fed the FO ($P < 0.05$; Fig. 1B).

3.2. Behavioural response to an external mechano-sensory stimulus

All individuals (9/9) responded to stimulus with a typical escape response in fish fed FO diet, while 7/9 and 8/9 responded in those fed DD and PO, respectively ($Chi^2 = 2.25$; $P > 0.1$; Fig. 2A). From the responding fish in each treatment, 33% presented a SB escape response while 67% displayed a DB escape response in those fed FO; 29% and 71%, respectively in those fed DD; and 13% and 87%, respectively in those fed PO ($Chi^2 = 1.05$; $P > 0.05$; Fig. 2B). However, these differences were not significant ($P > 0.05$) among treatments or among groups of fish. Similarly, no significant differences ($P > 0.05$) were recorded in distance covered ($P = 0.3$; Fig. 2C), turning rate during escape response ($P = 0.9$; Fig. 2D) and escape timing ($P = 0.1$; Fig. 2E). However, there was a tendency for escape latency to be the longest in fish fed PO and DD diets ($P = 0.1$; Fig. 2E), which is in line with the significant correlation found between escape latency and individual brain n-6 DPA contents, although with a low r^2 value ($r^2 = 0.18$; $P = 0.03$; Fig. 3). In addition, a total of two of the fish (as mentioned above) showed no response and were not included in the original correlation test. Therefore, an additional correlation was performed by considering these fish conservatively, i.e. assigning them the highest latency we recorded (i.e. 210 ms), which lead to a more significant correlation value ($P = 0.01$; $r^2 = 0.23$). No significant differences were observed for activity basal ($P = 0.8$) and after stimulation ($P = 0.6$) levels (Fig. 4).

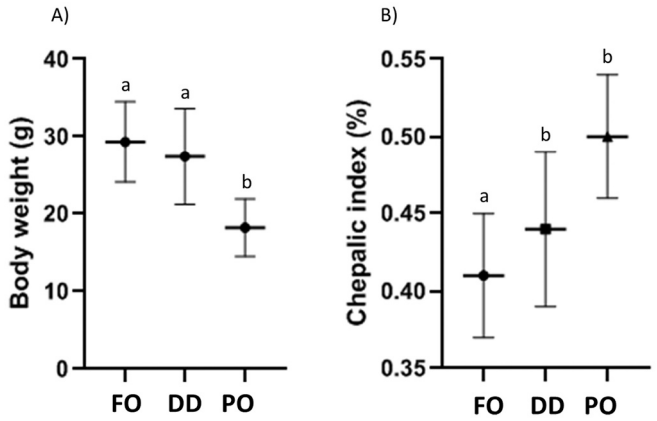

Fig. 1. Growth (A) and cephalic index (B) of gilthead sea bream fed the different experimental diets for 5 months. Different letters above bar errors denote significant differences among the experimental groups ($P < 0.05$).

3.3. Brain fatty acid composition

No differences were observed in brain lipid content, either at 2.5 or 5 months of feeding with the experimental diets (Tables 4 and 5).

After 2.5 months of feeding, brain fatty acid profiles of sea bream were similar among fish fed the different diets, regardless the dietary differences (Table 4). As in diet, FO fish had more EPA, EPA/ARA and EPA/DHA and DD fish had a higher content in n-6 DPA. However, PO fish was not lower in n-3, n-3 LC-PUFA or EPA + DHA as it occurred in the diet. Moreover, 18:2n-9, 18:3n-6 and 20:3n-6, fatty acids that are products from FADS2 activity, were higher in PO fish, despite their low dietary values ($P < 0.05$; Tables 4). These tendencies in brain fatty acid profiles were even stronger after 5 months of feeding the experimental diets (Table 5). Thus, products of FADS2, as well as products of elongases, were high in brain of fish fed the PO diet (18:2n-9, 18:3n-6, 20:3n-6, 20:4n-6). Besides, the FADS2 substrates 18:1n-9 and 18:2n-6 were low, contrary to their high dietary content ($P < 0.05$; Tables 4 and 5). Furthermore, PO fish also had the highest DHA content in brain, despite the lowest contents of this fatty acid in PO diet (Table 5). Accordingly, the EPA level in brain of PO fish was similar to that of sea bream fed the other two diets, regardless the lowest EPA content in PO diet (Table 5). Interestingly, brain of sea bream fed the DD diet, not only was the highest in n-6 DPA, in agreement with its high dietary levels, but it was also highest in ARA after 2.5 months of feeding (Table 4) and higher than in fish fed the FO diet after 5 months (Table 5), regardless the low ARA contents in DD diet.

