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Abstract (294 words)

Hydrocarbons contamination and hypoxia are two stressors that can coexist in coastal ecosystems. At present, few studies evaluated the combined impact of these stressors on fish physiology and behavior. Here, we tested the effect of the combination of hypoxia and petrogenic hydrocarbons on the anti-predator locomotor performance of fish. Specifically, two groups of European sea bass (*Dicentrarchus labrax*) were exposed to clean water (Ctrl) or oil-contaminated water (Oil). Subsequently, fish of both groups were placed in normoxic (norx) or hypoxic (hyp) experimental tanks (i.e. four groups of fish were formed: Ctrl norx, Ctrl hyp, Oil norx, Oil hyp). In these tanks, escape response

was elicited by a mechano-acoustic stimulus and recorded with a high speed camera. Several variables were analyzed: escape response duration, responsiveness (percentage of fish responding to the stimulation), latency (time taken by the fish to initiate a response), directionality (defined as away or toward the stimulus), distance-time variables (such as speed and acceleration), maneuverability variables (such as turning rate), escape trajectory (angle of flight) and distancing of the fish from the stimulus. Results revealed (i) effects of stressors (Ctrl hyp, Oil norx and Oil hyp) on the directionality; (ii) effects of Oil norx and Oil hyp on maneuverability and (iii) effects of Oil hyp on distancing. These results suggest that individual stressors could alter the escape response of fish and that their combination could strengthen these effects. Such an impact could decrease the probability of prey escape success. By investigating the effects of hydrocarbons (and the interaction with hypoxia) on the anti-predator behavior of fish, this work increases our understanding of the biological impact of oil spill. Additionally, the results of this study are of interest for oil spill impact evaluation and also for developing new ecotoxicological tools of ecological significance.

Keywords: oil, oxygen, teleost, escape response, locomotion

Capsule:

This work evaluates the effects of hypoxia and hydrocarbons on the anti-predator locomotor performance of fish. Impact were observed on directionality, maneuverability and distancing

1. Introduction

Coastal ecosystems are of high ecological relevance mainly due to their status of nursery for marine organisms and to their biodiversity (Worm et al., 2006; Diaz et al., 2003).

They also have been described as highly productive areas contributing to the economic value of marine environments (Costanza et al., 1997). However, these ecosystems are under multiple anthropogenic stressors. Among these stressors, hypoxia is in constant expansion since the sixties and nowadays there are more than 400 hypoxic coastal ecosystems distributed world-wide (Diaz and Rosenberg, 2008). Thus, a large body of literature has been devoted to testing the effects of hypoxia on several organisms (e.g. Domenici et al., 2007; Cheung et al., 2008; Kodama and Horiguchi, 2011; Cook et al., 2014; Rabalais and Turner, 2013).

Petrogenic hydrocarbons are also considered as some of the most important stressors of coastal ecosystems and are present all over the world (GESAMP, 2007). The effects of petroleum on marine flora and fauna have been extensively described at different levels of biological organization (recently reviewed by Beyer et al., 2016 and Bejarano and Michel, 2016). Most of the current research has investigated the effects of hydrocarbons at the cellular level through the assessment of biomarkers (eg: Milinkovitch et al., 2011, a, b, c, 2015; Danion et al., 2014; Dussauze et al., 2015, a, b; Geraudie et al., 2016) and more recently, studies were conducted at the individual and the population levels (e.g. Davoodi and Claireaux, 2007; Milinkovitch et al., 2012 and Langangen et al., 2017, respectively).

Although hypoxia and petroleum can coexist in coastal ecosystems, only few studies evaluated the biological effects of their interaction on fish physiology and behavior. Among these, experimental studies were conducted (i) by challenging organisms with extremely severe levels of hypoxia and/or contamination (Claireaux et al., 2013; Dasgupta et al., 2015) or (ii) by assessing biological effects at the sub-individual level (Negreiros et al., 2011), so that predictions of impacts on the fitness of the organism were uncertain. More recently, Mager et al., (2018) showed that an exposure to hypoxia alone or combined with crude oil elicited significant decreases in critical swimming speed to a similar extent.

Given the limited knowledge on the biological effects of hypoxia and petroleum and considering the main goal of the Marine Directive (i.e. to achieve Good Environmental Status of EU marine waters by 2020), this study aims at testing the biological effects of hypoxia in fish contaminated by realistic exposure of petrogenic hydrocarbons. Specifically, this is the first study to test the cumulative and separated effects of hypoxia and petroleum on behavioral and kinematic parameters of the fish escape response. Fish escape responses have been described as "high accelerations, often accompanied by a change in direction" (Domenici et al., 2011) aimed at avoiding predators. This response is therefore fundamental for survival and is consequently an important proxy of fitness (Walker et al., 2005). Thus, by measuring alterations of behavioral and locomotor parameters at the individual level, this study also aims at providing an insight of the impact of oil and hypoxia on fish fitness. In parallel, chemical parameters - the polycyclic aromatic hydrocarbons (PAHs), the total petroleum hydrocarbons (TPHs) and the PAHs biliary metabolites concentrations – will permit to link the biological effects observed in fish with the exposure and the incorporation of contaminants. TPHs were measured since they represent the whole hydrocarbons *i.e.* the polar compounds, the saturated and the polycyclic hydrocarbons. PAHs were measured since they are considered to be the most toxic compounds among hydrocarbons in accordance with the literature (review in Neff, 2002) and the United States Environmental Protection Agency (USEPA).

As a biological model, *Dicentrarchus labrax* was used, because of its key role in the coastal ecosystems (Barbault, 1995), its sensitivity to hypoxia (Terova et al., 2008) and hydrocarbons (Gravato and Santos, 2003; Claireaux et al., 2018) and its economic importance, since this species represents world aquaculture and capture productions of 156000 tons and 8000 tons, respectively (FAO, 2014).

2. Materials and Methods

2.1. Experimental animals

Juvenile European seabass (*Dicentrarchus labrax)* were provided by Aquanord Ichtus (Gravelines, France). During 3 weeks, 90 fish were kept in 6 tanks of 300 L (15 fish per tank; tanks similar than those used for the experiment) under natural photoperiod, with open circulation of sea water (free of nitrites and nitrates). Temperature, salinity, pH and dissolved oxygen concentration were monitored every day and values were respectively 20.12 ± 0.19 °C, 35 ± 0.1 ‰, 7.95 ± 0.01 and 93.59 ± 0.62 % of air saturation (mean ± standard error of the mean). Fish were fed daily with fish food (Neostart, Le Gouessant) and starved for 48 hours before the experiment. Fish, in their second year, showed an average length and weight of 16.44 ± 0.08 cm and 49.79 ± 0.64 g, respectively. Fifty six fish were used for the experiment.

2.2. Exposure to contaminants

A Brazilian crude oil, containing 0.5 % of asphaltenes and 13.2 % of wax (% of weight) compounds, was used for the experiment. The PAHs concentration of the oil is presented in **supplementary material**. The viscosity measured at 20ºC is 35574 mPa.s. The flash and pour points are respectively -2.5 ºC and -28ºC.

The set-up for contamination has already been extensively described in the literature (e.g. Milinkovitch et al., 2011a, b; Claireaux et al., 2013; Milinkovitch et al., 2013 a, b). Briefly, the static water system permits to maintain a dispersion of oil in the water column. It is composed of a funnel (creating a vortex) linked to a water pump, so that petroleum and/or seawater are continuously sucked up from the surface and expelled through the pump at the bottom of the tank.

