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### ► To cite this version:

Marie Vagner, Salomé Ducos, Nicolas Graziano, Fabienne Le Grand, Antoine Bideau, et al.. Omega-long chain highly unsaturated fatty acid content in tissue correlates with escape response efficiency in golden gray mullet. *Frontiers in Fish Science*, 2024, 2, pp.1394423. 10.3389/frish.2024.1394423 . hal-04675052

**HAL Id: hal-04675052**

**<https://hal.science/hal-04675052v1>**

Submitted on 22 Aug 2024

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RECEIVED 01 March 2024

ACCEPTED 16 April 2024

PUBLISHED 14 May 2024

## CITATION

Vagner M, Ducos S, Graziano N, Le Grand F,  
Bideau A, Dubillot E and Lefrançois C (2024)  
Omega-3 long chain highly unsaturated fatty  
acid content in tissue correlates with escape  
response efficiency in golden gray mullet.  
*Front. Fish Sci.* 2:1394423.  
doi: 10.3389/frish.2024.1394423

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# Omega-3 long chain highly unsaturated fatty acid content in tissue correlates with escape response efficiency in golden gray mullet

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Omega-3 long-chain highly unsaturated fatty acids (n-3 HUFA) are poorly synthesized *de novo* by marine fish, but are necessary to maintain their metabolic and physiological performance. Consequently, fish must find them in their diet. However, the availability of these lipid compounds is predicted to decrease in relation to global changes in ocean waters. The aim of this study was to experimentally determine the effects of an n-3 HUFA-deficient diet on the escape performance of *Chelon auratus*, a microphytobenthos grazer of high ecological importance. Fish were fed either a standard n-3 HUFA diet or an n-3 HUFA-deficient diet for 2 months. At the end of this conditioning period, several variables (latency, responsiveness, initial direction of escape, response duration, distance covered, maximum acceleration and velocity, and turning rate) related to escape performance were characterized in the tested fish. The results showed that a lower dietary n-3 HUFA content caused a significant reduction in the proportion of these fatty acids in fish muscle, both in membranes and in reserves. While the FA composition of the brain also reflected that of the diet in reserves, the membrane fatty acid composition of the brain remained similar between the two dietary groups, suggesting the preservation of neural and cognitive functions of the fish. Neither growth rate nor escape response variables were significantly different between the two diets. However, some escape response variables (e.g., latency, duration, maximum velocity and acceleration, and turning rate) were highly correlated with fish n-3 HUFA content in brain or muscle, and these correlations were tissue specific. While response duration and latency seemed to be more correlated with n-3 HUFA content in the brain membrane, other escape response variables such as turning rate, maximum velocity, cumulative distance, and maximum acceleration seemed to be correlated with n-3 HUFA content in the lipid reserve, both in the brain and in the muscle. Our results indicate that the reduction of n-3 HUFA in the dietary sources of marine fish appears to reduce the performance of the escape response, suggesting some impact of n-3 HUFA dietary changes with global change on predator-prey relationships within the marine ecosystem.

## KEYWORDS

fish, n-3 long chain polyunsaturated fatty acids, EPA, DHA, acceleration, velocity, turning rate, response duration

## 1 Introduction

Long chain highly unsaturated fatty acids (20–22 carbon atoms) of the n-3 series, also known as n-3 HUFA (or omega-3 HUFA), are the major constituents of the cell membranes of all living organisms (1). They play an essential structural role by allowing the maintenance of membrane fluidity, which then determines the conformation and function of proteins embedded in cell membranes (2). As a result, they determine the proper functioning of the cells and tissues on which the physiological and behavioral performance of organisms depends (e.g., growth, locomotion, and reproduction), contributing to their fitness and, in cascade, to the functioning of the ecosystem. Among these n-3 HUFA, EPA (eicosapentaenoic acid 20:5n-3), and DHA (docosahexaenoic acid 22:6n-3) are particularly important in fish, and have been shown to be essential for maintaining their growth, survival, welfare and development (1, 3). In particular, DHA is required for normal development of the nervous system, and has been shown to play a key role in highly active sites of brain (4, 5).

Despite the biological importance of these lipid compounds, they can only be synthesized to a limited extent by animals (6). In the natural environment, they are mainly produced at the first level of the food chain, i.e., by marine microalgae (7). Thus, most marine consumers acquire n-3 HUFA from their diet, directly from microalgae or through successive predation (1), which allows the transfer of these lipids into the food web, up to humans (8–12).

Recent studies have linked the effects of global changes (e.g., increased temperature, decreased salinity, dissolved oxygen in water) to a decrease in n-3 HUFA production at the base of the food chain, resulting from a shift in microalgal communities with the dominance of species that produce less n-3 HUFA (10, 13–15). A recent study predicted that EPA content in marine phyto- and zooplankton would decline in the next century by 2%, ranging from 0.7 to 28.5%, depending on the geographic location (16). An exploratory analysis also calculated, through linear regression models, that global n-3 HUFA production would be reduced by 8.2% for EPA and 27.8% for DHA with an increase in water temperature of 2.5°C (14). This would reduce the availability of n-3 HUFA to consumers, such as fish, with consequences for their cell membrane composition and functionality and, in cascade, on their physiological and behavioral performance (17–20).

Among fish performances that contribute to ecosystem functioning, the escape response performance is critical in the predator-prey relationships. Escape response is an innate behavior of primordial interest in maintaining the selective value (i.e., the probability of survival and transmission of its alleles to its offspring) of the individual. It is a reflex response, that corresponds to a sudden acceleration in response to a stimulus (e.g., attack by a predator). It is triggered by the activation of Mauthner cells and associated neurons cells until a “decision threshold” of excitation is reached (21) in response to a visual or acoustic stimulus (22, 23). Escape duration is extremely short (between 5 and 40 ms on average; 22, 24) because the large size of Mauthner cells and their axons result in very rapid transmission of the action potential along the spinal cord. This transmission leads to a contraction of the lateral muscles, usually on the side opposite the stimulus, and results in a characteristic C-sharp curved shape of the fish, called

stage 1. This stage 1 is often followed by a second stage (stage 2), which corresponds to the contraction of the muscles on the opposite side, resulting in a change of direction of the fish. The escape response is considered complete when no more sudden changes in direction are observed (25). The presence or absence of a stage 2 has been used to classify responses as double or single bend, respectively (26–28). The success of an escape response depends not only on behavior of the predator, but also on the performance of the predated individual (e.g., velocity and acceleration), its maneuverability (radius of rotation of the animal's body) and its reaction time (latency) (29). Kinematic patterns of escape responses have been shown to depend on environmental conditions (e.g., dissolved oxygen conditions, temperature), ontogenetic stage and species (23, 28, 30, 31).

However, despite the important role that n-3 HUFA play in the vertebrate nervous system (1, 4, 32), and in particular the relationship found between a DHA deficiency and cognitive deficits (4, 33–35), still little is known about the specific effect of n-3 HUFA on the escape response in fish. To our knowledge, only four studies have reported the effect of an n-3 HUFA-deficient diet on the escape response of fish, all of them on sea bream *Sparus aurata*, three of them investigating the response of larvae to a visual and sound stimulus (35–37) and a recent one on the effect of a mechanical stimulus on the escape response of juveniles (38). A lower dietary n-3 HUFA content (about 5 times less n-3 HUFA than in the control diet, which was about 18% of total fatty acids, TFA) was associated with reduced burst swimming speed and a delayed response compared to sea bream larvae fed a higher dietary n-3 HUFA content (36). This was also associated with lower levels of DHA measured in the eyes and brain, suggesting impaired functional development of the brain and vision. Dietary n-3 HUFA deficiency (about 2 times less n-3 HUFA than in the control diet, which was about 26% of TFA) also reduced the number of larvae responding to a visual stimulus, and this was associated with a reduced immune-positive neuronal response in Mauthner cells, indicating lower acetylcholine production (37). In addition, DHA, rather than EPA, was shown to increase the swimming speed of escaping larvae, which may be mediated, at least in part, by an increase in neuronal activity in the Mauthner cell (35). More recently, Carvalho et al. (38) reported no effect of a dietary n-3 HUFA deficiency (about three times less n-3 HUFA than in the control diet, which was about 12% TFA) on locomotor performance or escape response in juveniles. However, they did find a slight, but non-significant, increase in latency in fish fed the low n-3 HUFA diet, suggesting a slower reactivity to the stimulus in these fish, which may be related to a slower rate of translation of the neural signal to activate the escape response. Thus, studies reporting the effect of n-3 HUFA on the escape response in fish are scarce, involve only one species, and suggest that this effect may depend on several factors, including ontogenetic stage.

In order to improve our knowledge on this subject, the present study aims to measure and analyze the effect of dietary n-3 HUFA content on the escape response of the golden mullet (*Chelon aurata*), a euryhaline fish that feeds partly on microalgae and is very common in coastal areas where it comes to feed at high tide (39, 40). Several variables characteristic of the escape response (i.e., responsiveness, latency, type of escape response, duration,

directionality and kinematics in terms of maximum acceleration, maximum velocity, cumulative distance, and turning rate) were compared between two groups of fish fed contrasting levels of n-3 HUFA in the diet. Our hypothesis was that a dietary n-3 HUFA deficiency (about 8 times less than in the control diet, which was about 20% TFA) would alter the escape response of juvenile mullet by altering the structure and functionality of (i) the brain and hence the Mauthner cells, and (ii) the lateral muscle tissue involved in contraction during the escape response.

## 2 Materials and methods

### 2.1 Ethic statement

The collection and handling of the animals were carried out under the jurisdiction of the Departmental Service of Fisheries and the Animal Care Committee of France (#12886), respectively (N° APAFIS 2017122208475172).