3.4. Telencephalon gene expression

No significant differences were observed for the relative expressions

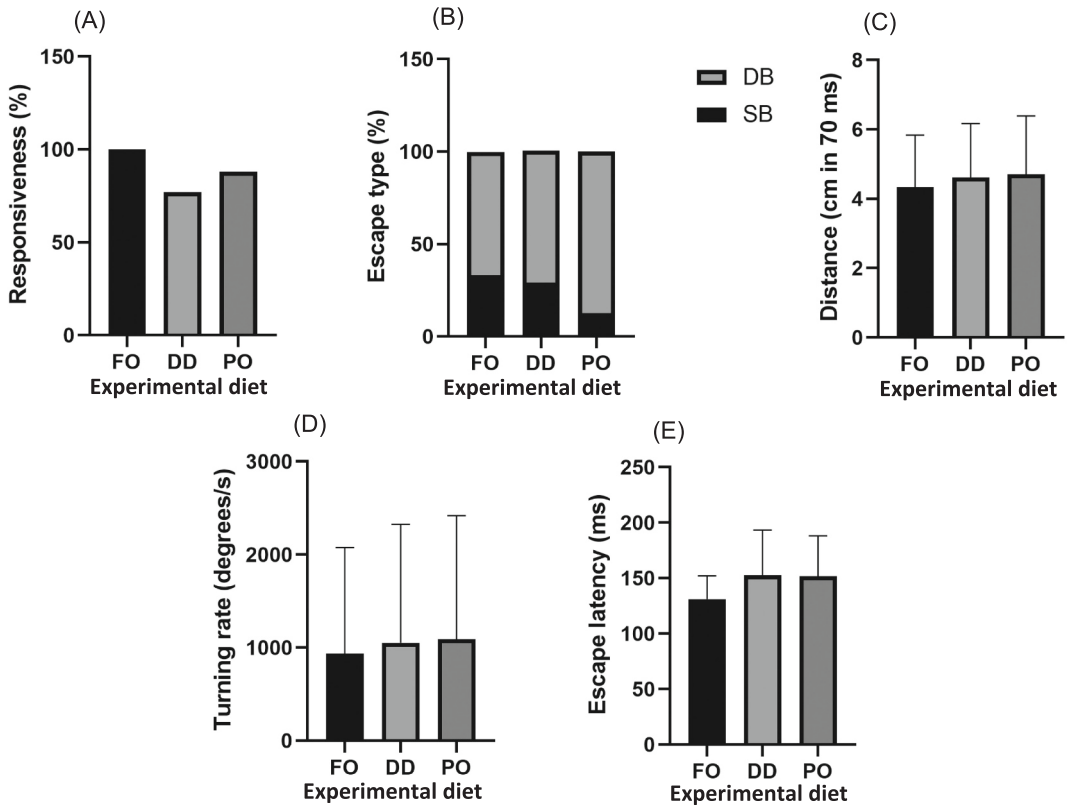

Fig. 2. Behavioural response to an external mechano-sensory stimulus of gilthead sea bream fed the different experimental diets for 5 months. (A) responsiveness; (B) escape response type; (C) distance covered after stimulus; (D) turning rate during escape; (E) escape latency.

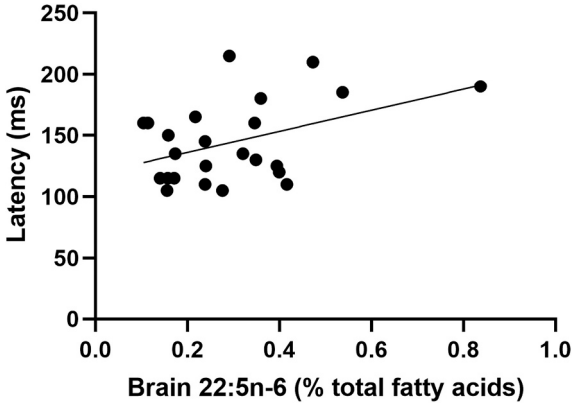

Fig. 3. Linear correlation between brain n-6 docosapentaenoic acid and escape latency ($P = 0.03$).

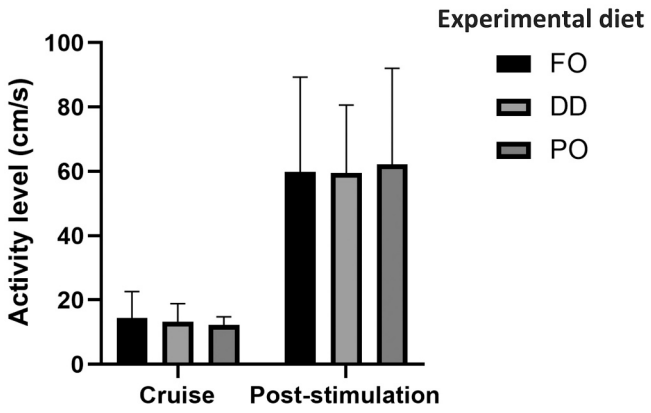

Fig. 4. Activity basal and after stimulation level in gilthead sea bream fed the different experimental diets for 5 months.

of *egr1* or *c-fos* among fish fed the experimental diets (Fig. 5). However, sea bream fed the PO diet showed an upregulation of telencephalon *mRNA* levels of *neurod6*, *bdnf* and *nos1* ($P < 0.05$) compared with those fed FO, but not with those fed DD diet (Fig. 5). Additionally, a tendency to present higher relative expression of *fads2* was also noted in fish fed PO ($P = 0.1$), particularly when compared with those fish fed DD (with the highest dietary DHA) (Fig. 5). Indeed, significant correlations were found between individual telencephalon DHA content and the respective *mRNA* levels of these genes (*neurod6*: $r^2 = 0.23$, $P = 0.01$; *bdnf*: $r^2 = 0.27$, $P = 0.01$; *nos1*: $r^2 = 0.21$, $P = 0.02$; *fads2*: $r^2 = 0.24$, $P = 0.01$; Supplementary material).

4. Discussion

Essential fatty acids, in particular the n-3 LC-PUFA DHA and EPA, are of key importance in fish metabolism, health and development. After a long period of feeding of 5 months, gilthead sea bream fed the PO diet, with low EPA and DHA levels, showed reduced growth compared to those fed FO or DD diets, with high DHA. In fact, only 2.5 months of feeding was sufficient to reduce growth in sea bream fed PO (Carvalho et al., 2020a), suggesting a marginal deficiency in these fish, in agreement with other marine species, for which these fatty acids are essential (reviewed in Izquierdo, 2005). Furthermore, this EFA-deficiency was likely to be more related to a dietary DHA deficiency, since the low inclusion of EPA in DD diet did not cause a decrease in growth performance in fish juveniles, when compared to those fed FO (with high EPA

Table 4
Brain lipid content (% wet weight - ww) and fatty acid composition (% total fatty acids) of gilthead sea bream fed the different experimental diets after 2.5 months.