The oil treatment was made by pouring 15 g of oil into the funnel of the set-up while the control one was made by pouring 15 g of seawater. According to Dussauze et al. (2015a), a waiting period of 6 hours was used in order to obtain a homogenous solution (same

concentration measured at three different depths within the experimental tanks). After this period, every day, two juvenile fish were introduced in the oil treatment tank and two juvenile fish were introduced in the control treatment tank, for an 18 hours exposure. The operation was repeated 14 times (7 times with the oil treatment and 7 times with control treatment with randomized order) so that 28 fish were exposed to the oil treatment and 28 fish to the control treatment. A 300 watts HYDOR™ resistance was used to maintain the temperature at 19.89 ± 0.13 °C. In parallel, pH and dissolved oxygen were maintained at 8.09 ± 0.01 and 99.9 ± 0.47 % of air saturation, respectively.

2.3. Total petroleum hydrocarbons (TPHs) measurement

The TPHs concentrations were measured for all treatments at the beginning of the trial and after 18 h, using a method described in Fusey and Oudot (1976). Briefly, three samples of 125 mL of contaminated or uncontaminated seawater were collected at T=0h and at T=18h on the tank bottom. Then, samples were extracted using 25 mL of dichloromethane (Carlo ErbaReactifs, France) permitting a separation of the organic and aqueous phases. After being dried on anhydrous sulphate, extracts were analyzed using a UV spectrophotometer (UVeVisspectrophometer, Unicam, France) at 390 nm. Analyses were conducted at Cedre (Centre de Documentation de Recherche et d'Expérimentations sur les Pollutions Accidentelles des Eaux), a laboratory with agreement ISO 9001 and ISO 14001. In accordance with Cedre, limit of quantification is below 1 mg/L.

2.4. Escape response under hypoxia or normoxia

2.4.1. Experimental set-up and protocol

After the contamination period (described in 2.2), oil-contaminated and uncontaminated fish were all directly and individually transferred in experimental setups enabling the exposure to

144 hypoxia or normoxia. Consequently, our experiment was designed to obtain 4 experimental
145 groups of fish: (i) uncontaminated fish under normoxia (Ctrl norx); (ii) uncontaminated fish
146 under hypoxia (Ctrl hyp); (iii) oil-contaminated fish under normoxia (Oil norx); (iv) oil-
147 contaminated fish under hypoxia (Oil hyp). Under these hypoxic or normoxic environment,
148 fish were tested for escape responses.

149 The experimental set-up designed to control oxygen levels and to elicit an escape response is
150 described in **supplementary material**. An oxygen regulator (OXY-REG, Loligo systems,
151 Denmark), placed at the interface between an oxygen probe measuring the oxygen level in a
152 tank (1 meter of diameter, 0.56 m of depth, filled with 0.2 m of water depth) and a solenoid
153 valve (Gas control set, Loligo systems, Denmark) managing the bubbling of nitrogen (from a
154 gas bottle) in a seawater gas equilibration column, was used to monitor the oxygen level.
155 Nitrogen permits the extraction of oxygen from the water. The gas equilibration column was
156 linked to a water pump (EHEIM 600, UK) situated in the tank and permitting a constant flow
157 of deoxygenated water between the tank and the column. In order to avoid stress in the fish,
158 the water pump, the oxygen probe and a thermic resistance (average seawater temperature
159 during the experiment: 20.04 ± 0.04 °C) were isolated, behind a plastic partition, from the rest
160 of the tank (where the fish experienced the oxygen decrease and was tested for escape
161 response). For the hypoxic conditions, the regulator was set-up at 23 % of air saturation:
162 above this value, nitrogen was bubbled in the gas equilibration column. The oxygen decrease
163 was obtained in three hours (**see supplementary material**) in order to obtain a progressive
164 decline (Lefrançois and Domenici (2006), and then oxygen varied between 23.7% and 22.3%
165 with a homogenous concentration of oxygen in the tank. The same setup was used to test the
166 fish in normoxic condition, but in this case air was bubbled instead of nitrogen so that oxygen
167 concentration was always higher than 95 % of air saturation. After three hours, a subsequent

168 30 min period of steady-state oxygen level was conducted for both hypoxic and normoxic
169 conditions.

170 Then, fish were startled (within a 1 hour period) by dropping a PVC cylinder (2.8 cm of
171 diameter, 10 cm length, 23 grams) in the water from a height of 91 cm. The stimulus, held
172 above the tank by an electromagnet, fell through a black PVC pipe (8 cm diameter, 90 cm
173 length situated at 1 cm of the water surface) in order to prevent the fish from seeing the
174 stimulus before it hits the water, thus allowing the measurement of the response latency to a
175 mechanical rather than visual stimulus (Dadda et al., 2010). The drop of the stimulus was
176 triggered by the experimenter when the fish was at a distance longer than 25 cm (representing
177 1.5 times the mean of fish body length) (i) from the edge of the tank (to avoid wall effects,
178 Eaton and Emberley, 1991), and (ii) from the point of impact of the stimulus with the water
179 surface. A high-speed camera (Casio Exilim FH 100) positioned at 180 cm above the tank
180 recorded the escape response at 240 frames s^{-1}. Among the 56 fish tested, only 41 videos (Ctrl
181 norx=11, Ctrl hyp=9, Oil norx=9, Oil hyp=12) were feasible for analysis. This was due to the
182 fact that some fish did not leave the edge of the tank or that the responses were elicited when
183 they were too close to this area. After stimulation, fish were euthanized using a lethal dose of
184 eugenol (4-allyl-2-methoxyphenol, Sigma Aldrich, USA). Gallbladder was excised and frozen
185 at -80°C for fixed wave fluorescent analysis of bile.

186 **2.4.2. Analysis of escape response**

187 Escape response tracking was conducted using the WIN Analyse software and taking into
188 account the digitized coordinates of the tip of the head and of the center of mass. In
189 accordance with Lefrançois and Domenici (2006), the center of mass (CM) was estimated to
190 be at 38 % (from the tip of the head) of the total fish length and positioned thanks to the

191 software. In order to analyze the escape response, the two stages of the fast-start escape
192 response (S1 and S2, as described in **supplementary material**) were considered.

193 The first stage (S1) has been described as a phase in which the fish forms a C-shape
194 (Domenici and Blake, 1997). S1 begins (t1) with the first detectable movement of the head. It
195 ends with the beginning of stage 2 (t2), when the fish changes the turning direction of the
196 anterior body midline (the line from the center of mass to the head). Stage 2 ends when the
197 fish changes again the turning direction (t3) (Domenici and Blake, 1997). Considering this
198 kinematic sequence, several variables were analyzed for each treatment (Ctrl norx, Ctrl hyp,
199 Oil norx, Oil hyp) (Lefrancois and Domenici 2006, Marras et al 2011):

200 - Escape response duration, *i.e.* the time for the fish to accomplish the whole escape response
201 (S1 and S2 stages) *i.e.* the time between t1 and t3 as described above.

202 -Responsiveness. *i.e.* the percentage of fish (for each treatment) responding to the stimulus
203 with a fast start escape response.