### 2.2 Fish conditioning

Juvenile mullets ( $n = 23$ ) were caught in the Pertuis charentais area, near La Rochelle ( $46^{\circ}12'14.4''N$   $1^{\circ}11'43.7''W$ ) the 7th of November 2017, and transported to the LIENSs laboratory (UMR 7266 CNRS- La Rochelle Université, France), where all the experiments were conducted. Fish were kept for 7 weeks in a common 300 L ( $98 \times 98 \times 58$  cm) closed-circuit experimental tank equipped with a biological filter (Eheim professional 3 2080, Eheim, Deizisau, Germany), and thermoregulated (TECO TR20, Conselice, Italy). Temperature ( $13 \pm 0.2^{\circ}C$ ) and salinity ( $28.7 \pm 0.1$ ) were monitored daily (TetraCon<sup>®</sup> 325, Laboratoires Humeau, La Chapelle-sur-Erdre, France) and were kept similar to that of the sampling site. The oxygen concentration was monitored once a week, and was maintained at  $93.3 \pm 4.4\%$  saturation. Fish were maintained under a 12:12 photoperiod, and fed daily with commercial pellets (Le Gouessant<sup>®</sup>, Lamballe, France).

In January 2018, all the fish were starved for 24 h, before being anesthetized (Ethyl 3-aminobenzoate methanesulfonate, MS-222, Sigma-Aldrich, St Quentin-Fallavier, France;  $0.1 \text{ g L}^{-1}$ ) in seawater, individually marked with transponders (diameter 2 mm, length 12 mm, M120; biolog-id, Réseumatique, Bernay, France) injected into the muscle at the base of the dorsal fin using a sterile injector, weighed (mean  $\pm$  standard deviation:  $34.3 \pm 6.5$  g), and randomly assigned to one of the four replicate 300 L holding tanks ( $n = 5-6$  fish per tank). Fish were acclimated for 4 weeks in their new tanks, during which they were fed commercial pellets (Le Gouessant<sup>®</sup>, Lamballe, France) twice a day, to a total of 2% of their biomass, and reared at the same temperature and salinity as before (temperature:  $13 \pm 0.2^{\circ}C$ ; salinity:  $28.7 \pm 0.1$ ).

In February 2018, fish were progressively acclimated to  $20^{\circ}C$  over a period of about 2 weeks at a rate of  $0.5^{\circ}C$  per day. This temperature was chosen because it is the temperature at which whole organism performance declined in mullet fed on the same low n-3 HUFA diet, but it is still within their natural thermal range, as  $20^{\circ}C$  reflects the mean summer temperature in the

natural environment of mullet (17–19). In particular, the dietary n-3 HUFA deficiency used in the present study has been estimated to significantly reduce the n-3 HUFA content in biological membranes and has been shown to impair the aerobic performance of golden gray mullet at  $20^{\circ}C$  (18). This temperature was chosen in order to be able to compare our results with our previous studies carried out in the same species, as we know that the effect of dietary fatty acids on physiological performance in ectotherms can depend on ambient temperature, due to homeoviscous adaptation. Homeoviscous adaptation is the process by which organisms adjust their membrane lipid composition in response to temperature to maintain membrane fluidity and functionality (2).

Fish were left another 2 weeks period of acclimation to insure physiological adjustment to the change in temperature. Temperature ( $19.9 \pm 1^{\circ}C$ ) and salinity ( $29.6 \pm 1.1$ ) were daily measured in the four experimental tanks.

Following this period of acclimation to water temperature, fish were switched to the experimental diets for about 2 months (mean  $\pm$  standard error of the mean sem =  $61 \pm 2$  days, range: 45–73 days, depending on which day each individual was further tested to measure its escape response performance; see Section 2.3.1). This duration was chosen because it has been shown to be sufficient to detect differences in the membrane fatty acid composition between diet treatments (41). Fish were randomly allocated to the treatment (two tanks per dietary treatment): fish had their food progressively switched to either a standard n-3 HUFA diet (SH diet;  $n = 11$ ) or a deprived n-3 HUFA diet (LH diet;  $n = 12$ ). The experimental diets to commercial diet ratio was increased every 2nd day from 25:75%, 50:50%, 75:25%, and finally to 100:0%. The experimental diets were formulated at Ifremer-PFOM (UMR 6539 LEMAR, Plouzané, France) and manufactured at INRAE (Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement, Donzax, France). Differences in n-3 HUFA contents between diets were achieved by replacing fish oil (rich in n-3 HUFA) of the SH diet with rapeseed oil (poor in n-3 HUFA) in the LH diet (see ingredients in Table 1). Experimental diets were isocaloric and isolipidic. SH and LH diets contained 17.5 and 1.2% EPA + DHA per total fatty acid mass of the diets, respectively, which represents about 15-fold difference in EPA and DHA content between diets (Table 1). The EPA and DHA content in the SH diet cover the needs of several fish species (41, 42), although the needs for the golden gray mullet are not well-known.

Body mass did not differ between fish groups subsequently assigned to the two food treatments (SH:  $31.3 \pm 1.8$  g; LH:  $34.9 \pm 1.5$  g,  $t$ -test:  $t_{21} = -1.510$ ,  $p = 0.146$ ). Body mass was measured every 2–3 weeks and rations were recalculated to adjust for growth.

Individuals were fed daily for 2 months at 2% of their biomass, split into two meals during the day (morning and late afternoon). The feed was automatically distributed by an electronic feeder (JBL AutoFood, Neuhofen, Germany).

### 2.3 Escape response measurement

#### 2.3.1 Experimental protocol

As only two fish per day could be tested for escape response, the duration of the diet treatment varied between

TABLE 1 (A) Ingredient (in g 100 g<sup>-1</sup>) and (B) fatty acid (FA) composition expressed as a percentage of FA mass per total FA mass (% mean  $\pm$  standard error of the mean;  $n = 3$ ) in the standard (SH) and low (LH) n-3 highly unsaturated fatty acid (n-3 HUFA) diets (made at INRAE, Donzac, France).

(A)		
Ingredients <sup>a</sup>	SH diet	LH diet
Fish meal LT <sup>a</sup> 94	17	17
Casein	30	30
Rapeseed oil	2	10
Fish oil	8	0
Precooked starch	30	30
Vitamin mixture <sup>b</sup>	8	8
Mineral mixture <sup>c</sup>	4	4
Betaine	1	1
(B)		
FA	SH diet	LH diet
14:0	4.30 $\pm$ 0.35	0.41 $\pm$ 0.03
15:0	0.61 $\pm$ 0.05	0.04 $\pm$ 0.04
16:0	18.67 $\pm$ 1.39	6.10 $\pm$ 0.45
17:0	0.71 $\pm$ 0.04	0.09 $\pm$ 0.01
18:0	4.59 $\pm$ 0.36	1.91 $\pm$ 0.16
$\Sigma$ SFA	29.27 $\pm$ 2.20	10.13 $\pm$ 1.78
16:1n-7	4.83 $\pm$ 0.19	0.44 $\pm$ 0.01
18:1n-9	25.04 $\pm$ 0.41	56.74 $\pm$ 0.51
18:1n-7	2.56 $\pm$ 0.63	0.05 $\pm$ 0.09
20:1n-9	1.82 $\pm$ 0.14	4.94 $\pm$ 3.62
$\Sigma$ MUFA	36.57 $\pm$ 1.02	63.35 $\pm$ 3.36
18:2n-9	0.20 $\pm$ 0.05	0.57 $\pm$ 0.29
18:2n-6	5.06 $\pm$ 0.63	15.13 $\pm$ 3.15
18:3n-3	2.42 $\pm$ 0.43	6.49 $\pm$ 1.78
18:4n-3	0.80 $\pm$ 0.13	0.11 $\pm$ 0.11
20:4n-6 (ARA)	1.38 $\pm$ 0.12	0.27 $\pm$ 0.06
20:5n-3 (EPA)	6.63 $\pm$ 1.04	0.39 $\pm$ 0.12
22:5n-6	0.51 $\pm$ 0.04	0.00 $\pm$ 0.00
22:5n-3	1.64 $\pm$ 0.15	0.88 $\pm$ 0.53
22:6n-3 (DHA)	10.95 $\pm$ 1.09	0.79 $\pm$ 0.23
$\Sigma$ PUFA	31.75 $\pm$ 3.47	24.93 $\pm$ 4.29
$\Sigma$ n-6	7.91 $\pm$ 0.67	15.56 $\pm$ 3.14
$\Sigma$ n-3	23.18 $\pm$ 2.87	8.69 $\pm$ 1.49
$\Sigma$ EPA+DHA	17.58 $\pm$ 2.13	1.18 $\pm$ 0.36
DHA/EPA	1.66 $\pm$ 0.09	2.05 $\pm$ 0.07
$\Sigma$ n-3 HUFA	19.95 $\pm$ 2.08	2.30 $\pm$ 0.22

<sup>a</sup>Sources: fish meal LT 94: Norse (Fyllingsdalen, Norway); casein: Sigma-Aldrich (Germany); rapeseed oil: Système U (Créteil, France); fish oil: pure cod oil Cooper (Melun, France); precooked starch: Prégéflo Roquette Frères (Lestrem, France); vitamin mixture (INRA Jouy-en-Josas, France).

<sup>b</sup>Vitamin mixture (g kg<sup>-1</sup> vitamin mix): retinyl acetate 1; cholecalciferol 2.5; D $\alpha$ -tocopheryl acetate 5; menadione 1; thiamine-HCl 0.1; riboflavin 0.4; D-calcium pantothenate 2; pyridoxine-HCl 0.3; cyanocobalamin 1; niacin 1; choline 200; ascorbic acid (ascorbyl polyphosphate) 5; folic acid 0.1; D-biotin 1; mesoinositol 30.