	FO	DD	PO
Lipids (%ww)	7.88 ± 0.59	8.62 ± 0.50	8.65 ± 1.04
Fatty acid (% total fatty acids)			
14:0	0.48 ± 0.02	0.21 ± 0.11	0.31 ± 0.05
14:1n-7	0.01 ± 0.00	0.07 ± 0.06	0.02 ± 0.00
14:1n-5	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
15:0	0.08 ± 0.01	0.08 ± 0.01	0.0 ± 0.01
15:1n-5	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
16:0ISO	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
16:0	14.56 ± 0.81^{ab}	16.49 ± 2.54^{a}	12.55 ± 0.21^{b}
16:1n-7	1.82 ± 0.10	1.77 ± 0.24	1.47 ± 0.04
16:1n-5	0.05 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
16:2n-4	0.04 ± 0.00^{a}	0.04 ± 0.01^{ab}	0.02 ± 0.00^{b}
17:0	0.07 ± 0.00^{a}	0.05 ± 0.01^{ab}	0.04 ± 0.00^{b}
16:3n-4	0.13 ± 0.01^{a}	0.11 ± 0.01^{ab}	0.10 ± 0.00^{b}
16:3n-3	0.03 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
16:3n-1	4.77 ± 0.34	4.11 ± 0.59	4.18 ± 0.27
16:4n-3	0.69 ± 0.04	0.61 ± 0.06	0.67 ± 0.05
16:4n-1	0.40 ± 0.03	0.36 ± 0.03	0.37 ± 0.03
18:0	11.22 ± 0.63	12.01 ± 0.98	9.92 ± 0.38
18:1n-9	22.73 ± 1.24	22.42 ± 0.64	22.18 ± 1.09
18:1n-7	1.63 ± 0.11	1.54 ± 0.07	1.51 ± 0.07
18:1n-5	0.05 ± 0.00	0.07 ± 0.01	0.04 ± 0.00
18:2n-9	0.47 ± 0.03^{b}	0.47 ± 0.00^{b}	0.63 ± 0.02^{a}
18:2n-6	3.89 ± 0.27	3.82 ± 0.30	5.70 ± 1.02
18:2n-4	0.04 ± 0.00^{a}	0.02 ± 0.01^{b}	0.02 ± 0.00^{b}
18:3n-6	0.23 ± 0.03^{b}	0.23 ± 0.04^{b}	0.45 ± 0.03^{a}
18:3n-4	0.08 ± 0.02	0.04 ± 0.01	0.05 ± 0.01
18:3n-3	0.76 ± 0.07	0.54 ± 0.06	0.85 ± 0.23
18:3n-1	0.01 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
18:4n-3	0.43 ± 0.01	0.31 ± 0.05	0.37 ± 0.02
18:4n-1	0.13 ± 0.01	0.11 ± 0.02	0.13 ± 0.01
20:0	0.31 ± 0.03	0.29 ± 0.04	0.29 ± 0.01
20:1n-9	0.13 ± 0.01	0.11 ± 0.01	0.13 ± 0.02
20:1n-7	0.76 ± 0.05	0.62 ± 0.06	0.69 ± 0.07
20:1n-5	0.14 ± 0.01	0.12 ± 0.00	0.10 ± 0.01
20:2n-9	0.26 ± 0.01	0.26 ± 0.01	0.35 ± 0.02
20:2n-6	0.15 ± 0.01	0.16 ± 0.01	0.21 ± 0.02
20:3n-9	0.05 ± 0.02	0.07 ± 0.05	0.07 ± 0.02
20:3n-6	0.28 ± 0.02^{b}	0.34 ± 0.06^{b}	0.61 ± 0.01^{a}
ARA (20:4n-6)	1.31 ± 0.07^{b}	1.92 ± 0.02^{a}	1.48 ± 0.09^{b}
20:3n-3	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
20:4n-3	0.19 ± 0.01	0.10 ± 0.05	0.15 ± 0.01
EPA (20:5n-3)	3.25 ± 0.33^{a}	1.98 ± 0.17^{b}	2.43 ± 0.13^{ab}
22:1n-11	0.27 ± 0.04	0.17 ± 0.03	0.21 ± 0.04
22:1n-9	0.46 ± 0.09	0.41 ± 0.05	0.41 ± 0.03
22:4n-6	0.14 ± 0.01	0.22 ± 0.04	0.16 ± 0.01
DPA (22:5n-6)	0.10 ± 0.02^{b}	1.01 ± 0.20^{a}	0.27 ± 0.14^{b}
22:5n-3	1.22 ± 0.11^{a}	0.63 ± 0.09^{b}	0.98 ± 0.05^{ab}
DHA (22:6n-3)	26.07 ± 2.72	25.93 ± 2.80	29.70 ± 1.71
ΣSFA	26.75 ± 1.46	29.14 ± 3.19	23.17 ± 0.51
ΣMUFA	28.07 ± 1.59	27.36 ± 0.53	26.79 ± 1.34
Σn-9	23.97 ± 2.25	23.63 ± 1.20	23.65 ± 1.93
Σn-6	6.11 ± 0.47	7.70 ± 0.91	8.88 ± 1.88
n-6 LC-PUFA	1.83 ± 0.08	3.43 ± 0.38	2.57 ± 0.11
Σn-3	32.70 ± 5.53	30.19 ± 5.47	35.25 ± 2.89
n-3 LC-PUFA	30.79 ± 5.46	28.71 ± 5.25	33.34 ± 3.22
EPA + DHA	29.32 ± 3.04	27.91 ± 2.91	32.13 ± 1.84
EPA/ARA	2.48 ± 0.21^{a}	1.03 ± 0.08^{c}	1.64 ± 0.03^{bc}
EPA/DHA	0.12 ± 0.00^{a}	0.08 ± 0.01^{b}	0.08 ± 0.00^{b}

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acids. Different superscript letters denote significant differences among the experimental groups ($P < 0.05$).

Table 5
Brain lipid content (% wet weight) and fatty acid composition (% total fatty acids) of gilthead sea bream fed the different experimental diets after 5 months.