204 -Latency *i.e.* the time between the stimulus hitting the water surface (t0) and the first
205 detectable movement of the head of the fish (t1)

206 -Directionality, defined as “away or “toward” depending if the first detectable movement of
207 the head was orientated “away” or “toward” the stimulus.

208 -Distance-time variables (cumulative distance, straight distance, average speed, average
209 acceleration, maximum speed, maximum acceleration) are measured within the fixed time of
210 the escape response duration (i.e. the mean total escape response duration of all treatments, in
211 this study 55.4 ms) in order to avoid any bias due to differences in escape response duration in
212 accordance with previous authors (Webb, 1976; Domenici and Blake, 1993). Cumulative
213 distance is total distance achieved by the fish during the whole escape response duration by

summing all the distances covered between frames. Straight distance is defined as the linear distance between the CM at t1 and the CM at t3. Average speed is the cumulative distance divided by the duration of the escape response. Maximum speed is the peak value of the speed vs. time curve. The speed curve is obtained using a five-point polynomial regression procedure (Lanczos, 1956). Average acceleration is the mean of the accelerations obtained during the escape response. Maximum acceleration is the peak value (obtained with the procedure of Lanczos, 1956).

-Maneuverability variables: S1 turning angle is the difference of orientation (in degrees) between the fish anterior body midlines at t1 and at t2. Similarly, S2 angle is the difference of orientation between the fish anterior body midlines at t2 and at t3. S1 and S2 average turning rates are calculated as S1 and S2 angles divided by the duration of each stage, respectively. S1 and S2 maximum turning rates are the peak values of the derivative of S1 and S2 angles *vs.* time curve (obtained with the procedure of Lanczos, 1956), respectively.

-Escape trajectory is expressed in degree and ranged between 0 and 360° (Domenici et al. 2011a, 2011b). This circular data is measured as the angle between the line passing through the stimulus position and CM at t0 and the anterior body midline at t3. By convention, stimulus position was always considered to be on the right side of the fish at the onset of the response (Domenici and Blake 1993).

-Distancing (**supplementary material**) is defined as the distance between the CM of the fish and the stimulus at t3 minus the distance between the CM of the fish and the stimulus at t1. Like distance time variables, distancing is measured within the fixed time of the escape response duration (i.e. 55.4 ms). A negative value implies a fish getting closer to the stimulus while positive values implies a fish distancing itself from the stimulus during the escape response.

2.5. Fixed wavelength fluorescence analysis of bile

Bile samples were diluted (1:500) in absolute ethanol (Sigma Aldrich, France) and assays, in quartz cuvettes (Sigma Aldrich, USA) on a spectrofluorimeter (SAFAS Flx-Xenius, Monaco), were conducted for five fixed wavelength fluorescence (FF). According to Aas et al. (2000), naphthalene-derived metabolites were detected for the emission:excitation wavelength pairs 290:335 , pyrene-derived metabolites for 341:383 and benzo[a] pyrene-derived metabolites for 380:430. Moreover, fluorene type derived metabolites were detected at 275:328 and phenanthrene type derived metabolites at 250:370, according to Kopecka-Pilarczyk and Correia (2009) and Szlinder-Richert et al. (2014), respectively. Obtained values were expressed as arbitrary units of fluorescence.

2.6. Statistical analysis

A χ^2 test of homogeneity was used to test the effect of the 4 treatments (Ctrl norx, Ctrl hyp, Oil norx, Oil hyp) on the responsiveness of the fish. A χ^2 test of independence was used to determine if treatments have an effect on directionality. To do so, we considered the proportion of “away” and “toward” responses for each treatment and tested if it differs significantly from random (i.e. from a 50:50 ratio). After confirmation of the homoscedasticity, a two-way analysis of variance was employed in order to test the effect of the treatments on escape response duration, latency, distance time variables (cumulative distance, straight distance, speeds and accelerations), S1 and S2 turning angles, S1 and S2 turning rates, distancing and fluorescence intensity. When ANOVA was significant for any of the factors (i.e. for “contamination” and “oxygen level” factors) and for the interaction of these factors, a Fisher post-hoc test was used for intergroup comparisons. In order to analyze the effect of treatments on escape trajectories, circular statistics (Rayleigh test, Batschelet,

1981) were employed to highlight significant non-random circular distribution of trajectories for each group of fish. Subsequently, a two-way ANOVA for circular data (based on Harrison and Kanji, 1988; Berens, 2009) were conducted in order to compare the different angles of escape trajectories for the different group of fish. A Mann-Whitney U test was used to highlight statistical differences between the TPH concentrations at T=0h and at T=18h. Statistica™ software was used for all analysis except for circular statistics (escape trajectories) for which Oriana™ software was used. Significance was accepted at $P<0.05$. Results are expressed as mean ± standard error of the mean, except for escape trajectories (expressed as mean ± 95% confidence interval).

3. Results

3.1. Hydrocarbon exposure and organism incorporation

For the seven replicates of exposure to oil treatment, Total Petroleum Hydrocarbons (TPH) mean concentration was 16.4 ± 0.3 mg/L at T=0h and 19.4 ± 0.7 mg/L at T=18h (data not shown). Statistical analysis did not show significant difference between these two concentrations ($P>0.05$). For the seven replicates of exposure to control treatment, no TPHs were detected in the water column.

For relative concentrations of all types of biliary PAH metabolites, a significant effect of the factor "contamination" was observed with P value always lower than 0.01. In line with this, post-hoc tests show significantly higher concentrations of all types of biliary PAH metabolites for contaminated fish (Oil norx and Oil hyp), when compared to uncontaminated fish (Ctrl norx and Ctrl hyp) with P value always lower than 0.01 (**Figure 1**). The oxygen level used during the escape response trials did not influence the concentration of PAHs metabolites (of

all types) since post-hoc statistical tests revealed no difference between Ctrl norx and Ctrl hyp as between Oil norx and Oil hyp (P>0.05).

3.2. Fast start performance

3.2.1. Escape response duration, responsiveness, latency and distance-time performances

Escape response duration, responsiveness and escape latency did not differ statistically among the treatments (P>0.05). In the same line, distance-time parameters (cumulative distance, straight distance, average and maximum speeds as well as average and maximum accelerations) were not impacted by hypoxia nor by petroleum (P>0.05) **(see table in supplementary material).**

3.2.2. Maneuverability, directionality and escape trajectories

Maneuverability parameters were measured for S1 and S2. S1 turning angles (**Figure 2A**) ranged between 58.6 ± 5.4 degrees in Ctrl hyp and 76.6 ± 3.7 degrees in Oil hyp. S1 average turning rates (**Figure 2B**) ranged between 2408.2 ± 75.4 in Oil hyp and 2013.0 ± 186.4 degrees.s^{-1} in Oil norx group. S1 maximum turning rate (**Figure 2C**) was 2944.4 ± 133.5 degrees.s^{-1} for the control group. For these parameters, no effects of stressors was observed (P>0.05).

S2 turning angles (**Figure 2D**) ranged between 57.9 ± 9.9 degrees in uncontaminated fish under normoxia (Ctrl norx) to 26.0 ± 7.5 degrees in contaminated fish under normoxia (Oil norx). A Two-way ANOVA revealed an effect of the stressor “contamination" (P<0.05) on this variable. The Post-hoc tests showed that, when compared to Ctrl norx group, contaminated fish (*i.e.* Oil norx and Oil hyp) showed significant lower S2 turning angles (P<0.05). On the other hand, no statistical difference (P>0.05) was found between uncontaminated fish under normoxia (Ctrl norx) and uncontaminated fish under hypoxia (Ctrl

hyp). Similarly, no significant difference (P>0.05) was observed for this variable between the two groups of contaminated fish (Oil norx and Oil hyp).