<sup>c</sup>Mineral mixture (g kg<sup>-1</sup> mineral mix): KCl 90; KI 0.04; CaHPO<sub>4</sub>·2H<sub>2</sub>O 500; NaCl 40; CuSO<sub>4</sub>·5H<sub>2</sub>O 3; ZnSO<sub>4</sub>·7H<sub>2</sub>O 4; CoSO<sub>4</sub> 0.02; FeSO<sub>4</sub>·7H<sub>2</sub>O 20; MnSO<sub>4</sub>·H<sub>2</sub>O 3; CaCO<sub>3</sub> 215; MgOH 124; Na<sub>2</sub>SeO<sub>3</sub> 0.03; NaF 1.

processing batches (mean  $\pm$  SEM = 61  $\pm$  2 days, range: 45–73 days).

The day before each escape response measurement day, one fish from each treatment was randomly netted before the afternoon meal and individually placed in an isolation tank similar to the acclimation tanks (Length: 98  $\times$  Width: 98  $\times$  Height: 58 cm) and maintained at 20°C with a water depth of  $\sim$ 10 cm. The fish were thus fasted for more than 24 h before being exposed to a startle stimulus.

The next morning, the two fish that had been starved for 24 h in isolation tanks were individually placed in two experimental thermoregulated tanks (Length 75  $\times$  Width: 75  $\times$  Height: 49 cm; 19.9  $\pm$  0.4°C) with a water depth of  $\sim$ 11 cm (Figure 1), 2 h before the test, which was considered sufficient for the mullet to recover from the transfer (28, 37, 38, 43). After this 2 h period, the fish seemed to have stopped exploring the new tank and showed a calm behavior. To avoid a possible time of day effect on the escape response, all fish were tested in the morning (44).

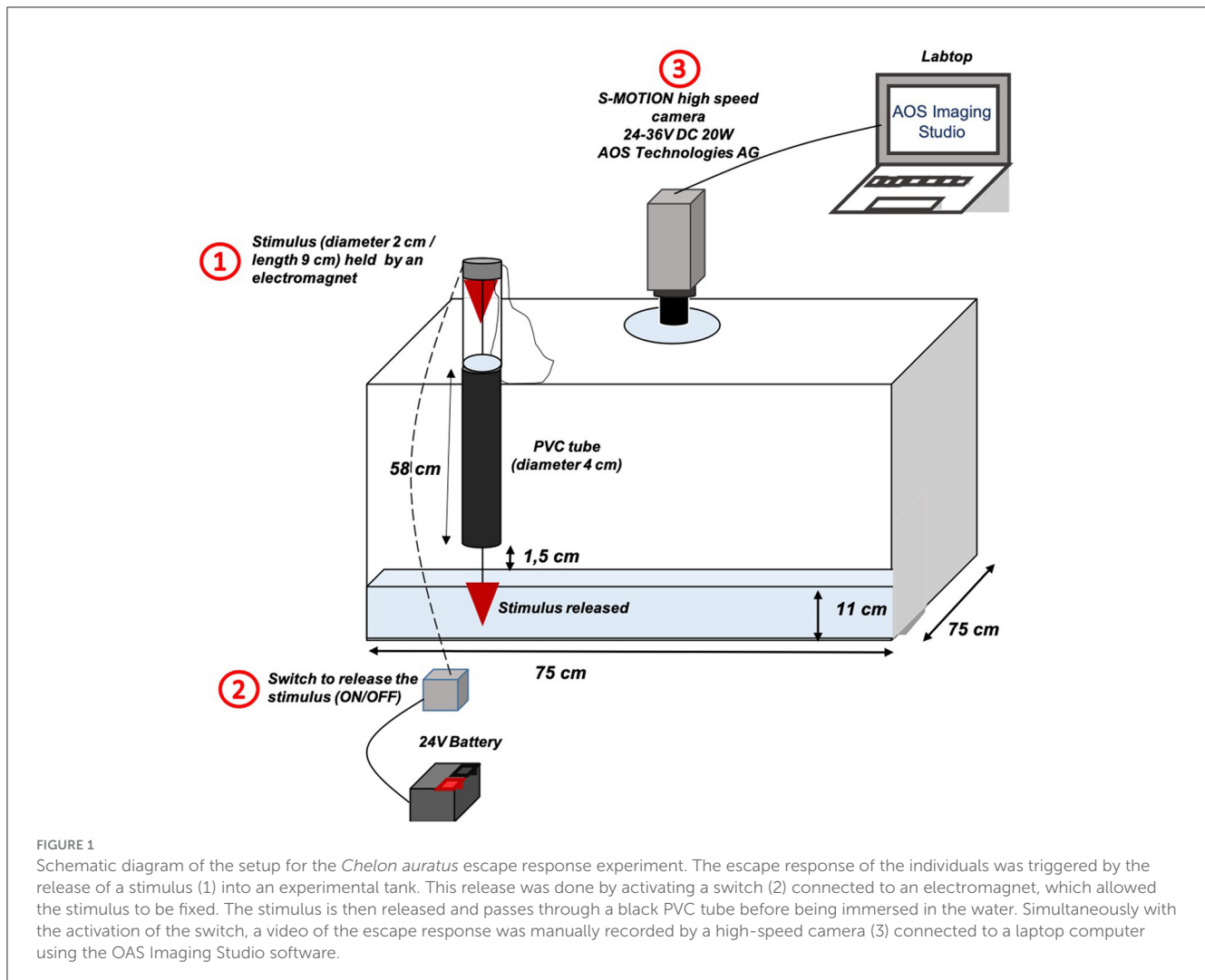
The experimental tanks were covered with an opaque lid and surrounded by a black tarpaulin to keep the fish in a quiet environment as much as possible. The escape response of the individuals was triggered by the release of a stimulus (diameter: 2 cm; length: 9 cm). The stimulus was held above the experimental tank by an electromagnet. When released, the stimulus passed through a black PVC tube (length: 58 cm; diameter: 4 cm) before dropping into the water, without touching the bottom of the tank. The black tube ensures that the stimulus is not seen by the fish as it falls, mimicking an aerial attack by predator in a naturally turbid or shallow environment (28), which is commonly frequented by mullets. This tube is also essential for determining the exact moment of stimulation (i.e.,  $t_0$ ). The escape response was recorded using a high-speed camera (S-MOTION, 500 fps-1, 24–36 V DC 20 W, AOS Technologies AG) connected to a laptop with OAS Imaging Studio software.

The protocol was formulated with three requirements to trigger the stimulus (28): (1) it is necessary that the fish was positioned at more than 1 body length from the nearest wall in order to avoid influencing its escape direction, (2) its velocity must be lower than 2 body lengths s<sup>-1</sup>, and (3) there must be no waves on the water surface. Based on the video analyses, we were able to verify that the distance between the impact of the stimulus on the water surface and the center of mass (CM) of the animal was not significantly different between the two experimental dietary groups ( $t = -0.91939$ ;  $df = 15$ ;  $p = 0.3724$ ). The CM was positioned on the videos according to Lefrançois et al. (28) on the same species, and corresponds to a dorsal point located 1/3 of the body length from the snout, which generally corresponds to the center of the segment between the two pectoral fins.

### 2.3.2 Video analysis and measured variables

Responsiveness was calculated for both experimental conditions (SH and LH) and corresponds to the proportion of fish that responded to the stimulation with a sudden acceleration (in %). Individuals showing no movement at the time of stimulation were considered as non-responders. Only the videos of fish showing an escape response were then analyzed using the WINanalysis software, which allows a decomposition of the





different frames of the video (1 frame = 2 ms). The variables analyzed for each individual were:

- The latency (ms), corresponding to the time interval between the impact of the stimulus on the surface ( $t_0$ ) and the first escape movement ( $t_1$ ).
- The direction of escape, which was classified as “away” or “toward” depending on whether the first detectable movement of the head was oriented away or toward the stimulus, respectively (25).
- Total escape duration (ms), which was determined as the sum of stages 1 and 2 as described in Lefrançois et al. (28).
- The distance-time variables were calculated over the mean escape duration over which the kinematic variables were measured, i.e., 62 ms. For each video sequence, the x and y co-ordinates of the CM were digitized exported to WINanalyze software to perform automatic tracking of the fish’s movements. We then evaluated:
  - The maximum acceleration  $A_{max}$ .
  - The maximum velocity  $V_{max}$ .
  - The cumulative distance  $D_{cum}$ .
  - $D_{cum92}$ , which was calculated as the cumulative distance over the duration of the mean latency added to the mean duration of the total escape response, i.e., over a total of 92 ms (28).
- The turning rate ( $^{\circ} \text{ms}^{-1}$ ), which was calculated as the angle (in  $^{\circ}$ ) of the line passing through the CM and the tip of the head of each individual, between the start of the escape response and the end of stage 1 (31, 38). The higher it is, the greater the maneuverability of the fish.

After stimulus release and video recording of the two fish, individuals were individually netted and anesthetized (MS-222, Sigma-Aldrich, St Quentin-Fallavier, France;  $0.1 \text{ g L}^{-1}$ ) in seawater. Fish were electronically identified (transponder), weighed and measured as described above. They were sacrificed by a prolonged anesthesia and dissected on ice to remove muscle and whole brain (five lobes) for further fatty acid analyses. Samples of white skeletal

muscle were taken between the lateral and dorsal lines of dorso-ventral symmetry and between the head of the fish and its dorsal fin. All biological tissues collected were weighed (g), and stored in liquid nitrogen before being stored at  $-80^{\circ}\text{C}$  until fatty acid analysis. The liver was removed and weighed for assessment of the hepatosomatic index HSI using Equation (1):

$$HSI = \frac{(W_{liver} \times 100)}{(W_{fish} - W_{liver})} \quad (1)$$

where  $W_{liver}$  is the liver weight and  $W_{fish}$  is the fish weight.