	FO	DD	PO
Lipids (%ww)	11.30 ± 2.70	12.18 ± 2.45	11.65 ± 3.18
Fatty acid (% total fatty acids)			
14:0	0.90 ± 0.22	0.86 ± 0.24	0.74 ± 0.30
14:1n-7	0.02 ± 0.00	0.02 ± 0.01	0.03 ± 0.01
14:1n-5	0.04 ± 0.01^{b}	0.03 ± 0.00^{b}	0.10 ± 0.03^{a}
15:0	0.15 ± 0.03^{a}	0.15 ± 0.02^{a}	0.09 ± 0.02^{b}
15:1n-5	0.02 ± 0.03	0.01 ± 0.00	0.02 ± 0.01
16:0ISO	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.01 ± 0.01^{b}
16:0	17.28 ± 2.31	15.97 ± 1.05	18.00 ± 1.63
16:1n-7	1.98 ± 0.27^{a}	1.93 ± 0.41^{a}	1.22 ± 0.33^{b}
16:1n-5	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.02
16:2n-6	0.01 ± 0.01^{ab}	0.00 ± 0.00^{b}	0.01 ± 0.01^{ab}
16:2n-4	0.10 ± 0.02^{a}	0.10 ± 0.04^{a}	0.03 ± 0.02^{b}
17:0	0.09 ± 0.02^{a}	0.09 ± 0.02^{a}	0.04 ± 0.01^{b}
16:3n-4	0.15 ± 0.02^{a}	0.14 ± 0.03^{a}	0.07 ± 0.01^{b}
16:3n-3	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.02
16:3n-1	0.69 ± 0.22^{b}	0.59 ± 0.26^{b}	1.22 ± 0.17^{a}
16:4n-3	0.32 ± 0.12^{b}	0.27 ± 0.14^{b}	0.69 ± 0.30^{a}
16:4n-1	0.05 ± 0.03^{b}	0.07 ± 0.04 ^{ab}	0.10 ± 0.02^{a}
18:0	9.85 ± 2.55^{b}	8.77 ± 2.49^{b}	14.76 ± 1.23^{a}
18:1n-9	27.64 ± 3.21^{a}	27.68 ± 4.89^{a}	16.11 ± 2.91^{b}
18:1n-7	2.01 ± 0.20^{a}	2.00 ± 0.25^{a}	1.58 ± 0.27^{b}
18:1n-5	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
18:2n-9	0.53 ± 0.07^{b}	0.47 ± 0.08^{b}	0.66 ± 0.04^{a}
18:2n-6	8.64 ± 1.73^{a}	9.50 ± 2.69^{a}	3.80 ± 1.91^{b}
18:2n-4	0.07 ± 0.01^{a}	0.07 ± 0.02^{a}	0.02 ± 0.00^{b}
18:3n-6	0.18 ± 0.03^{b}	0.17 ± 0.04^{b}	0.21 ± 0.09^{a}
18:3n-4	0.06 ± 0.01^{a}	0.07 ± 0.03^{a}	0.03 ± 0.01^{b}
18:3n-3	2.02 ± 0.50^{a}	2.19 ± 0.71^{a}	0.64 ± 0.41^{b}
18:3n-1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
18:4n-3	0.29 ± 0.07^{ab}	0.31 ± 0.10^{a}	0.18 ± 0.11^{b}
18:4n-1	0.05 ± 0.01	0.05 ± 0.01	0.24 ± 0.61
20:0	0.20 ± 0.03^{a}	0.21 ± 0.04^{a}	0.12 ± 0.02^{b}
20:1n-9	0.12 ± 0.03^{a}	0.14 ± 0.03^{a}	0.06 ± 0.03^{b}
20:1n-7	1.29 ± 0.29^{a}	1.42 ± 0.38^{a}	0.48 ± 0.16^{b}
20:1n-5	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.04
20:2n-9	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.02
20:2n-6	0.34 ± 0.08	0.39 ± 0.11	0.33 ± 0.64
20:3n-9	0.02 ± 0.01^{a}	0.01 ± 0.00^{a}	0.01 ± 0.01^{b}
20:3n-6	0.11 ± 0.01^{b}	0.11 ± 0.01^{b}	0.18 ± 0.02^{a}
ARA (20:4n-6)	0.94 ± 0.11^{c}	1.26 ± 0.36^{b}	1.64 ± 0.25^{a}
20:3n-3	0.11 ± 0.01^{a}	0.11 ± 0.03^{a}	0.06 ± 0.01^{b}
20:4n-3	0.19 ± 0.05^{ab}	0.21 ± 0.04^{a}	0.09 ± 0.02^{c}
EPA (20:5n-3)	3.42 ± 0.61	3.19 ± 0.47	2.93 ± 0.57
22:1n-11	0.39 ± 0.07^{a}	0.43 ± 0.11^{a}	0.14 ± 0.07^{b}
22:1n-9	0.21 ± 0.03	0.25 ± 0.04	0.21 ± 0.06
22:4n-6	0.06 ± 0.02^{b}	0.07 ± 0.01^{ab}	0.08 ± 0.03^{ab}
DPA (22:5n-6)	0.16 ± 0.04^{c}	0.49 ± 0.16^{a}	0.29 ± 0.05^{b}
22:5n-3	0.72 ± 0.18^{a}	0.65 ± 0.15^{a}	0.41 ± 0.06^{b}
DHA (22:6n-3)	18.19 ± 3.50^{b}	19.16 ± 6.19^{b}	32.02 ± 5.54^{a}
ΣSFA	28.47 ± 4.57^{b}	26.04 ± 3.19^{b}	33.75 ± 1.98^{a}
ΣMUFA	33.95 ± 3.84^{a}	34.13 ± 5.99^{a}	20.14 ± 3.57^{b}
Σn-9	28.49 ± 3.18^{a}	28.50 ± 4.85^{a}	17.08 ± 2.95^{b}
Σn-6	10.09 ± 1.70^{a}	11.61 ± 2.24^{a}	6.20 ± 1.84^{b}
n-6 LC-PUFA	1.60 ± 0.14^{b}	2.33 ± 0.41^{a}	2.51 ± 0.61^{a}
Σn-3	23.28 ± 3.95^{b}	23.95 ± 5.74^{b}	36.44 ± 5.70^{a}
n-3 LC-PUFA	22.63 ± 3.85^{b}	23.32 ± 6.00^{b}	35.51 ± 3.57^{a}
EPA + DHA	21.61 ± 3.77^{b}	22.35 ± 5.86^{b}	34.95 ± 5.69^{a}
EPA/ARA	3.66 ± 0.65^{a}	2.74 ± 0.88^{b}	1.81 ± 0.40^{c}
EPA/DHA	0.19 ± 0.03^{a}	0.18 ± 0.06^{a}	0.09 ± 0.02^{b}

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acids. Different superscript letters denote significant differences among the experimental groups ($P < 0.05$).

and DHA). Although both DHA and EPA compete for the incorporation into cell membranes phospholipids, the former has a higher function as growth promoter and stress resistance than the latter (Watanabe et al., 1989; Watanabe and Kiron, 1994), explaining the effect in growth performance associated to dietary DHA rather than EPA.

Indeed, the present results suggest a selective incorporation and retention of DHA over EPA into brain lipids of sea bream juveniles. Accordingly, in mammals, DHA and ARA are activated and incorporated into phospholipid cell membranes, while EPA is catabolised (Chen and Bazinet, 2015). This could explain the lower efficiency of EPA compared to DHA in regulating membrane phospholipids fluidity, important for neural cells growth, health and normal function (Bowen and Clandinin, 2002). The results provided in the literature suggest a similar mode of action in fish and highlight the higher biological importance of DHA