S2 average and maximum turning rates for Ctrl norx groups were 1623.6 ± 121.7 and 2229.2 ± 162.6 degrees.s^{-1}, respectively and two-way ANOVA revealed an effect of oil (P<0.05). More specifically, a similar pattern of results was observed for S2 average and maximum turning rates (**Figure 2E and 2F**) as for S2 angle. Indeed, post-hoc tests showed that turning rates were significantly lower for contaminated groups, when compared to Ctrl norx group (P<0.05) while no significance was observed when comparing contaminated fish under normoxia with contaminated fish under hypoxia (P>0.05).

With respect to directionality (**Figure 3**), only uncontaminated fish under normoxia (Ctrl norx) showed a non-random response with 81.8 % of the fish swimming away from the stimulus (P<0.05). All treatments (*i.e.* hypoxia, petroleum and hypoxia + petroleum) altered such a response since statistical analysis showed random responses (P>0.05) for Ctrl hyp (with 33.3 % of fish swimming away), Oil norx (66.7 % away) and Oil hyp (41.7 % away).

For escape trajectories (**Figure 4**), Rayleigh tests highlighted a significantly non-random circular distribution (P<0.05) for the Ctrl norx while the distribution was significantly random for the other groups(P>0.05).

The mean escape trajectories of Ctrl norx and Ctrl hyp groups were 104.1 ± 31.2 (95% confidence interval) and 97.8 ± 45.4 (95% confidence interval) degrees, respectively. Contaminated fish (*i.e.* Oil norx and Oil hyp) showed average values of 179.3 ± degrees and 56.8 degrees, respectively. Two-way ANOVA for circular data did not show significant difference between fish groups.

3.2.3. Distancing (Figure 5)

Uncontaminated fish under normoxia (Ctrl norx) distance themselves from the stimulus by 26.8 ± 8.11 mm. Distancing for uncontaminated fish under hypoxia (Ctrl hyp) and for contaminated fish under normoxia (Oil norx) were respectively 6.1 ± 10.0 mm and 21.4 ± 10.8 mm. Oil hyp fish get closer to the stimulus during escape response with a negative value of distancing (-14.4 ± 12.6 mm). Statistics revealed no effect of the contamination nor the hypoxia but an effect of the interaction of stressors with a significance between Oil hyp and Ctrl groups ($P<0.05$).

4. Discussion

4.1. Exposure to hydrocarbons in normoxic or hypoxic conditions and metabolites

Fish were exposed to stable Total petroleum hydrocarbons (TPHs) concentrations of dispersed oil, varying between 16.4 mg/L (at T=0h) and 19.4 mg/L (at T=18h). Such concentrations of dispersed oil are consistent with estimations already reported in the field. For instance, Lewis and Daling (2001) estimated that naturally dispersed oil concentrations in the water column would decrease from 10 to 1 mg/L in 12 hours when the oil is released in the open ocean with moderate meteorological conditions (wind at 10 $m.s^{-1}$) while the same authors reported TPH concentrations of 50 mg/L, few hours after the Braer oil spill, declining to 5 mg/L, in ten days. In the same line, oxygen level used to simulate hypoxic exposure (23%) is consistent with the fact that coastal marine fish species are still present in hypoxic areas with 27% a.s. but are poorly encountered under 20% of air saturation, choosing a strategy of hypoxic avoidance (Pihl et al., 1991) and consequently not suffering the effect of hypoxia.

Measurement conducted on the petroleum used in this study revealed a majority of light parents and alkylated PAHs (2-3 rings PAHs such as Naphtalene, Benzo(b)thiophene, Biphenyl, Acenaphtylene, Acenaphtene, Fluorene) as well as 4 rings PAHs (such as

Phenanthrene, Anthracene, Dibenzothiophene, Fluoranthene, Pyrene, Benzo[a]anthracene, Chrysene). On another hand, heavier PAHs concentrations (Benzo[b+k]fluoranthene, Benzo[e]pyrene, Benzo[a]pyrene, Perylene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene, Benzo[g,h,i]perylene) are low relatively to lighter compounds.

While the relative concentration of biliary PAHs metabolites estimates the metabolism of detoxication of PAHs, it also has been used in experimental and field studies as an indirect marker of PAHs body burden (reviewed in Amiard and Amiard-Triquet, 2008). In our study, all metabolites of PAHs were higher in contaminated fish than in control ones, confirming the incorporation of the several types of PAHs. The results of light PAHs (naphthalene, fluorene) were expected since they are considered as highly soluble in seawater and consequently available for incorporation by organisms (Neff, 2002). Similarly, our results concerning 4 ring PAHs are in accordance with the literature since these PAHs are considered highly bioaccumulative (Canadian Environmental Guidelines, 1999; Achten and Andersson, 2015). More surprising is the significant incorporation of heavier PAHs (estimated with Benzo[a]pyrene metabolites relative concentration) since they have a low solubility in seawater (Neff, 2002) and represent a minor part of the petroleum used in this study (see paragraph above). This could be due to an incorporation through the ingestion, more than the respiration, of the drop of oil dispersed in the water column. The incorporation of these heavy PAHs (specially benzo[a]pyrene) is of interest since they are classified as the highest toxic PAH compounds (Neff, 2002). When we compared Oil norx and Oil hyp conditions, we found that hypoxia, used during the escape response test, did not affect the concentration of PAHs metabolites. This suggests that the hydrocarbons body burden was not modulated by the hypoxic period following contamination. On this basis, we suggest that the interactive effects of hypoxia plus oil (described below) are not due to a differentiated hydrocarbons body burdens but rather to the biological impacts of both stressors on the organism.

4.2. Effects of hypoxic conditions and hydrocarbons on responsiveness and response latency

Taking into account the timing of the events in C-start escape response, the first variable observed are the response latency and the responsiveness. In our study, neither hypoxia nor the oil had an effect on response latency and responsiveness. Our results on the effect of hypoxia on latency are in line with previous study by Lefrançois et al. (2005) and Lefrançois and Domenici (2006). Indeed, these authors showed no effect of hypoxia on latency at 50 % and 20 % of air saturation in golden grey mullet and European seabass, respectively. Significant effects were only observed at 10% of air saturation (severe hypoxia) in golden grey mullet. Since in our study we never reached such severe level of hypoxia, we cannot determine whether there is an effect of severe hypoxia on latency like in previous works. Regarding effect of hypoxia on responsiveness, previous studies (Lefrançois et al., 2005 and Lefrançois and Domenici, 2006) found significant effects in both seabass and mullet at 10% of air saturation, but no effect at 20 % and 50 % of air saturation, in line with our study conducted at 23 % air saturation. Probably, if conducted at severe levels of hypoxia (e.g. 10 % of air saturation) our study would have revealed effects of hypoxia on responsiveness.