The Fulton index (FI) was determined using Equation (2) (45):

$$FI = \frac{W}{L^3} \times 100 \quad (2)$$

where  $W$  is the fish weight in g and  $L$  the total fish length in cm.

The specific growth rate (SGR,  $\% \text{ day}^{-1}$ ) was calculated for each fish using Equation (3):

$$SGR = \frac{(\ln \text{ final weight} - \ln \text{ initial weight})}{\text{duration of dietary conditioning}} \times 100 \quad (3)$$

where weight was in g, and duration of dietary conditioning was in days.

## 2.4 Lipid analysis

### 2.4.1 Lipid extraction

Lipids from fish muscle and brain (LH:  $n = 12$ ; SH:  $n = 11$  per tissue) were extracted according to the method of Mathieu-Resuge et al. (46). Briefly, tissues were ground to a fine homogeneous powder in liquid nitrogen. Lipids from 200 to 250 mg of tissue powder were then extracted in 6 ml of chloroform/methanol mixture (2:1; v/v). To ensure complete lipid extraction, samples were sonicated at  $4^{\circ}\text{C}$  for  $2 \times 5$  min. Lipids from experimental diets ( $n = 3$  per diet) were extracted using the same method. Pellets were ground in a mortar and the powder was transferred to chloroform/methanol (2:1; v/v). All lipid extracts were then stored at  $-20^{\circ}\text{C}$  in a nitrogen atmosphere until further analysis.

### 2.4.2 Separation of neutral lipids and polar lipids

Fatty acids of biological membranes are attached to a polar head group, collectively called polar lipids (PL), in contrast to fatty acids of energy stores that form triglycerides, which are called neutral lipids (NL). Brain and muscle lipids were separated into NL and PL fractions by solid-phase extraction following the method described in Mathieu-Resuge et al. (46). Briefly, an aliquot of the total lipid extract (1/6) was evaporated to dryness under nitrogen, recovered with three washes of 0.5 ml of chloroform/methanol (98:2; v/v) and applied to the top of a silica gel column ( $40 \times 4$  mm, silica gel 60A 63–200  $\mu\text{m}$ ) previously heated at  $450^{\circ}\text{C}$  and deactivated with 6 wt %  $\text{H}_2\text{O}$ . NL were eluted with 10 mL of chloroform/methanol (98:2; v/v) and PL were eluted with 20 mL of methanol. After the addition of 2.3  $\mu\text{g}$  of an internal standard (tricosanoic acid C23:0) to each of the lipid fractions, they were stored at  $-20^{\circ}\text{C}$  in a nitrogen atmosphere.

### 2.4.3 Lipid transesterification

Lipid transesterification was performed as described in Mathieu-Resuge et al. (46). Both neutral and polar lipid fractions were evaporated to dryness under nitrogen. Fatty acid methyl esters (FAME) were obtained by adding 800  $\mu\text{L}$  of  $\text{H}_2\text{SO}_4$ /methanol (3.4%; v/v) and heating at  $100^{\circ}\text{C}$  for 10 min. FAME were then extracted in 800  $\mu\text{L}$  of hexane and washed three times with 1.5 ml of distilled water saturated with hexane.

### 2.4.4 Gas chromatography analysis of FAME

FA composition was analyzed by GC coupled with Flame-Ionization Detector (GC-FID), as described by Mathieu-Resuge et al. (46), using a Varian CP8400 gas chromatograph. After splitless mode injection, FAMES were separated in parallel on two different columns (DBWAX 30 m  $\times$  0.25 mm ID  $\times$  0.2  $\mu\text{m}$  and DB5 30 m  $\times$  0.25 mm ID  $\times$  0.2  $\mu\text{m}$ , Agilent). FAMES were identified by comparison of their retention times with those of commercial standards (Supelco 37 Component FAME Mix, PUFA No. 1 and No. 3, and Bacterial Acid Methyl Ester Mix, Sigma) and laboratory prepared standard mixtures. The internal standard (C23:0) allowed the calculation of FA content ( $\mu\text{g mg}^{-1}$  muscle wet mass). For each PL and NL fraction, the fatty acid proportion was then determined as the percentage of fatty acid mass per total mass of fatty acids in each fraction.

## 2.5 Statistical analyses

All statistical analyses were carried out with Rstudio<sup>®</sup> software (Version 1.1.423; Boston, USA).

It was possible to measure an escape response on seven out of 11 SH fish and on 10 out of 12 LH fish. The reason why some fish could not have been tested is that they were not correctly positioned in the tank within the time limit of the experiment (8:00–13:00) to be stimulated, or displayed an extremely long latency (382 and 224 ms), which did not seem to correspond to escape responses by acoustic-mechanical stimulation (on average between 5 and 40 ms; 24) but rather by visual stimulation (47).

The distance between the CM and the point at which the stimulus touched the water surface has been compared between the two dietary groups using a  $t$ -test, and was not significantly different between treatment (LH:  $319.22 \pm 47.49$  mm; SH:  $380.78 \pm 52.55$  mm;  $t$ -test,  $p = 0.37$ ,  $F = 15$ ), indicating that fish were randomly placed in the tank before stimulation.

Responsiveness was compared between the two dietary groups using a G-test (R package *DescTools*). The escape direction was statistically compared to the theoretical value of 50% of “away” and “toward” response using a binomial test (31, 48).

The effect of diet on other escape response variables (latency, turning rate, duration, and kinematics in terms of maximum acceleration, maximum velocity, cumulative distance, and turning rate), as well as on growth and condition parameters was statistically tested using linear model (lm; 49). The models' conditions of application have been visually inspected on residuals with quantile-quantile plots. The processing batch (testing day) and fish tanks were included as potential covariate in initial analysis.

Each of these variables were not significant, so were removed from all the final models.

The fatty acid composition of brain and muscles were compared between the two-dietary groups using a two-way ANOVA (diet and tissue). When an interaction occurred, a Tukey HSD test was performed.

Our whole dataset (escape responses variables and fatty acid composition) was explored with PCA presented in [Supplementary material](#) (R package *Factoshiny*). Correlations between variables were then tested using Spearman correlations (R package *ggplot2*, *ggpubr*, *gridExtra*, *cowplot*, and *gridGraphics*), as Spearman correlation works with both linear and monotonic relationships.

## 3 Results

### 3.1 Fish biometric and condition variables

All individuals gained weight during the experiment, as shown by a positive SGR, and the SGR was not different between the two groups ([Table 2](#)). Final body mass did not differ between SH and LH fish ([Table 2](#)), eliminating body mass as a confounding source of differences in escape response between the dietary treatments. Fish from the two experimental groups (LH and SH) did not differ significantly neither in other growth traits considering length, height, and width, nor in body condition indices (HSI and FI, [Table 2](#); linear model;  $p \geq 0.05$ ).

### 3.2 Effect of dietary treatment on fatty acid composition of brain and muscle

The fatty acid composition of the NL and PL fractions of fish brain and muscle is shown in [Table 3](#).

PL was the major lipid fraction of brain tissue, with a concentration about 5 times higher in brain than in muscle, whereas muscle was equally composed of NL and PL.

DHA was preferentially incorporated into PL rather than in NL in both muscle ( $F = 41.69$ ;  $p < 0.001$ ) and brain ( $F = 41.69$ ,  $p < 0.001$ ).

EPA was also preferentially incorporated into PL rather than in NL in muscle ( $F = 27.12$ ,  $p < 0.001$ ), but not in brain where it was preferentially incorporated in NL ( $F = 177.4$ ,  $p < 0.001$ ).

In muscle, the fatty acid composition reflected that of the diet. Muscle from LH fish had lower levels of EPA and DHA contents, as well as lower DHA/EPA,  $\Sigma$  EPA + DHA and  $\Sigma$  n-3 HUFA in both the PL and NL fractions compared to SH fish. In the brain, the dietary fatty acid composition was reflected in the NL fraction, but not in the PL fraction, where we observed that EPA, DHA,  $\Sigma$  n-3 HUFA, and  $\Sigma$  PUFA contents remained similar between the two dietary groups. In addition, the DHA/EPA ratio was significantly the highest in the PL brain of LH fish ([Table 3](#)).

Overall, strong inter-individual variability in fatty acid composition was observed within each dietary group, with some LH individuals showing similar EPA and DHA proportions to SH fish, especially in brain PL, brain NL and muscle NL.

## 3.3 Escape response performance

### 3.3.1 Responsiveness, latency, escape response duration, and directionality

Responsiveness did not differ between the two groups, with a total of 83% of the LH individuals responding to the stimulus, vs. 78% for the SH group (Chi-test:  $G = 0.10$  e-31,  $df = 1$ ,  $p = 0.75$ ).

Among the responding fish, a total of 60% of the LH fish and 86% of the SH fish escaped away from the stimulus. These values were not statistically different from the random theoretical value of 50% of “away” and “toward” responses (LH binomial test;  $p = 0.377$ ; SH binomial test:  $p = 0.06$ ; [31](#)).

The latency did not differ between the two groups (lm;  $F = 0.66$ ;  $df = 1$ ;15;  $p = 0.43$ , [Figure 2](#)) and was  $27.4 \pm 4.8$  ms for the LH group, vs.  $et. 32.9 \pm 4.0$  ms for the SH group (mean  $\pm$  sem).