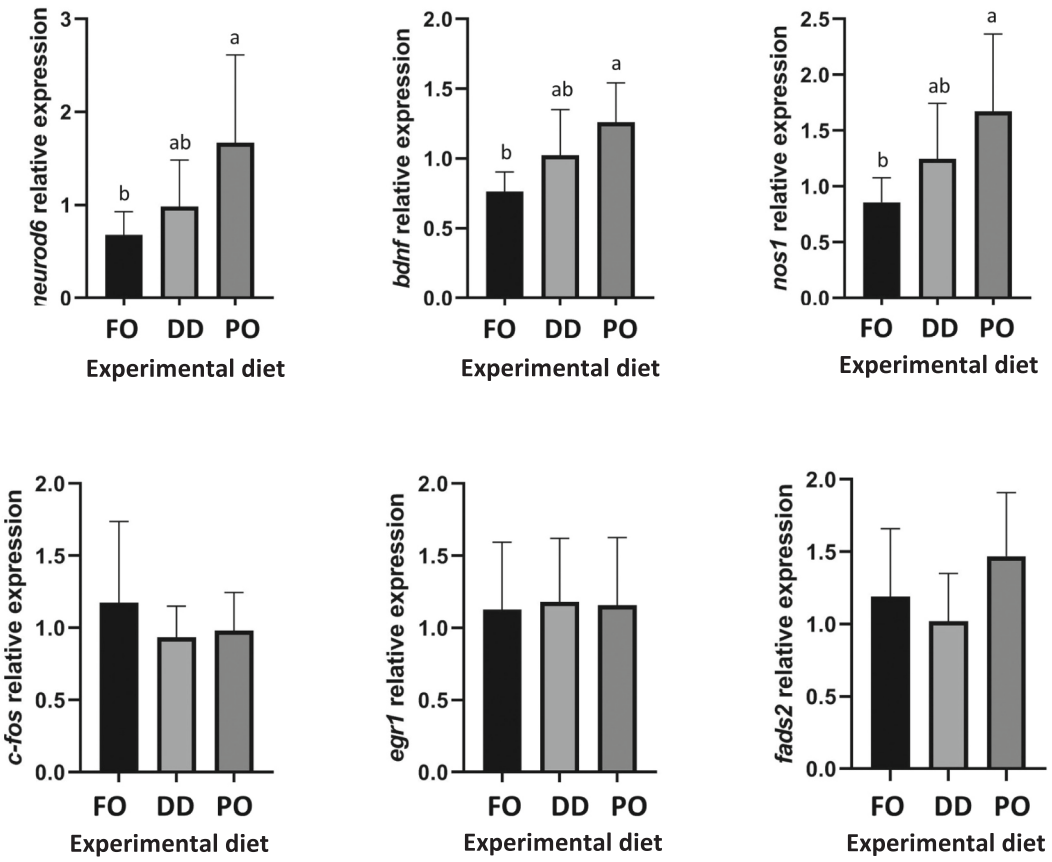

Fig. 5. Relative gene expression ($2^{-\Delta\Delta ct}$) of neural markers in brain of gilthead sea bream fed the different experimental diets after 5 months; *neurod6, neurogenic differentiation factor 6; bdnf, brain-derived neurotrofic factor; nos1, nitric oxid synthase 1 (neuronal); egr1, early growth response gene; fads2, fatty acyl desaturase 2.*

and, possibly ARA, compared to EPA as modulator of neural function (Mourente, 2003). Indeed, contrary to the lowest dietary levels, brain contents of these two FA were the highest in brains of fish fed PO diets, which was evidenced more at 5 months of feeding than 2.5, suggesting a very high retention capacity of sea bream brains when fed EFA-deficient diets for prolonged time. This, added to the highest transcriptional levels of telencephalon *fads2*, as well as with the increased contents of other products of FADS2 activity (18:2n-9, 20:2n-9, 18:3n-6 and 20:3n-6) suggests an activation of the biosynthetic pathways in response to a DHA deficiency, which seem to be stronger over the time. This increase in desaturation products is also consistent with the decrease of the desaturation substrates in these fish, particularly of 18:1n-9 and 18:2n-6. It is well recognised that marine species have lower ability to endogenously synthesize LC-PUFA from their C_{18} precursors due to a limited activity of desaturases and elongases, for instance FADS2 (Izquierdo and Koven, 2011). Many studies reported an upregulation of *fads2* expression or synthesis of its products in many tissues of fish fed low EFA diets, for instance in alternative diets where FO was replaced by EFA-devoid ingredients (Houston et al., 2017; Carvalho et al., 2018, 2020b), but few studies have focused on the expression of this gene in brain. Indeed, brain is often the organ with the highest *fads2* transcription in many marine fish (Monroig et al., 2013). In gilthead sea bream, FADS2 have both Δ6 and Δ8 desaturase activities, but weak Δ5 activity (Monroig et al., 2011). This is the main reason why in most of the studies there is an increased in Δ6 products but the failure in their further desaturation to ARA, EPA or DHA to meet the requirements. In agreement, in the freshwater striped snakehead (*Channa striata*), juveniles fed a diet low in FO and high in linseed oil, brain *fads2* was upregulated (Kuah et al., 2015). In contrast, expression of *fads2* was increased in liver but not in brain in meager fed diets where FO was replaced by VO (Silva-Brito et al., 2016). These differences suggest the species and tissue-specificity of FADS2 expression and activity in fish in response to dietary FA composition but could possibly be also influenced by the duration of the deficiency period (being 2 months in the latter study compared to a prolonged period of 5 months in our study). Interestingly, the high brain ARA contents observed in fish fed DD diet, contrary to the lower dietary levels of this FA might also indicate a retroconversion of the high dietary n-6 DPA to ARA (Carvalho et al., 2020a).

Furthermore, this high retention capacity of DHA to compensate dietary deficiency could, at least partially, explain the lack of effects in behavioural response among sea bream fed the different diets. For instance, in sea bream larvae, high dietary DHA boosted escape behaviour, which was partly related with the increase in the neural activity of the Mauthner cells (Benítez-Santana et al., 2014). Besides, escape swimming speed was slower in fish fed low EPA and DHA levels, in response to a similar stimulus to that of the present study (Benítez-Santana et al., 2014). Essential fatty acid deficiency was also related to an impaired visual ability in larvae of this species (Benítez-Santana et al., 2007) and herring (Bell et al., 1995), and to a delayed development of normal behaviour in yellowtail (Masuda et al., 1998). These results suggest that fish larvae are more susceptible to an EFA-deficiency than juveniles, probably due to their higher requirement for the faster growth rates and the incomplete neuronal development without selective retention mechanisms for dealing with nutritional deficiency. Brains of fish juveniles are fully developed, being a very conservative tissue and, consequently, less sensitive to a nutritional EFA-deficiency, which could explain that neural circuits are not primarily affected and most of vital brain function is preserved. Indeed, in the above-mentioned studies with sea bream larvae, a clear reduction in brain EPA and, particularly, DHA contents was noticed (Benítez-Santana et al., 2007, 2014), whereas the opposite was noticed in the present study. In

agreement with this finding, in rainbow trout and European sea bass (*Dicentrarchus labrax*) juveniles, brain tissue showed the highest DHA concentration, despite the low dietary concentration of this FA, displaying the greatest capacity to regulate and preserve brain functionality (Skalli et al., 2006; Benedito-Palos et al., 2010; Kuah et al., 2015). This may allow higher percentages of replacement of FO with alternative lipid sources with lower n-3 LC-PUFA contents in juveniles than in larvae diets, without affecting fish behaviour and brain function.

Indeed, in the present study, an upregulation of genes involved in neurogenesis and neural activity, including *neurod6*, *bdnf* and *nos1* was demonstrated in telencephalons of fish fed PO diet that was positively correlated with brain DHA content (highly retained in these fish). These results suggest an apparent increase in neurogenesis and neural activity in those fish. Despite the important roles that EFA play in nervous system in vertebrates, studies on the effect of EFA on modulation of neurogenesis and neural plasticity in fish juveniles are scarce. In agreement, Turkmen et al. (2017) demonstrated for the first time in fish an increased proliferative activity of retinal ganglion cells (neural cells) associated to an increment of dietary LC-PUFA, particularly DHA, by increasing BrdU positive cells, in gilthead sea bream larvae. Also, DHA is known to promote neurogenesis and neural differentiation in mammals (Kawakita et al., 2006; Katakura et al., 2009).