Our study showed no effect of hydrocarbons (interacting or not with hypoxia) on latency and responsiveness. Comparison with previous studies is limited since little is known on the effect of hydrocarbons on fish behaviour. Gonçalves et al. (2008) and Johansen et al. (2017) revealed an effect of hydrocarbons upon spontaneous locomotor activity in juvenile sea bream (*Sparus aurata*) and upon sheltering and shoaling in coral reef fishes, respectively, however no study has yet investigated the effect of hydrocarbons on escape response. Other pollutants such as metallic compounds have been shown to affect responsiveness and latency in seabass (Faucher et al., 2006, 2008) and in zebrafish (Weber, 2006). In seabass, these authors linked this alteration with the damage of the hair cells of the neuromasts of the lateral line, involved

406 in the detection and in the initiation of the escape response through mechano-sensory
407 detection. In our study, the absence of an effect on responsiveness and latency suggests that
408 the mechano-acoustic detection as well as the firing of the escape response was not impaired
409 by this stressor. Comparison between these studies and our suggests that effects depend on the
410 type of contaminant. Indeed, while trace elements impact the trigger of the escape response,
411 organic contaminants such as hydrocarbons do not. Thus, other studies investigating the
412 impact of other organic contaminants (such as PCBs or pesticides) on escape response could
413 be of interest.

414 **4.3. Impacts on directionality, maneuverability and escape trajectory**

415 The first detectable movement of fish head indicates the directionality - "away" from or
416 "toward" the stimulus – taken by the fish. While it is known that fish may turn towards an
417 object falling on the water that may represent a food source (Wohl and Schuster, 2007), the
418 characteristics of the stimulus used here (see 2.4.1.) are such that it was most likely
419 representing a threat to avoid, rather than an attractive potential food stimulus for the fish.
420 Consequently, in control condition, the fish statistically escape away from the stimulus. Our
421 study found an effect of all stressors (hypoxia, oil and oil plus hypoxia) on directionality.
422 Indeed, fish under hypoxia and/or contamination did not statistically turn away from the
423 stimulus. Following the first movement of the head, escape response is divided in two stages
424 (S1 and S2). Our study showed a significant effect, during the S2 stage, on the angle taken by
425 the fish as well as on average and maximum turning rates for fish exposed to oil and to oil
426 plus hypoxia.
427 The altered directionality and maneuverability of fish under stressors could have further
428 consequences such as affecting the escape trajectory of the fish. In line with this possibility,
429 circular distribution of the escape trajectories showed that control fish showed statistically
430 uniform trajectories while fish under hypoxia and/or contamination took random ones

suggesting an impairment of the stressors. Thus, our results show a high variability of angles of escape trajectories for fish exposed to stressors. This high variability could be the reason why we did not find any significant difference when comparing the means of angle of trajectories between the different groups.

Concerning the effect of hypoxia on the directionality, maneuverability and escape trajectory, our study is in accordance with Lefrançois et al. (2005) and Lefrançois and Domenici (2006). Indeed, these authors also showed that hypoxia (i) decreases the ability of grey mullet and seabass to escape away from the stimulus (ii) had no effect upon the turning angle and turning rates during S1 nor S2 (iii) did not influence the escape trajectory.

Regarding the effect of contaminant, to our knowledge, no study investigated the impact on fish escape trajectory and only one study has focused on the effect of ammonia on directionality and maneuverability (McKenzie et al., 2008). McKenzie et al. (2008) showed no effect of the ammonia on directionality, and an impact on S1 maximum turning rate. This discrepancy between their work and our is not surprising since chemical nature and consequently chemical properties of the contaminants are different.

After measuring the effect of oil plus hypoxia on directionality, maneuverability and escape trajectory, our results showed that the impact of both stressors was not statistically higher than the effect of oil alone.

While we already showed no impact of oil on the trigger of escape response (*i.e.* on responsiveness and response latency, see above), we also demonstrate that stressors impact the directionality, the maneuverability and the escape trajectory of the fish. We suggest that the impact of contaminant on the directionality, the maneuverability and the escape trajectory is probably due to an alteration of the functional integrity of the nervous system. In this line of thought, Vignet et al. (2017) recently showed that PAHs diet lead in zebrafish to changes in

brain monoamines concentrations and to a consequent behavioral disruption. Along the same lines, Rastgar et al. (2015) showed an increase of serotonin and dopamine in the pituitary and the hypothalamus of *Acanthopargus latus* after an exposure to benzo[a]pyrene. The cholinergic system is also altered in fish *Pomatoschistus microps* exposed to PAHs (Vieira et al., 2008).

4.4 Effects on distance, time and distance-time variables

Along the two stages of the escape response (S1 and S2), escape response duration, distance (cumulative and straight) and distance-time (speed and acceleration) variables did not show any alteration due to hydrocarbons and/or hypoxia. Regarding hypoxia, in line with our results, Lefrançois and Domenici (2006) also showed no alteration of these parameters when seabasses were exposed to 50 or 20 % of air saturation. These findings are consistent with the fact that fish locomotor performance during escape response is mainly fueled by the anaerobic metabolism and is therefore not impacted by the decrease of water oxygenation. However, for mullets, alteration was observed at 10% of air saturation (Lefrançois et al., 2005). At this extreme level of hypoxia, authors suggest that the alteration of locomotor performance could be due to exhaustion, i.e. to a depletion of intramuscular ATP (adenosine triphosphate) preventing the anaerobic metabolism processes.

Regarding oil effects, while Gonçalves et al. (2008) and Johansen et al (2017) revealed an effect of hydrocarbons upon spontaneous locomotor activity in juvenile sea bream *Sparus aurata* and upon sheltering and shoaling in coral reef fishes, no study investigated the effect of hydrocarbons on anti-predator locomotor behavior. Studies investigating the effect of heavy metals on distance-time variables of anti-predator escape response showed slight effects of these contaminants. Indeed, Rice et al. (2011) showed no effect of lead exposure on escape response duration and on maximum speed (when zebrafish larvae were stimulated with

a single mecano-acoustic signal) and Weber (2006) showed effect of mercury on larvae zebrafish maximum speed only for the highest (and non-environmentally relevant) concentrations. Therefore, based on our results and previous work, various kinds of contaminants such as heavy metal and hydrocarbons do not seem to impact the distance-time variables of escape response. However, Breckels and Neff (2010) highlight an effect on burst swimming speed of brown bullheads fish *in situ* exposed in a river highly contaminated with several pollutants. This suggests that contaminants other than heavy metals and hydrocarbons are likely to alter the distance-time variables.

4.5. Distancing

At the end of the escape response, the position of the fish permits to evaluate the distancing (*i.e.* the distance change between the fish and the stimulus, due to the escape response). To our knowledge, this variable has not yet been studied. While the straight and the cumulative distances both represent measurements of the distance completed during the escape response, they did not estimate how much the fish increase the distance from the stimulus. Indeed, a fish could show elevated straight and cumulative distances but get closer to the stimulus, for example if its escape trajectory is orientated toward the stimulus. Therefore distancing is an integrative parameter of ecological significance since it integrates several aspects of the escape response (angle of flight and distance covered by the fish). In our study, hypoxic fish under contamination was the only group significantly affected (when compared to normoxic control fish). The negative distancing for this group show that fish were closer to the stimulus at the end of the escape response than at the beginning. Such a result is likely to be the consequence of the altered directionality and maneuverability (discussed above) observed for this group, since other parameters (cumulative and straight distances, distance-time variables, response latency and responsiveness) were not significantly affected neither by contaminants nor by hypoxia. The significant effect of the interaction oil plus hypoxia, and on the other

hand the lack of effect due to hypoxia or oil alone, strongly suggests a synergistic effect of these stressors.