Irrespective of the escape phase considered (stage 1, stage 2 or both; [Figure 2](#)), the duration was not significantly different between the two groups (lm for total duration:  $F = 4.44$ ,  $df = 1$ ;14,  $p = 0.053$ ; stage 1 duration:  $F = 0.72$ ,  $df = 1$ ;15,  $p = 0.41$ ; stage 2 duration:  $F = 2.81$ ,  $df = 1$ ;14,  $p = 0.12$ ), with the total duration of the escape response ranging from 36 to 128 ms (mean  $73 \pm 10$  ms) for the LH group and from 32 to 64 ms ( $43 \pm 6$  ms) for the SH group.

### 3.3.2 Kinematic variables and turning rates

All fish, except one SH fish, responded with a double bend response, characterized by a C-shape resulting from the contraction of the muscles on one side of the body (stage 1), which is followed by a return flip of the tail, presumably as a result of a contralateral contraction (stage 2; [31](#)).

Stage-1 turning rate was not affected by diet (lm:  $F = 2.30$ ;  $df = 1$ ;15;  $p = 0.15$ ; [Figure 2](#)) and was  $2.56 \pm 0.66$  degrees  $\text{min}^{-1}$  (mean  $\pm$  sem) for SH fish and  $2.24 \pm 0.13$  degrees  $\text{min}^{-1}$  (mean  $\pm$  sem) for LH fish.

Diet had no effect on cumulative distance, maximum velocity, or maximum acceleration ([Figures 2E–G](#)). Cumulative distance traveled was not different between the two dietary groups, whether or not latency was included in the mean total duration of the escape response (lm: Dcum:  $F = 1.02$ ,  $df = 1$ ; 15,  $p = 0.33$ ; Dcum92:  $t = 0.77$ ,  $df = 1$ ;15,  $p = 0.39$ ). Mean Dcum was  $7.2 \pm 0.2$  cm for SH fish and  $6.6 \pm 0.4$  cm for LH fish, and mean Dcum92 was  $12.0 \pm 1.0$  cm for SH fish and  $11.0 \pm 0.9$  cm for LH fish. Mean maximum velocity was  $1.84 \pm 0.09$  m  $\text{s}^{-1}$  for SH fish and  $1.68 \pm 0.12$  m  $\text{s}^{-1}$  for LH fish (lm:  $F = 0.81$ ,  $df = 3$ ; 13,  $p = 0.51$ ). The mean maximum acceleration of SH fish was  $117.8 \pm 6.3$  m  $\text{s}^{-1}$  vs.  $103.2 \pm 6.8$  m  $\text{s}^{-1}$  for LH fish (lm:  $F = 0.71$ ,  $df = 3$ ;13,  $p = 0.56$ ; [Figure 2](#)).

Overall, all the escape response variables presented in [Figure 2](#) showed a higher inter-individual variability in the LH group than in the SH group, which may have prevented the determination of a significant difference between these two dietary groups. We therefore investigated the correlations between the proportions of n-3 HUFA (EPA+DHA+DPA), EPA, DPA, DHA, EPA+DHA, and the DHA/EPA ratio in fish tissues (brain and muscle) and fraction (PL and NL) and the measured escape response variables.



**TABLE 2** Fish growth and body condition variables in both experimental groups, at the end of the 2-month conditioning period (LH: low n-3 HUFA diet,  $n = 12$  and SH: standard n-3 HUFA diet,  $n = 11$ ), mean  $\pm$  standard error.

Groups	SGR (%)	Weight (g)	TL (cm)	SL (cm)	Height (cm)	Width (cm)	FI	HSI
LH	18.0 $\pm$ 2.0	40.6 $\pm$ 1.5	16.6 $\pm$ 0.2	14.1 $\pm$ 0.2	2.9 $\pm$ 0.06	1.9 $\pm$ 0.04	0.88 $\pm$ 0.01	1.6 $\pm$ 0.1
SH	19.0 $\pm$ 3.0	36.4 $\pm$ 2.0	16.0 $\pm$ 0.2	13.5 $\pm$ 0.3	2.8 $\pm$ 0.07	1.8 $\pm$ 0.05	0.89 $\pm$ 0.03	1.2 $\pm$ 0.1
Statistics (linear model)	$F = 0.21$	$F = 2.76$	$F = 4.297$	$F = 4.549$	$F = 2.563$	$F = 0.56$	$F = 0.13$	$F = 3.39$
	$df = 1,19$	$df = 1,19$	$df = 1,19$	$df = 1,19$	$df = 1,19$	$df = 1,19$	$df = 1,19$	$df = 1,19$
	$p = 0.65$	$p = 0.11$	$p = 0.05$	$p = 0.05$	$p = 0.13$	$p = 0.5$	$p = 0.72$	$p = 0.08$

SGR, specific growth rate calculated over the dietary conditioning period for each fish; TL, total length; SL, standard length; FI, Fulton index; HSI, hepato-somatic index.

**TABLE 3** Composition of fatty acids of interest as a percentage of total fatty acids in neutral lipids (NL) or polar lipids (PL), expressed as mean  $\pm$  standard deviation in brain and muscle of fish fed the standard n-3 HUFA diet (SH fish,  $n = 11$ ) and those fed the low n-3 HUFA diet (LH fish,  $n = 12$ ).

	Brain		Muscle		Statistics		
	SH	LH	SH	LH	Diet	Tissue	Diet x tissue
NL	4.73 $\pm$ 1.88	8.24 $\pm$ 5.06	5.97 $\pm$ 3.47	5.61 $\pm$ 2.46	ns	ns	ns
20:5n-3 (EPA)	6.7 $\pm$ 1.1	5.4 $\pm$ 1.2	4.8 $\pm$ 1.7	2.3 $\pm$ 1.2	***	***	ns
22:5n-3 (DPA)	1.7 $\pm$ 0.9 <sup>ab</sup>	1.5 $\pm$ 1.0 <sup>a</sup>	1.9 $\pm$ 0.4 <sup>a</sup>	0.9 $\pm$ 0.3 <sup>b</sup>	*	ns	.
22:6n-3 (DHA)	7.5 $\pm$ 2.6	4.9 $\pm$ 1.6	5.4 $\pm$ 1.9	1.6 $\pm$ 0.4	***	***	ns
$\Sigma$ EPA+DHA	14.2 $\pm$ 2.5	10.3 $\pm$ 2.1	10.1 $\pm$ 3.2	3.9 $\pm$ 1.6	***	***	ns
DHA/EPA	1.2 $\pm$ 0.5	0.9 $\pm$ 0.3	1.2 $\pm$ 0.4	0.8 $\pm$ 0.3	**	ns	ns
$\Sigma$ SFA	35.3 $\pm$ 3.8	32.8 $\pm$ 2.9	33.9 $\pm$ 6.7	28.3 $\pm$ 4.9	ns	**	ns
$\Sigma$ MUFA	30.3 $\pm$ 6.6	35.2 $\pm$ 5.1	40.4 $\pm$ 2.0	48 $\pm$ 3.6	***	***	ns
$\Sigma$ PUFA	28.2 $\pm$ 7.4	25.4 $\pm$ 4.3	21.9 $\pm$ 5.9	20.5 $\pm$ 3.4	***	***	ns
n-3 HUFA	15.9 $\pm$ 2.4 <sup>a</sup>	11.8 $\pm$ 2.4 <sup>b</sup>	12.0 $\pm$ 3.6 <sup>b</sup>	4.8 $\pm$ 1.8 <sup>c</sup>	***	***	*
PL	14.99 $\pm$ 4.10	16.15 $\pm$ 3.29	3.74 $\pm$ 0.93	3.30 $\pm$ 1.21	ns	***	ns
20:5n-3 (EPA)	2.4 $\pm$ 0.4 <sup>a</sup>	2.1 $\pm$ 0.4 <sup>a</sup>	9.8 $\pm$ 1.0 <sup>b</sup>	5.7 $\pm$ 3.6 <sup>c</sup>	***	***	**
22:5n-3 (DPA)	2.3 $\pm$ 2.3	0.8 $\pm$ 0.5	5.6 $\pm$ 0.5	4.0 $\pm$ 2.1	**	***	ns
22:6n-3 (DHA)	21.0 $\pm$ 3.8 <sup>a</sup>	21.9 $\pm$ 4.2 <sup>a</sup>	20.8 $\pm$ 2.2 <sup>a</sup>	10.1 $\pm$ 6.8 <sup>b</sup>	***	***	***
$\Sigma$ EPA+DHA	23.3 $\pm$ 4.1 <sup>a</sup>	24.1 $\pm$ 4.5 <sup>ab</sup>	30.6 $\pm$ 2.6 <sup>b</sup>	15.8 $\pm$ 10.3 <sup>c</sup>	***	ns	***
DHA/EPA	8.9 $\pm$ 1.1 <sup>a</sup>	10.4 $\pm$ 1.3 <sup>b</sup>	2.1 $\pm$ 0.3 <sup>c</sup>	1.6 $\pm$ 0.5 <sup>c</sup>	ns	***	***
$\Sigma$ SFA	33.3 $\pm$ 3.5	33.7 $\pm$ 4.5	30.9 $\pm$ 4.0	35.3 $\pm$ 12.6	ns	ns	ns
$\Sigma$ MUFA	26.8 $\pm$ 1.9 <sup>a</sup>	26.9 $\pm$ 1.5 <sup>a</sup>	18.9 $\pm$ 1.5 <sup>b</sup>	28.8 $\pm$ 9.8 <sup>a</sup>	**	.	**
$\Sigma$ PUFA	30.0 $\pm$ 3.8 <sup>a</sup>	29.8 $\pm$ 5.1 <sup>a</sup>	45.6 $\pm$ 3.6 <sup>b</sup>	31.6 $\pm$ 14.0 <sup>a</sup>	**	***	**
n-3 HUFA	25.6 $\pm$ 3.7 <sup>a</sup>	24.9 $\pm$ 4.7 <sup>a</sup>	36.2 $\pm$ 2.8 <sup>b</sup>	19.8 $\pm$ 12.4 <sup>a</sup>	***	ns	***

NL and PL concentrations are expressed in  $\mu$ g of FA per mg of wet tissue.

DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid.

The sums of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), EPA+DHA, n-3 HUFA (EPA+DPA+DHA) are also presented as a percentage of total FA, as well as the EPA/DHA ratio. The results of the two-way ANOVA and *post-hoc* Tukey test are presented according to their significance level with = 0.05, \* = 0.01 \*\* = 0.001 and \*\*\* = 0. Tukey test: Values not sharing a common letter within a row are significantly different. Further details on fatty acid composition of the tissues are given in [Supplementary Table 1](#).

### 3.3.3 Relationships between escape response variables and brain fatty acid composition in fish

Spearman correlation tests showed significant correlations between escape response variables and n-3 HUFA proportion in PL of brain ([Figure 3](#)). More specifically, escape response duration was negatively correlated with n-3 HUFA proportion ([Figure 3A](#)), latency was positively correlated with EPA ([Figure 3B](#)), and turning rate and maximum velocity were both negatively correlated with DHA/EPA ratio ([Figures 3C, D](#)).

We also observed significant correlations between escape response variables and brain FA composition in NL, that were different from those observed in brain PL ([Supplementary Figure 2B](#), [Figure 4](#)). Cumulative distance was positively correlated with EPA. Turning rate and maximum velocity were both positively correlated with n-3 HUFA content. Maximum velocity was also positively correlated with the sum of EPA+DHA.

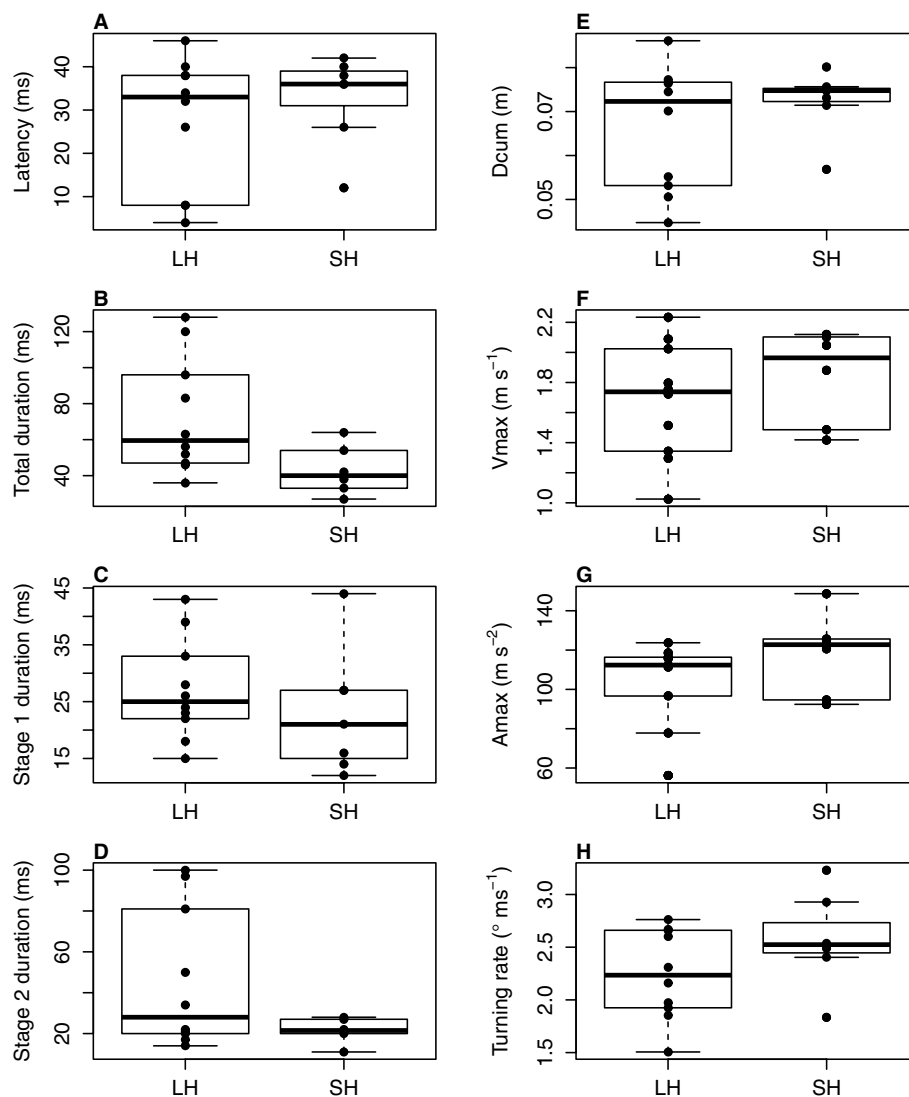


FIGURE 2

Escape response variables of mullets fed two contrasting diets (LH diet: low n-3 HUFA diet,  $n = 10$ ; SH diet: standard n-3 HUFA diet,  $n = 7$ ). Latency in ms (A), escape response duration in ms (B: total duration; C: duration of stage 1; D: duration of stage 2), cumulative distance Dcum in m (E); maximum velocity Vmax in  $m s^{-1}$  (F); maximum acceleration Amax in  $m s^{-2}$  (G); turning rate in  $^{\circ} ms^{-1}$  (H). Dcum, Amax and Vmax were calculated over the mean escape duration during which kinematic variables were measured, i.e., 62 ms.

### 3.3.4 Relationships between escape response variables and muscle fatty acid composition in fish

The duration of the escape response was negatively correlated with DHA and n-3 HUFA in PL of the muscle (Figure 5).

In terms of muscle NL (Figure 6), turning rate and maximum acceleration were both positively correlated with EPA, and n-3 HUFA content. In addition, the turning rate was positively correlated with DPA and DHA.

## 4 Discussion

After 2 months of nutritional conditioning at 20°C, a lower dietary n-3 HUFA content caused a significant reduction of the proportion of these fatty acids in fish muscle, in both PL and NL fractions. While the FA composition of the brain also reflected that of the diet in NL, PL fatty acid composition in brain remained

similar between both dietary groups, suggesting the preservation of neural and cognitive functions of fish (38, 50–52).

Greater variability was measured in the LH group compared to the SH group for most of the escape response variables measured, and fatty acid composition was highly variable between individuals within each dietary group. This may have prevented the detection of a significant difference between these two groups. However, our results show strong correlations between some escape response variables and n-3 HUFA content in fish tissue, regardless of diet. In particular, we showed that a low dietary n-3 HUFA content in fish tissue would alter the escape response by prolonging its duration, reducing maximum velocity and acceleration, and reducing turning rate. Lower levels of EPA content in fish tissue would also reduce both latency and cumulative distance traveled. These correlations seemed to be highly dependent on the tissue (muscle or brain), and the lipid fraction (PL or NL) considered.

It is interesting to note that the values of Dcum, Vmax, and Amax obtained in our study are in the lower range of that found