Despite the selective incorporation and retention of DHA in fish brains, there is non-significant tendency for an increase in fish escape latency in response to the external stimulus in sea bream fed DD or PO diets. These results suggest a slower reactivity to the stimulus in these fish, which might be related to a lower speed in the translation of the neural signal to activate escape response. Furthermore, this activation response may be controlled by alternative neural commands (i.e. non-Mauthner cells), since it is known that non-Mauthner cell responses show longer latencies than Mauthner cell responses (Domenici and Hale, 2019; Hecker et al., 2020). In line with this tendency, a significant ($P = 0.03$) correlation was observed between escape and n-6 DPA in brain, which showed the highest concentration in fish fed with PO diet and, particularly DD diet, reflecting the dietary composition. The effect of excessive n-6 DPA in fish brain is poorly studied because this FA is not found in high concentrations in fish tissues or farmed fish fed FO or VO diets. However, with the increase of new emergent ingredients, for instance microalgae, often with high n-6 DPA contents in some species, this concern might arise in the future. Therefore, the present study suggests, for the first time in fish, a possible influence of high neural n-6 DPA on the behavioural response of fish. However, this effect needs further corroboration and deserves more investigation in the future. In mammals, n-6 DPA competes with DHA for the incorporation in neural phospholipids and, therefore, an increase in n-6 DPA is observed in DHA-deficient individuals, which is correlated with poorer cognitive function and mental health in general (Garcia-Calatayud et al., 2005). This is because DPA and DHA present different biochemical structures, with DPA lacking a double bond at the end of the chain methyl, affecting fluidity of cell membranes and inducing alterations in neurotransmission which may lead to behavioural deficits (Wainwright, 2002; Eldho et al., 2003; Aïd et al., 2003; Garcia-Calatayud et al., 2005).

5. Conclusions

The present study showed that FO replacement by PO in diets for juvenile gilthead sea bream, namely the reduction in EPA and DHA, stimulates neurogenesis and neural activity in sea bream upregulating *neurod6*, *bdnf* and *nos1*, possibly in relation to the increased DHA content in brain. These results also corroborate the importance of DHA for neural function. In addition, the low dietary EPA and DHA in PO diet activated LC-PUFA synthesis pathways in brain, as denoted by an increased telencephalic *fads2* expression and the presence of desaturation and elongation products in brain fatty acid profiles. Although this increased LC-PUFA synthesis was not sufficient to maintain fish growth, it protected neural circuits and locomotor behavioural response, which was not primarily affected by EFA-deficiency, as shown by the lack of effects of PO in fish when performed a behaviour test. Moreover, a slight but significant positive linear relation between n-6 DPA in brain on non-locomotor behaviour (escape latency) of fish was observed for the first time. Therefore, more investigation is needed to corroborate this possible effect of n-6 DPA on fish behaviour as alternative dietary lipid sources arise in the industry, in agreement with the negative effect of n-6 DPA on behavioural aspects that has been previously recorded in mammals.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737873.

References

Aïd, S., Vancassel, S., Poumès-Ballihaut, C., Chalon, S., Guesnet, P., Lavialle, M., 2003. Effect of a diet-induced n-3 PUFA depletion on cholinergic parameters in the rat hippocampus. J. Lipid Res. 44 (8), 1545–1551.

Alfonso, S., Sadoul, B., Gesto, M., Joassard, L., Chatain, B., Geffroy, B., Bégout, M.L., 2019. Coping styles in European sea bass: the link between boldness, stress response and neurogenesis. Physiol. Behav. 207, 76–85.

Arechavala-Lopez, P., Diaz-Gil, C., Saraiva, J.L., Moranta, D., Castanheira, M.F., Nuñez-Velázquez, S., Grau, A., 2019. Effects of structural environmental enrichment on welfare of juvenile seabream (*Sparus aurata*). Aquacult. Reports 15, 100224.

Arechavala-Lopez, P., Nazzaro-Alvarez, J., Jardí-Pons, A., Reig, L., Carella, F., Carrassón, M., Roque, A., 2020. Linking stocking densities and feeding strategies with social and individual stress responses on gilthead seabream (*Sparus aurata*). Physiol. Behav. 213, 112723.

Association of Official Analytical Chemists (AOAC), 2000. Official Methods of Analysis, 17th ed. Association of Official Analytical Chemists, Gaithersburg, MD, USA.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C., Sargent, J.R., 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus L.*). Lipids 30 (5), 443–449.

Benedito-Palos, L., Navarro, J.C., Kaushik, S., Pérez-Sánchez, J., 2010. Tissue-specific robustness of fatty acid signatures in cultured gilthead sea bream (*Sparus aurata L.*) fed practical diets with a combined high replacement of fish meal and fish oil. J. Anim. Sci. 88 (5), 1759–1770.

Benítez-Santana, T., Masuda, R., Carrillo, E.J., Ganuza, E., Valencia, A., Hernández-Cruz, C.M., Izquierdo, M.S., 2007. Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. Aquaculture 264 (1–4), 408–417.

Benítez-Santana, T., Juárez-Carrillo, E., Betancor, M.B., Torrecillas, S., Caballero, M.J., Izquierdo, M.S., 2012. Increased Mauthner cell activity and escaping behaviour in seabream fed long-chain PUFA. Br. J. Nutr. 107 (2), 295–301.