Conclusion

The impact of hydrocarbons, interacting with hypoxia, has never been investigated on fish escape response. Here, we demonstrated that single and combined stressors (i.e. oil, hypoxia, oil plus hypoxia) affect fish directionality. This suggests that stressors alter the ability of fish to escape away from the predator. This tactical error of directionality may have lethal implication for the fish. Regarding the maneuverability of the fish, an impact of oil (interacting or not with hypoxia) was observed. However, it appears that the impact was not intense enough to induce effects on the angle of flight (escape trajectory angles) and the propulsive performances (measured through distance-time variables). Distancing, an integrative variable that measures if the distance from the threat increases or decreases after the completion of the escape response, showed alteration only when seabass were exposed to oil and to hypoxic conditions but no significant effects were observed when stressors were applied separately, suggesting that the synergy of the stressors causes an impact. This impact could have repercussions in prey-predator interactions, such as decreasing the probability of prey escape success. Such results suggest that oil contamination in hypoxic areas could have stronger ecological implication than oil spill in normoxic ones. These findings should be interpreted cautiously due to the experimental character of this study. However, they could be of great interest for a better understanding of the environmental effect of oil spill in coastal ecosystems. Additionally, this work is of interest in the aim to develop new ecotoxicological tools of ecological significance for the biomonitoring of oil-impacted areas.

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533 **5. References**

534 Aas E, Baussant T, Balk L, Liewenborg B, Andersen OK (2000) PAH metabolites in bile,
535 cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil
536 exposure: a laboratory experiment with Atlantic cod. Aquat. Toxicol. 51, 241–258.

537 Achten C, Andersson JT (2015) Overview of Polycyclic Aromatic Compounds (PAC).
538 Polycyclic Arom. Comp. 35, 177–186.

539 Amiard-Triquet C, Amiard JC (2008) Les biomarqueurs dans l'évaluation de l'état écologique
540 des milieux aquatiques. Lavoisier Tec et Doc, Paris

541 Barbault R (1995) Écologie des populations et des peuplements. Masson, Paris

542 Batschelet E (1981) Circular statistics in biology. Academic Press, NewYork

543 Bejarano AC, Michel J (2016). Oil spills and their impacts on sand beach invertebrate
544 communities: A literature review. Environ. Pol. 218, 709-722.

545 Berens P. (2009) CircStat: A MATLAB Toolbox for Circular Statistics. J. Stat. Software.
546 doi:10.18637/jss.v031.i10.

547 Beyer J, Trannum HC, Bakke T, Hodson PV, Collier TK (2010). Environmental effects of the
548 Deepwater Horizon oil spill: A review. Mar. Pol. Bul. 110, 28–51.

549 Breckels RB, Neff BD (2010) Pollution-induced behavioural effects in the brown bullhead
550 (*Ameiurus nebulosus*). Ecotox. 19, 1337–1346

551 Canadian environmental guideline (1999). Canadian Sediment Quality Guidelines for the
552 Protection of Aquatic Life. POLYCYCLIC AROMATIC HYDROCARBONS (PAHs). 1299,
553 1-16

554 Cheung SG, Chan HY, Liu CC, Shin PK (2008). Effect of prolonged hypoxia on food
555 consumption, respiration, growth and reproduction in marine scavenging gastropod *Nassarius*
556 *festivus*. Mar. Pol. Bul. 57, 280–286.

557 Claireaux G, Théron M, Prineau M, Dussauze M, Merlin FX, Le Floch S (2013) Effects of oil
558 exposure and dispersant use upon environmental adaptation performance and fitness in the
559 european sea bass, *Dicentrarchus labrax*. Aquat. Toxicol. 131, 160–170.

560 Claireaux G, Queau P, Marras S, Le Floch S, Farrell AP, Nicolas-Kopec A, Lemaire P,
561 Domenici P (2018). Avoidance Threshold to Oil Water-Soluble Fraction by a Juvenile Marine
562 Teleost Fish. Environ. Toxicol. Chem. 37, 854-859
563

564 Cook DG, Brown EJ, Lefevre S, Domenici P, Steffensen JF (2014). The response of striped
565 surfperch *Embioto calateralis* to progressive hypoxia: Swimming activity, shoal structure, and
566 estimated metabolic expenditure. J. Exp. Mar. Biol. Ecol. 460, 162–169.

567 Costanza R, d'Arge R, de Groot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S,
568 O'Neill RV, Paruelo J, Raskin RG, Sutton P, Van den Belt M (1997). The value of the world's
569 ecosystem services and natural capital. Nature 387, 253–260.

570 Dasgupta S, Huang IJ, McElroy AE (2015) Hypoxia Enhances the Toxicity of Corexit
571 EC9500A and Chemically Dispersed Southern Louisiana Sweet Crude Oil (MC-242) to

572 Sheepshead Minnow (*Cyprinodon variegatus*) Larvae. Plos One. doi:
573 10.1371/journal.pone.0128939.

574 Dadda M, Koolhaas WH, Domenici P (2010) Behavioural asymmetry affects escape
575 performance in a teleost fish. Biol. Lett. 6, 414–417

576 Danion M, Le Floch S, Lamour F, Quentel C (2014). EROD activity and antioxidant defenses
577 of sea bass (*Dicentrarchus labrax*) after an *in vivo* chronic hydrocarbon pollution followed by
578 a post-exposure period. Environ. Sci. Pollut. Res. 21, 13769–13778.

579 Davoodi F, Claireaux G (2007).Effects of exposure to petroleum hydrocarbons upon the
580 metabolism of the common sole *Solea solea*. Mar. Pol. Bul. 54, 928–934.

581 Diaz RJ, Cutter GR, Able KW (2003). The importance of physical and biogenic structure to
582 juvenile fishes on the shallow inner continental shelf. Estuar. 26, 12-20

583 Diaz RK, Rosenberg R (2008) Spreading Dead Zones and Consequences for Marine
584 Ecosystems. Science 321, 926-929

585 Domenici P, Blake RW (1991) The kinematics and performance of the escape response in the
586 angelfish (*Pterophyllum eimekei*). J. Exp. Biol. 156, 187–205

587 Domenici P, Blake RW (1993). Escape trajectories in angelfish (*Pterophyllum eimekei*). J.
588 Exp. Biol., 253-272.

589 Domenici P, Blake RW (1997) The kinematic and performance of fish fast-start swimming. J.
590 Exp. Biol. 200, 1165–1178.

591 Domenici P, Lefrançois C, Shingles A (2007). Hypoxia and the antipredator behaviours of
592 fishes. Phil. Trans. Royal Soc. 362, 2105-2121.

593 Domenici P, Blagburn JM, Bacon JP (2011) Animal escapology II: escape trajectory case
594 studies. J. Exp. Biol. 214, 2474-2494

595 Domenici P, Blagburn JM, Bacon JP (2011) Animal escapology I: theoretical issues and
596 emerging trends in escape trajectories. J. Exp. Biol. 214, 2463-2473.

597 Dussauze M, Danion M, Le Floch S, Lemaire P, Pichavant-Rafini K, Theron M (2015a).
598 Innate immunity and antioxidant systems in different tissues of sea bass (*Dicentrarchus*
599 *labrax*) exposed to crude oil dispersed mechanically or chemically with Corexit 9500. Ecotox.
600 Environ. Saf. 120, 270-278.