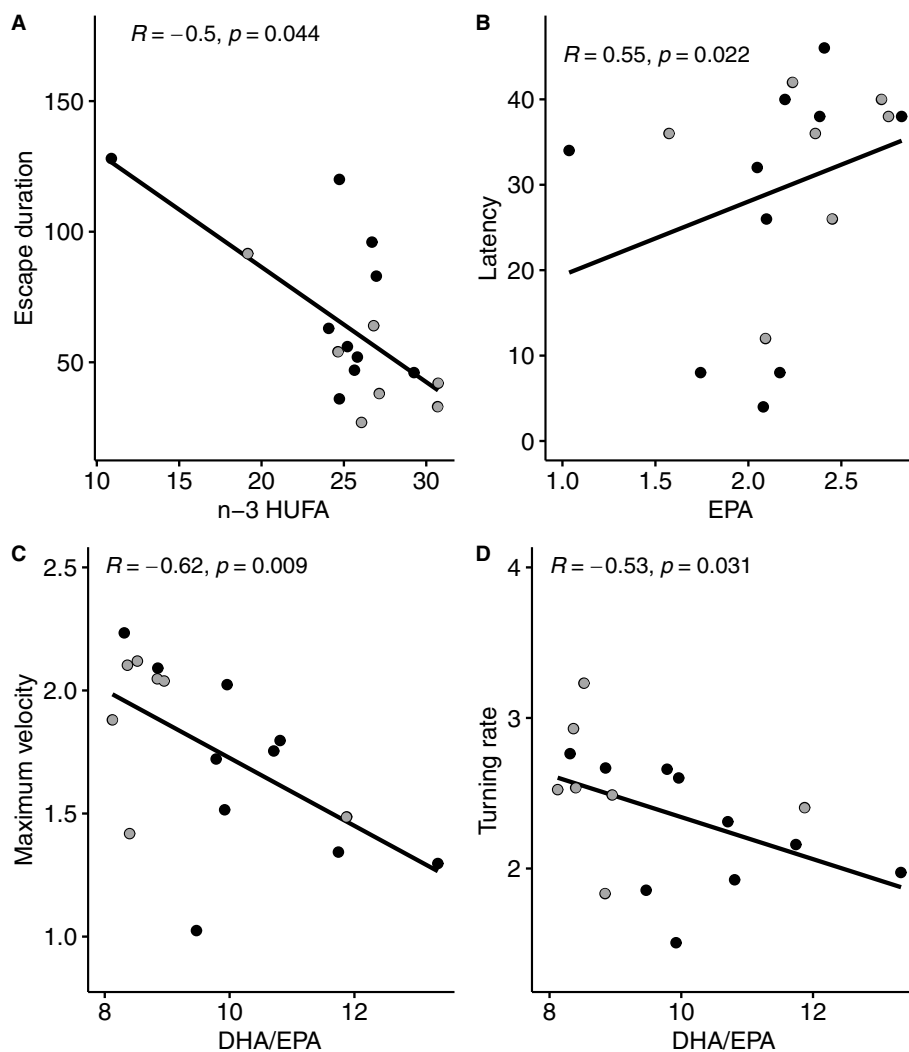


FIGURE 3

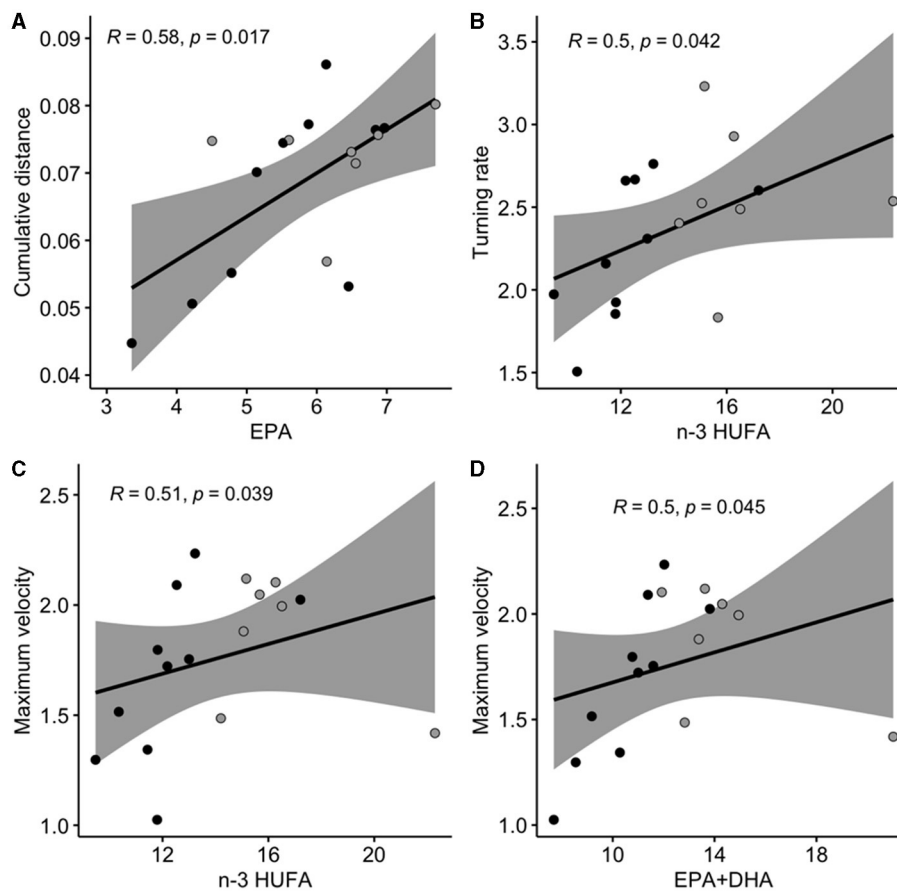
Significant relationships (Spearman correlation) measured between escape response variables and fatty acid composition in brain polar lipids (as % of total fatty acids in the polar fraction). Escape response duration (A) is expressed in ms, latency (B) in ms, maximum velocity (C) in  $\text{ms}^{-1}$  and turning rate (D) in  $^{\circ} \text{ms}^{-1}$ . Gray areas represent the 95% confidence interval. DHA, docosahexaenoic acid 22:6n-3; EPA, eicosapentaenoic acid 20:5n-3; n-3 HUFA is the sum of DHA, EPA, and DPA (docosapentaenoic acid 22:5n-3). Black dots represent individuals ( $n = 10$ ) fed the LH (low n-3 HUFA) diet, and dark gray dots represent individuals ( $n = 7$ ) fed the SH (standard n-3 HUFA) diet.

by Lefrançois et al. (28) in the same species. In particular, our values are in the same range as those found by these authors in fish exposed to 10% hypoxia without any surface access. However, these authors used longer fish than those tested here (about 21 vs. 16 cm). Acceleration, velocity and distance covered have been shown to be size-dependent and to increase with size in relation to changes in morphology (23). In addition, the mean turning rate was higher in our study, indicating a higher maneuverability of fish, which is in accordance with the higher maneuverability of smaller fish than of larger ones (23).

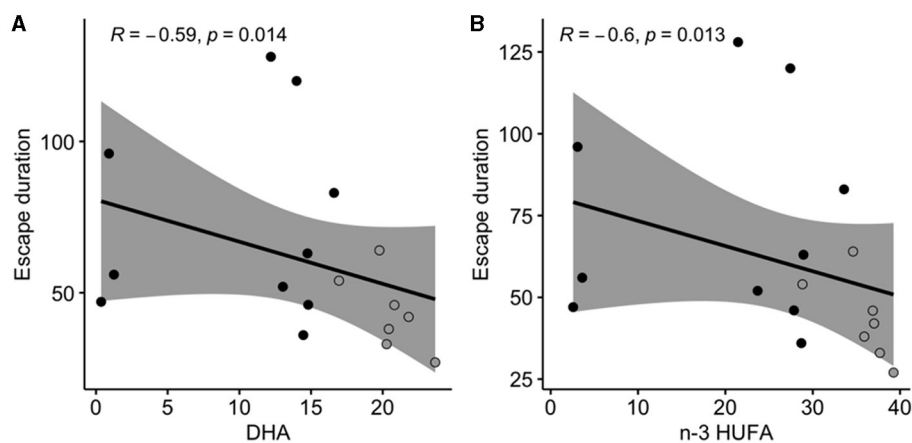
#### 4.1 Growth parameters

Biometric data (weight, total length, height, and thickness), and condition indices (HSI, FI) showed no significant differences between the two experimental groups. In a previous study carried

out on the same species, with the same diets, and at the same temperature, but during a 5-month period of feed conditioning (compared to 2 months in the present study), we found a higher growth rate in SH individuals than in LH individuals, which became apparent only at the end of the exposure period (17). Thus, it could be argued that if the fish in the present study had been fed their experimental diets for a longer period, they might have shown a different growth rate between the two groups. Looking at the large variability in several escape response variables in the LH group, it could also be argued that a longer period of dietary conditioning may have induced a difference in escape response performance between the two groups. In addition, we cannot rule out the possibility that the large variability in several escape response variables could be due to the individual personality of the fish, but we did not statistically measure the effect of this factor (53, 54). For example, Jones and Godin (54) reported a positive correlation between the duration of exploration of the



**FIGURE 4** Significant relationships (Spearman correlation) measured between escape response duration variables and fatty acid composition in brain neutral lipids (as % of total fatty acids in the neutral fraction). Cumulative distance (A) is expressed in cm, turning rate (B) in  $^{\circ} \text{ms}^{-1}$ , maximum velocity (C, D) in  $\text{ms}^{-1}$ . Gray areas represent the 95% confidence interval. DHA, docosahexaenoic acid 22:6n-3; EPA, eicosapentaenoic acid 20:5n-3; n-3 HUFA is the sum of DHA, EPA, and DPA (docosapentaenoic acid 22:5n-3). Black dots represent individuals ( $n = 10$ ) fed the LH (low n-3 HUFA) diet, and dark gray dots represent individuals ( $n = 7$ ) fed the SH (standard n-3 HUFA) diet.



**FIGURE 5** Significant relationships (Spearman correlation) measured between escape response duration (in ms) and DHA (docosahexaenoic acid 22:6n-3; A), or n-3 HUFA (sum of DHA, EPA eicosapentaenoic acid 20:5n-3, and DPA docosapentaenoic acid 22:5n-3; B) composition in muscle polar lipids (as % of total fatty acids in the polar fraction). Gray areas represent the 95% confidence interval. Black dots represent individuals ( $n = 10$ ) fed the LH (low n-3 HUFA) diet, and dark gray dots represent individuals ( $n = 7$ ) fed the SH (standard n-3 HUFA) diet.

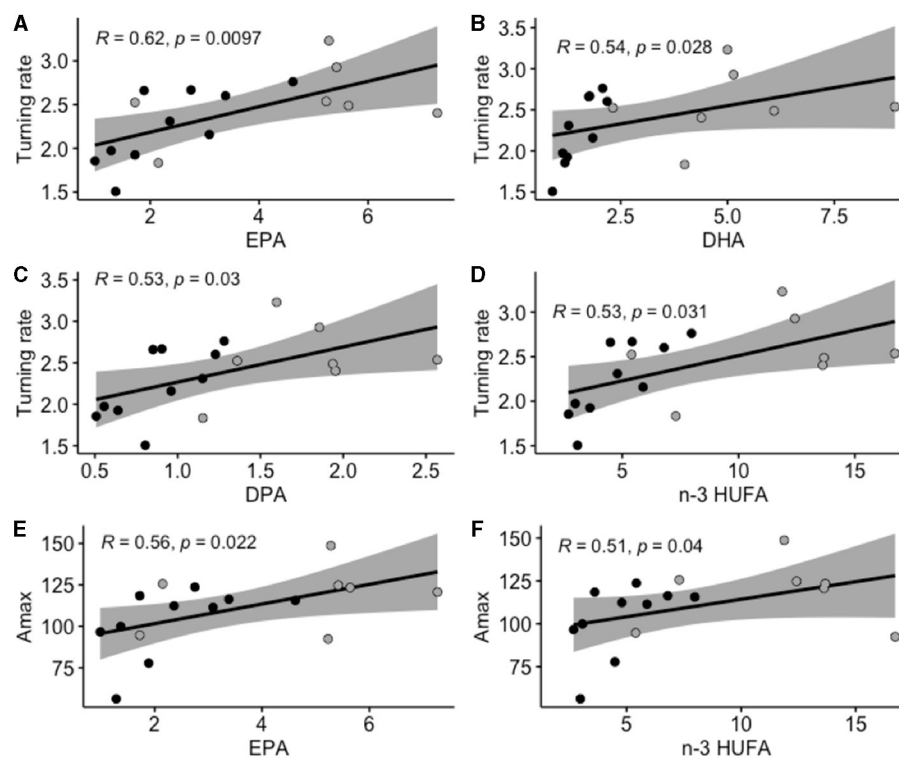


FIGURE 6

Significant relationships (Spearman correlation) measured between escape response variables (turning rate and maximum acceleration) and fatty acid composition in muscle neutral lipids (as % of total fatty acids in the neutral fraction). Turning rate (T. rate; A–D) is expressed in  $^{\circ} \text{ms}^{-1}$ , and maximum acceleration (Amax; E, F) is expressed in  $\text{m s}^{-2}$ . Gray areas represent the 95% confidence interval. DHA, docosahexaenoic acid 22:6n-3; EPA, eicosapentaenoic acid 20:5n-3; n-3 HUFA is the sum of DHA, EPA, and DPA (docosapentaenoic acid 22:5n-3). Black dots represent individuals ( $n = 10$ ) fed the LH (low n-3 HUFA) diet, and dark gray dots represent individuals ( $n = 7$ ) fed the SH (standard n-3 HUFA) diet.