Benítez-Santana, T., Atalah, E., Betancor, M.B., Caballero, M.J., Hernández-Cruz, C.M., Izquierdo, M., 2014. DHA but not EPA, enhances sound induced escape behavior and Mauthner cells activity in *Sparus aurata*. Physiol. Behav. 124, 65–71.
Bourre, J.M., 2004. Roles of unsaturated fatty acids (especially omega-3 fatty acids) in the brain at various ages and during ageing. J. Nutr. 8, 163–174.
Bowen, R.A., Clandinin, M.T., 2002. Dietary low linolenic acid compared with docosahexaenoic acid alter synaptic plasma membrane phospholipid fatty acid composition and sodium–potassium ATPase kinetics in developing rats. J. Neurochem. 83 (4), 764–774.
Carvalho, M., Peres, H., Saleh, R., Fontanillas, R., Rosenlund, G., Oliva-Teles, A., Izquierdo, M., 2018. Dietary requirement for n-3 long-chain polyunsaturated fatty acids for fast growth of meagre (*Argyrosomus regius*, Asso 1801) fingerlings. Aquaculture 488, 105–113.
Carvalho, M., Montero, D., Rosenlund, G., Fontanillas, R., Ginés, R., Izquierdo, M., 2020a. Effective complete replacement of fish oil by combining poultry and microalgae oils in practical diets for gilthead sea bream (*Sparus aurata*) fingerlings. Aquaculture 529, 735696.
Carvalho, M., Montero, D., Torrecillas, S., Castro, P., Zamorano, M.J., Izquierdo, M., 2020b. Hepatic biochemical, morphological and molecular effects of feeding microalgae and poultry oils to gilthead sea bream (*Sparus aurata*). Aquaculture 532, 736073.
Cerqueira, M., Millot, S., Felix, A., Silva, T., Oliveira, G.A., Oliveira, C.C., Oliveira, R., 2020. Cognitive appraisal in fish: stressor predictability modulates the physiological and neurobehavioural stress response in sea bass. Proc. R. Soc. B 287 (1923), 20192922.
Chen, C.T., Bazinet, R.P., 2015. β-Oxidation and rapid metabolism, but not uptake regulate brain eicosapentaenoic acid levels. Prostaglandins Leukot. Essent. Fat. Acids 92, 33–40.
Christie, W.W., 1989. Gas Chromatography and Lipids: A Practical Guide. The Oily Press, Glasgow, UK.
Dadda, M., Koolhaas, W.H., Domenici, P., 2010. Behavioural asymmetry affects escape performance in a teleost fish. Biol. Lett. 6 (3), 414–417.
Domenici, P., Blake, R., 1997. The kinematics and performance of fish fast-start swimming. J. Exp. Biol. 200 (8), 1165–1178.
Domenici, P., Hale, M.E., 2019. Escape responses of fish: a review of the diversity in motor control, kinematics and behaviour. J. Exp. Biol. 222 (18), jeb166009.
Domenici, P., Claireaux, G., McKenzie, D.J., 2007. Environmental Constraints upon Locomotion and Predator–Prey Interactions in Aquatic Organisms: An Introduction.
Domenici, P., Turesson, H., Brodersen, J., Brönmark, C., 2008. Predator-induced morphology enhances escape locomotion in crucian carp. Proc. R. Soc. B Biol. Sci. 275 (1631), 195–201.
Eldho, N.V., Feller, S.E., Tristram-Nagle, S., Polozov, I.V., Gawrisch, K., 2003. Polyunsaturated docosahexaenoic vs docosapentaenoic acid differences in lipid matrix properties from the loss of one double bond. J. Am. Chem. Soc. 125 (21), 6409–6421.
Folch, J., Lees, M., Stanley, G.S., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226 (1), 497–509.
Furuita, H., Takeuchi, T., Uematsu, K., 1998. Effects of eicosapentaenoic and docosahexaenoic acids on growth, survival and brain development of larval Japanese flounder (*Paralichthys olivaceus*). Aquaculture 161 (1–4), 269–279.
Garcia-Calatayud, S., Redondo, C., Martin, E., Ruiz, J.I., Garcia-Fuentes, M., Sanjurjo, P., 2005. Brain docosahexaenoic acid status and learning in young rats submitted to dietary long-chain polyunsaturated fatty acid deficiency and supplementation limited to lactation. Pediatr. Res. 57 (5), 719–723.
Goldan, O., Popper, D., Karplus, I., 2003. Food competition in small groups of juvenile gilthead sea bream (*Sparus aurata*). Israeli J. Aquacul. - Bamidgeh 55 (2), 94–106.
Hecker, A., Schulze, W., Oster, J., Richter, D.O., Schuster, S., 2020. Removing a single neuron in a vertebrate brain forever abolishes an essential behavior. Proc. Natl. Acad. Sci. 117 (6), 3254–3260.
Houston, S.J., Karalazos, V., Tinsley, J., Betancor, M.B., Martin, S.A., Tocher, D.R., Monroig, O., 2017. The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n-3 long-chain PUFA content. Br. J. Nutr. 118 (12), 1010–1022.
IBM, 2020. Software IBM SPSS. https://www.ibm.com/es-es/analytics/spss-statistics-software (assessed on 15th december 2021).
Izquierdo, M., 2005. Essential Fatty Acid Requirements in Mediterranean Fish Species. Cahiers Options Méditerranéennes.
Izquierdo, M., Koven, W., 2011. In: Holt, J (Ed.), Larval Fish Nutrition. Wiley-Blackwell, John Wiley and Sons, Chichester, UK, pp. 47–84.
Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T., Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The Current Status of Fish Nutrition in Aquaculture. Tokyo Univ, Fisheries, Tokyo, pp. 221–232.
Joffre, C., Rey, C., Layé, S., 2019. N-3 polyunsaturated fatty acids and the resolution of neuroinflammation. Front. Pharmacol. 10, 1022.
Katakura, M., Hashimoto, M., Shahdat, H.M., Gamoh, S., Okui, T., Matsuzaki, K., Shido, O., 2009. Docosahexaenoic acid promotes neuronal differentiation by regulating basic helix–loop–helix transcription factors and cell cycle in neural stem cells. Neuroscience 160 (3), 651–660.
Kawakita, E., Hashimoto, M., Shido, O., 2006. Docosahexaenoic acid promotes neurogenesis in vitro and in vivo. Neuroscience 139 (3), 991–997.
Kinovea Project, 2016. https://www.kinovea.org/ (assessed on 15th December 2021).
Kuah, M.K., Jaya-Ram, A., Shu-Chien, A.C., 2015. The capacity for long-chain polyunsaturated fatty acid synthesis in a carnivorous vertebrate: functional characterisation and nutritional regulation of a Fads2 fatty acyl desaturase with Δ4 activity and an Elovl5 elongase in striped snakehead (*Channa striata*). Biochimica et Biophysica Acta (BBA)-molecular and cell biology of. Lipids 1851 (3), 248–260.
Lauritzen, L., Hansen, H.S., Jørgensen, M.H., Michaelsen, K.F., 2001. The essentiality of long chain n-3 fatty acids in relation todevelopment and function of the brain and retina. Prog. Lipid Res. 40, 1–94.