601 Dussauze M, Pichavant-Rafini K, Le Floch S, Lemaire P, Theron M (2015b). Acute toxicity
602 of chemically and mechanically dispersed crude oil to juvenile sea bass (*Dicentrarchus*
603 *labrax*): Absence of synergistic effects between oil and dispersants. Environ. Toxicol. Chem.
604 34, 1543-1551.

605 Eaton RC, Emberley DS (1991). How stimulus direction determines the trajectory of the
606 Mauthner-initiated escape response in a teleost fish. J. Exp. Biol. 161, 469-487.

607 FAO (2014). http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/fr#tcNA0053

608 Farell AP, 2011. Encyclopedia of fish physiology: from genome to environment academic
609 press, Ottawa, Canada.

610 Faucher K, Fichet D, Miramand P, Lagardère JP (2006) Impact of acute cadmium exposure
611 on the trunk lateral line neuromasts and consequences on the “C-start” response behaviour of
612 the sea bass (*Dicentrarchus labrax L.; Teleostei, Moronidae*). Aquatic Tox. 76, 278–294

613 Faucher K, Fichet D, Miramand P, Lagardère JP (2008) Impact of chronic cadmium exposure
614 at environmental dose on escape behaviour in sea bass (*Dicentrarchus labrax L.; Teleostei,*
615 *Moronidae*). Environ. Poll. 151, 148-157

616 Fusey P, Oudot J (1976) Comparaison de deux méthodes d'évaluation de la biodégradation
617 des hydrocarbures in vitro. Mater Org. (Berlin) 4, 241–51.

618 Geraudie P, Bakkemo R, Milinkovitch T, Thomas-Guyon H (2016) First evidence of marine diesel
619 effects on biomarker responses in the Icelandic scallops, *Chlamys islandica*. Environ. Sci. Pol. Res.
620 23, 504-512

621 GESAMP (2007) (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP Joint Group
622 of Experts on the Scientific Aspects of Marine Environmental Protection). 75, 1-96.

623 Gravato LC, Santos MA (2003) Genotoxicity biomarkers' association with B(a)P
624 biotransformation in *Dicentrarchus labrax*. Ecotoxicol. Environ. Saf. 55, 352–358.

625 Gonçalves R, Scholze M, Ferreira AM, Martins M, Correia AD (2008) The joint effect of
626 polycyclic aromatic hydrocarbons on fish behavior. Environ. Res. 108, 205–213

627 Harrison D, Kanji GK (1988) The development of analysis of variance for circular data. J.
628 Appli. Stat. 15, 197.

629 Husak JF (2006) Does survival depend on how fast you can run or how fast you do run?
630 Funct. Ecol. 20, 1080–1086.

631 Johansen JL, Allan BJM, Rummer JL, Esbaugh AJ (2017) Oil exposure disrupts early life-
632 history stages of coral reef fishes via behavioural impairments. Nature ecol. evol. 1, 1146–
633 1152

634 Kodama and Horiguchi (2011) Effects of hypoxia on benthic organisms in Tokyo Bay, Japan:
635 a review. Mar. Pol. Bul. 63, 215–220.

636 Kopecka-Pilarczyk J, Correia AD (2009) Biochemical response in gilthead seabream (*Sparus*
637 *aurata*) to in vivo exposure topyrene and fluorene. J. Exp. Mar. Biol. Ecol. 372, 49–57

638 Lanczos C (1956) Applied Analysis. Prentice Hall, Eaglewood Cliffs.

639 Langangen Ø, Olsen E, Stige LC, Ohlberger J, Yaragina NA, Vikebø FB , Bogstadb B,
640 Stenseth NC, Hjermann DØ (2017). The effects of oil spills on marine fish: Implications of
641 spatial variation in natural mortality. Mar. Pol. Bul. 119, 102-109.

642 Lefrançois C, Domenici P (2006) Locomotor kinematics and behaviour in the escape response
643 of European sea bass, *Dicentrarchus labrax* L., exposed to hypoxia. Mar. Biol. 149, 969–977

644 Lefrançois C, Shingles A, Domenici P (2005) The effect of hypoxia on locomotor
645 performance and behaviour during escape in *Liza aurata*. J. Fish Biol. 67, 1711–1729

646 Lewis A, Daling P (2001) Oil spill dispersants. (ed A. L. O. S. Consultant), pp. 28. SINTEF,
647 Staines.

648 Marras S, Killen SS, Claireaux G, Domenici P, McKenzie DJ (2011). Behavioural and
649 kinematic components of the fast-start escape response in fish: Individual variation and
650 temporal repeatability. J. Exp. Biol. 214, 3102-3110.

651 Mager EM, Pasparakis C, Stieglitz JD, Hoenig R, Morris JM, Benettia DD, Grosella M
652 (2018) Combined effects of hypoxia or elevated temperature and Deepwater Horizon crude
653 oil exposure on juvenile mahi-mahi swimming performance. Mar. Env. Res. 139, 129-135

McKenzie DJ, Shingles A, Claireaux G, Domenici P. Sublethal Concentrations of Ammonia Impair Performance of the Teleost Fast-Start Escape Response. Physiol. Biochem. Zool. 82, 353-36

Milinkovitch T, Sanchez W, Ndiaye A, Le Floch S, Thomas-Guyon H (2011a). Liver antioxidant responses and innate immune function in golden grey mullet (*Liza aurata*) following exposure to dispersed crude oil. Aquat.Toxicol. 101, 155–164.

Milinkovitch T, Godefroy J, Théron M, Thomas-Guyon H (2011b). Toxicity of dispersant application: biomarkers responses in gills of juvenile golden grey mullet (*Liza aurata*). Environ. Pol. 159, 2921–2928.

Milinkovitch T, Kanan R, Thomas-Guyon H, Le Floch S (2011c).Effects of dispersed oil exposure on the bioaccumulation of polycyclic aromatic hydrocarbons and the mortality of juvenile *Liza ramada*. Sci. Tot. Env. 409, 1643–1650.

Milinkovitch T, Lucas J, Le Floch S, Thomas-Guyon H, Lefrançois C (2012) Effect of dispersed crude oil exposure upon the aerobic metabolic scope in juvenile golden grey mullet (*Liza aurata*). Mar. Pol. Bul. 64, 865–871.

Milinkovitch T, Imbert N, Sanchez W, LeFloch S, Thomas-Guyon H (2013a) Toxicological effects of oil dispersant use in coastal waters: biomarker approach in the hearts of juvenile golden grey mullets (*Liza aurata*). Ecotoxicol. Environ. Saf. 88, 1–8.

Milinkovitch T, Lefrançois C, Thomas-Guyon H, Imbert N (2013b) Dispersant use as a response technique to oil spill: toxicological effectson fish cardiac performance. Fish Physiol. Biochem. 39, 257–262.

675 Milinkovitch T, Geraudie P, Camus L, Huet V, Thomas-Guyon H (2015) Biomarker
676 modulation associated with marine diesel contamination in the Iceland scallop (Chlamys
677 islandica). Environ. Sci. Pol. Res. 22, 19292-19296

678 Negreiros LA, Silva BF, Paulino MG, Fernandes MN, Chippari-Gomes AR (2011) Effects of
679 hypoxia and petroleum on the genotoxic and morphological parameters of *Hippocampus*
680 *reidi*. Comp. Biochem. Physiol. Part C 153, 408–414.