novel environment and the time taken to respond to the stimulus. The faster the fish explored the new tank, the slower they responded to the stimulus.

## 4.2 Responsiveness and direction of escape response were similar in both dietary groups

Neither responsiveness nor directionality (“toward” or “away” from the stimulus) was influenced by the n-3 HUFA composition of the diet.

The majority of individuals in both groups (SH: 78% and LH: 83%) responded with an escape response to the mechano-acoustic stimulation. Ecologically, the absence of an escape reflex response from an organism is directly related to a very high probability of its capture by a predator. The absence of a response in some individuals could be due to a lack of transmission of the detected stimulus to the muscle via the motor neuron of the excited Mauthner cell. It could also be related to the failure to reach a necessary threshold of stimulation. This threshold is known to depend on the intensity of the startle stimulus and on inter-individual variability in sensitivity (23). Here, as a standardized stimulus was used, and the distances between the tube and the fish were similar, it could be assumed that for some

individuals this threshold was not reached due to possible inter-individual sensitivity.

The direction of escape of all the individuals was predominantly in the opposite direction to the position of the stimulus, which is consistent with what has been already observed in previous work on the same species (28), and in the majority of studied species (23). Escaping in the opposite direction to the predator is expected to increase survival. This has usually been observed in almost 90% of cases (23), and always in more than 80% of cases (28, 55, 56). However, in the present study, only 60% of LH fish escaped in the opposite direction of the stimulus (compared to 86% of SH fish). This could be explained by the position of some fish when the stimulus fell. If the position of the fish (defined by the center of mass and the head) is relatively aligned with that of the stimulus, the fish will have approximately the same chance of survival regardless of which side it escapes from. When the escape directions were analyzed, one LH individual showed this very low angular position in relation to the stimulus. Another LH fish already showed a clear curvature of the body toward the stimulus at the time of stimulation, which explains its escape direction toward the stimulus.

The responsiveness and directionality of the escape response depend on the functionality of the Mauthner cell and the transmission in the M-cell circuits. Interestingly, the lack of significant difference in these variables between the two dietary groups was associated with a similar FA composition of brain



membranes (PL) in the two dietary groups. This similar FA composition between the two dietary groups would suggest that the composition, and thus the functionality, of the Mauthner cells responsible for the reflex response, was not altered by the n-3 HUFA dietary conditioning, and contributed to maintaining similar responsiveness and directionality between the two groups. This similar composition of brain membranes would result from a high selective retention of certain fatty acids in the brain, ensuring the protection of the neural circuit, despite the n-3 HUFA dietary deficiency. This is in line with other findings in striped snakehead (*Channa striata*; 52), sea bream (38, 51), and European seabass (*Dicentrarchus labrax*; 50), in which brain tissue showed the highest capacity to regulate DHA content in PL, despite the low dietary concentration of this FA, thus preserving brain functionality.

### 4.3 Latency, escape response duration, and kinematic variables are correlated with n-3 HUFA composition in fish tissue

While no differences were found when examining the effects of dietary treatment at the group level, inter-individual variation in brain FA composition was observed and correlated with some escape performance variables such as escape duration, latency, maximum velocity, and turning rate.

In particular, the higher the DHA/EPA ratio content in the brain membrane, the lower the maximum velocity and turning rate. This lower velocity and maneuverability of the fish would suggest a better success of predators in catching their prey. We also showed that the higher the EPA proportion in the brain membrane, the slower the reactivity of fish to the stimulus. This could also be detrimental to the survival of the fish, as a longer latency of the prey would increase the chances of the predator catching the prey. Although specific neurobiological studies may be required to clarify this point, it suggests that the level of EPA may influence the time course of the neural signal to initiate the escape response. However, it is important to note that, while a short response latency is beneficial for the fish to escape from the predator, it also induces a high state of readiness to escape (vigilance), which results in a high anaerobic metabolic demand to escape. As a consequence, this induces a longer aerobic recovery period after escape. Thus, there is a trade-off between the increased vigilance and the metabolic demand (57). The longer latency that correlates with higher EPA levels in brain PL may indicate lower vigilance as EPA increases, and thus a lower metabolic demand for the escape response, with a further shorter aerobic recovery period after escape. However, further studies would be needed to investigate this hypothesis.

The duration of the escape response also appeared to be highly dependent on the n-3 HUFA content in the membranes, and especially on DHA. The lower the n-3 HUFA content in muscle and brain membranes, the longer the response. A longer duration of the escape response may increase the energetic cost required for an individual to escape from a predator. The mean escape response duration of the LH individuals was almost 60% longer than that of the SH individuals, which was at the limit of significance. These values are well within the range of mean response durations

(between 32 and 200 ms for a panel of 14 different fish species) summarized by Domenici and Blake (25). Our results indicate that it is at stage 2 of the escape response that the differentiation (limit of significance) in the total duration of the escape response between the two experimental groups occurs, with LH individuals tending to perform a longer stage 2 than SH individuals. Stage 2 of the escape response corresponds to the contralateral activity of the muscle after activation of the Mauthner cells. Thus, our results would suggest that a lower content of n-3 HUFA, and especially DHA, in the muscle membrane may increase the duration of the contralateral activity.

While response duration and latency seemed rather correlated to n-3 HUFA content in the brain membrane, other escape response variables such as turning rate, maximum velocity, as well as cumulative distance and maximum acceleration appeared to be correlated with the n-3 HUFA content in the lipid reserve, in both in the brain and in the muscle. Lower levels of n-3 HUFA, and especially lower levels of EPA and DHA in the lipid reserve tended to reduce maximum velocity, turning rate, and maximum acceleration. As lipid reserves are usually used as energy stores, these results suggest the use of EPA and DHA as an energetic fuel to promote escape response performance. The escape response is primarily fuelled by anaerobic metabolism. During the early stage of the escape response, energy is provided by the breakdown of adenosine triphosphate (ATP) and creatine phosphate (PCr), whereas during later stages of the response, energy is provided by glycogenesis (58). Our results suggest that the depleted DHA and EPA stores caused by the depleted n-3 HUFA diet would reduce muscle ATP production, and in cascade, the ATP breakdown required to fuel the escape response. A similar hypothesis has been raised in some previous studies, which reported that long-term starvation (~4 weeks) reduced fatty acid stores in the lipid reserve in some fish species, decreasing ATP and PCr in the muscles, and consequently reducing their fast-start swimming performance, in terms of maximum velocity and acceleration, cumulative distance, and turning rate (59–62).

## 5 Conclusion

Our results suggest that n-3 HUFA content in reserves and membranes of both muscle and brain tissues is involved in the regulation of escape response performance. The reduction of n-3 HUFA, and especially EPA and DHA in dietary sources of marine fish seems to contribute to the reduced performance of the escape response, by reducing the maximum velocity and acceleration, and the turning rate, probably because it prevents to get the energy needed in muscle to maintain this performance. Also, a dietary reduction in n-3 HUFA would prolong the duration of the response, which may result in a higher energetic cost. However, this cost could be compensated by performing a lower velocity, acceleration and turning rate. Finally, a dietary reduction n-3 HUFA would slow down the reactivity of the fish. These results may suggest some impact of EPA and DHA dietary changes with global change on the predator-prey relationships within the marine ecosystem. However, the large inter-individual variability obtained in the depleted groups compared to the standard groups needs to be

further investigated to better understand the mechanisms involved in coping with this dietary depletion.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was approved by Departmental Service of Fisheries and the Animal Care Committee of France (#12886), respectively (N° APAFIS 2017122208475172). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

MV: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. SD: Conceptualization, Data curation, Investigation, Writing – original draft. NG: Data curation, Investigation, Writing – review & editing. FL: Data curation, Investigation, Methodology, Supervision, Writing – review & editing. AB: Investigation, Methodology, Writing – review & editing. ED: Methodology, Writing – review & editing. CL: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Writing – review & editing.

## Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work is a contribution to the project ECONAT Ax 1 -Ressources

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Marines Littorales: qualité et éco-valorisation, financed by the Contrat de Plan Etat-Région (CPER) and by the CNRS and the European Regional Development Fund (FEDER) through the project QUALIDRIS (Ressources Marines et Littorales: Qualité et Eco-valorisation).

## Acknowledgments

The authors would like to thank Christine Dupuy for her key role in coordinating the funding projects, and José Luis Zambonino Infante for formulating the diets.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frish.2024.1394423/full#supplementary-material>

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