Lund, I., Höglund, E., Ebbesson, L.O., Skov, P.V., 2014. Dietary LC-PUFA deficiency early in ontogeny induces behavioural changes in pike perch (*Sander lucioperca*) larvae and fry. Aquaculture 432, 453–461.
Marszalek, J.R., Lodish, H.F., 2005. Docosahexaenoic acid, fatty acid–interacting proteins, and neuronal function: breastmilk and fish are good for you. Annu. Rev. Cell Dev. Biol. 21, 633–657.
Martins, C.I., Galhardo, L., Noble, C., Damsgård, B., Spedicato, M.T., Zupa, W., Kristiansen, T., 2012. Behavioural indicators of welfare in farmed fish. Fish Physiol. Biochem. 38 (1), 17–41.
Masuda, R., Takeuchi, T., Tsukamoto, K., Ishizaki, Y., Kanematsu, M., Imaizum, K., 1998. Critical involvement of dietary docosahexaenoic acid in the ontogeny of schooling behaviour in the yellowtail. J. Fish Biol. 53 (3), 471–484.
Meager, J.J., Domenici, P., Shingles, A., Utne-Palm, A.C., 2006. Escape responses in juvenile Atlantic cod *Gadus morhua L.*: the effects of turbidity and predator speed. J. Exp. Biol. 209 (20), 4174–4184.
Melvin, S.D., Petit, M.A., Duvignacq, J.P., Sumpter, J.P., 2017. Towards improved behavioural testing in aquatic toxicology: acclimation and observation times are important factors when designing behavioural tests with fish. Chemosphere 180, 430–436.
Midttun, H.L., Vindas, M.A., Nadler, L.E., Øverli, Ø., Johansen, I.B., 2020. Behavioural effects of the common brain-infecting parasite Pseudoloma neurophilia in laboratory zebrafish (*Danio rerio*). Sci. Rep. 10 (1), 1–9.
Monroig, Ó., Li, Y., Tocher, D.R., 2011. Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 159 (4), 206–213.
Monroig, Ó., Tocher, D.R., Hontoria, F., Navarro, J.C., 2013. Functional characterisation of a Fads2 fatty acyl desaturase with Δ6/Δ8 activity and an Elovl5 with C16, C18 and C20 elongase activity in the anadromous teleost meagre (Argyrosomus regius). Aquaculture 412, 14–22.
Montero, D., Lalumera, G., Izquierdo, M.S., Caballero, M.J., Saroglia, M., Tort, L., 2009. Establishment of dominance relationships in gilthead sea bream *Sparus aurata* juveniles during feeding: effects on feeding behaviour, feed utilization and fish health. J. Fish Biol. 74 (4), 790–805.
Mourente, G., 2003. Accumulation of DHA (Docosahexaenoic Acid; 22: 6n-3) in Larval and Juvenile Fish Brain. The Big Fish Bang. Institute of Marine Research, Bergen, pp. 239–248.
Roy, J., Larroquet, L., Surget, A., Lanuque, A., Sandres, F., Terrier, F., Skiba-Cassy, S., 2020. Impact on cerebral function in rainbow trout fed with plant based omega-3 long chain polyunsaturated fatty acids enriched with DHA and EPA. Fish Shellfish Immunol. 103, 409–420.
Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols. Humana Press, Totowa, NJ, pp. 365–386.
Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J., Tocher, D.R., 1995. Requirement criteria for essential fatty acids. J. Appl. Ichthyol. 11 (3/4), 183–198.
Shah, M.R., Lutzu, G.A., Alam, A., Sarker, P., Chowdhury, M.K., Parsaeimehr, A., Daroch, M., 2018. Microalgae in aquafeeds for a sustainable aquaculture industry. J. Appl. Phycol. 30 (1), 197–213.
Shahidi, F., Ambigaipalan, P., 2018. Omega-3 polyunsaturated fatty acids and their health benefits. Annu. Rev. Food Sci. Technol. 9, 345–381.
da Silva Souza, J.G., Libeck, L.T., Virote, B.D.C.R., Egger, R.C., de Sá, G.C.R., Machado, G.J., Murgas, L.D.S., 2020. A method to analyze the relationship between locomotor activity and feeding behaviour in larvae of *Betta splendens*. Aquac. Int. 1–12.
Silva-Brito, F., Magnoni, L.J., Fonseca, S.B., Peixoto, M.J., Castro, L.F.C., Cunha, I., Gonçalves, J.F.M., 2016. Dietary oil source and selenium supplementation modulate Fads2 and Elovl5 transcriptional levels in liver and brain of meagre (*Argyrosomus regius*). Lipids 51 (6), 729–741.
Skalli, A., Robin, J.H., Le Bayon, N., Le Delliou, H., Person-Le Ruyet, J., 2006. Impact of essential fatty acid deficiency and temperature on tissues' fatty acid composition of European sea bass (*Dicentrarchus labrax*). Aquaculture 255 (1–4), 223–232.
Sokal, R.R., Rohlf, F.J., 1981. Biometry. Freeman, San Francisco.
Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. 11 (2), 107–184.
Tocher, D.R., 2015. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 449, 94–107.
Tukey, J.W., 1949. Comparing individual means in the analysis of variance. Biometrics 5, 99–114.
Turchini, G.M., Torstensen, B.E., Ng, W.K., 2009. Fish oil replacement in finfish nutrition. Rev. Aquac. 1 (1), 10–57.
Turesson, H., Satta, A., Domenici, P., 2009. Preparing for escape: anti-predator posture and fast-start performance in gobies. J. Exp. Biol. 212 (18), 2925–2933.
Turkmen, S., Castro, P.L., Caballero, M.J., Hernández-Cruz, C.M., Saleh, R., Zamorano, M.J., Izquierdo, M., 2017. Nutritional stimuli of gilthead seabream (*Sparus aurata*) larvae by dietary fatty acids: effects on larval performance, gene expression and neurogenesis. Aquac. Res. 48 (1), 202–213.
Wainwright, P.E., 2002. Dietary essential fatty acids and brain function: a developmental perspective on mechanisms. Proc. Nutr. Soc. 61 (1), 61–69.
Walker, J.A., Ghalambor, C.K., Griset, O.L., Kenney, D.M., Reznick, D.N., 2005. Do faster starts increase the probability of evading predators? Funct. Ecol. 19, 808–815.

Watanabe, T., Kiron, V., 1994. Prospects in larval fish dietetics. Aquaculture 124 (1–4), 223–251.
Watanabe, T., Thongrod, S., Takeuchi, T., Satoh, S., Kubota, S.S., Fujimaki, Y., Cho, C.Y., 1989. Effect of dietary n-6 and n-3 fatty acids on growth, fatty acid composition and histological changes of white fish *Coregonus lavaretus maraena*. Nippon Suisan Gakkaishi 55 (11), 1977–1982.
Webb, P., 1978. Fast-start performance and body form in seven species of teleost fish. J. Exp. Biol. 74 (1), 211–226.
Zhou, L., Chen, F., Ye, J., Pan, H., 2018. Selection of reliable reference genes for RT-qPCR analysis of *Bursaphelenchus mucronatus* gene expression from different habitats and developmental stages. Front. Genet. 9, 269.