681 Neff JM (2002) Polycyclic aromatic hydrocarbons in the aquatic environment: sources, fate
682 and biological effects. Applied Science publishers, London

683 Pihl L, Baden SP, Diaz RJ (1991) Effects of periodic hypoxia on distribution of demersal fish
684 and crustaceans. Mar. Biol. 108, 349-360

685 Rabalais NN, Turner RE (2013) Coastal Hypoxia: Consequences for Living Resources and
686 Ecosystems. American Geophysical Union, Washington DC.

687 Rastgar S, Movahedinia A, Savari A, Zanosi H, Rasoli M, Ardeshir RA (2015). Changes in
688 the Pituitary and hypothalamus Monoaminergic Neurotransmitters after Acute and Prolonged
689 Stress Exposure to Benzo(α)Pyrene in *Acanthopagrus latus*. SM J. Environ. Toxicol. 1003, 1-
690 4

691 Rice C, Ghorai JK, Zalewski K, Weber DN (2011) Developmental lead exposure causes
692 startle response deficits in zebrafish. Aquatic Tox. 105, 600– 608

693 Szlinder-Richert J, Nermer T, Szatkowska U (2014) PAH metabolites in European eels
694 (*Anguilla anguilla*) as indicators of PAH exposure: Different methodological approaches

695 Terova G, Rimoldi S, Corà S, Bernardini G, Gornati R, Saroglia M (2008) Acute and chronic
696 hypoxia affects HIF-1α mRNA levels in sea bass (*Dicentrarchus labrax*). Aquaculture 279,
697 150–159.

698 Vieira LR Sousa A, Frasco MF, Lima I, Morgado F, Guilhermino L (2008) Acute effects of
699 Benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus*
700 *microps (Teleostei, Gobiidae)*. Sci. Tot. Environ. 395, 87-100.

701 Vignet C, Trenkel VM, Vouillarmet A, Bricca G, Bégout ML, Cousin X (2017) Changes in
702 Brain Monoamines Underlie Behavioural Disruptions after Zebrafish Diet Exposure to
703 Polycyclic Aromatic Hydrocarbons Environmental Mixtures. Int. J. Mol. Sci. 18, 1-11.

704 Walker JA, Ghalambor CK, Griset OL, Kenney DM, Reznick DN (2005) Do faster starts
705 increase the probability of evading predators? Function. Ecol. 19, 808–815

706 Webb PW, Scales fast-start maneuvres. J. Exp. Biol. 214, 6, 875-877.

707 Weber DN (2006) Dose-dependent effects of developmental mercury exposure on C-start
708 escape responses of larval zebrafish *Danio rerio*. J. Fish Biol. 69, 75–94

709 Wöhl S, Schuster S (2007) The predictive start of hunting archer fish: a flexible and precise
710 motor pattern performed with the kinematics of an escape C-start. J. Exp. Biol. 210, 311-324

711 Worm B, Barbier EB, Beaumont N, Duffy JE, Folke C, Halpern BS, Jackson JBC, Lotze HK,
712 Micheli F, Palumbi SR, Sala E, Selkoe KA, Stachowicz JJ, Watson R (2006). Impacts of
713 Biodiversity Loss on Ocean Ecosystem Services. Science. 314, 787-790.

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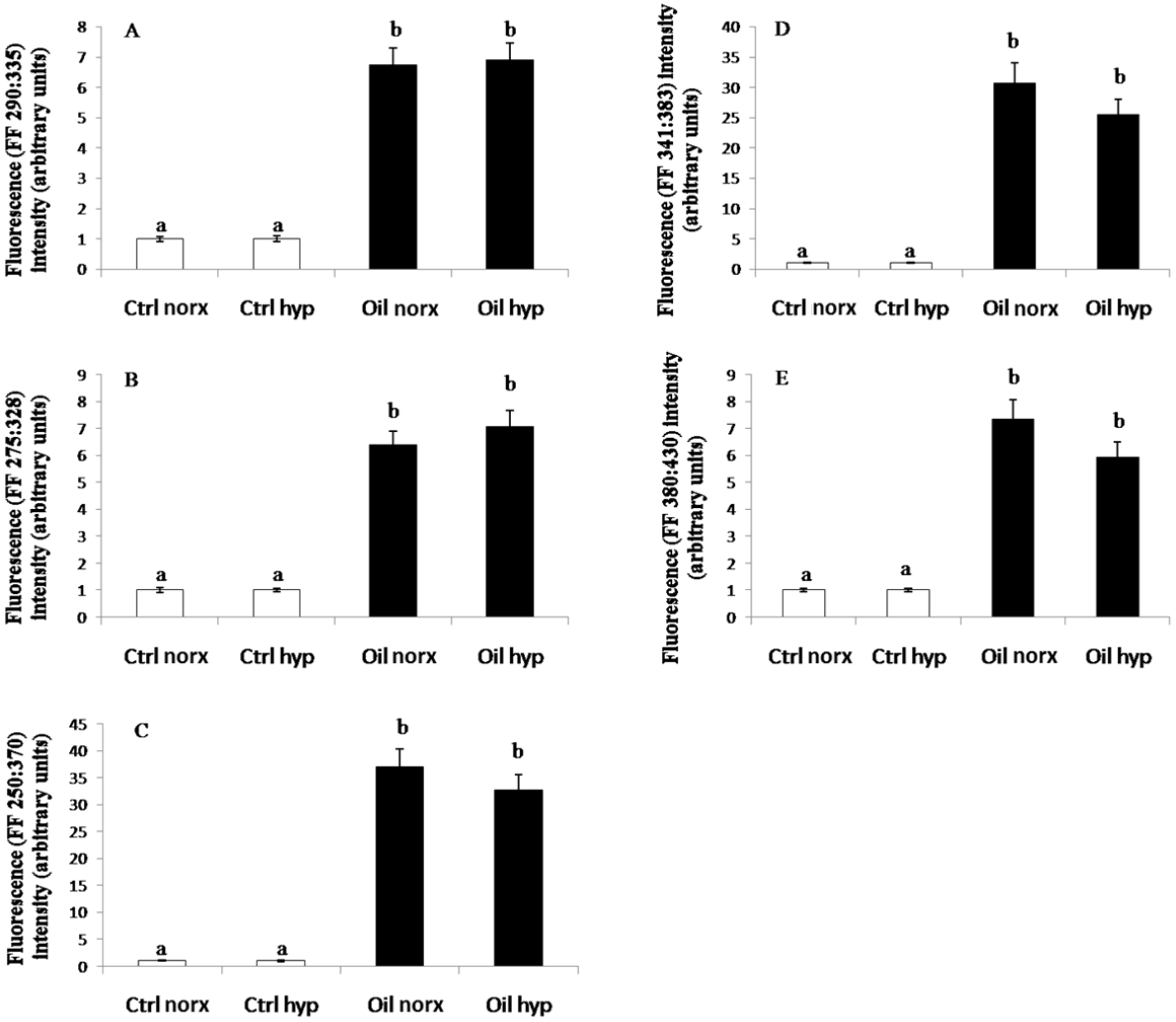


Figure 1: Relative concentration of biliary PAH metabolites measured by fixed wavelength fluorescence (FF) levels for each category of fish: fish exposed to seawater and challenged in normoxic condition (Ctrl norx); fish exposed to seawater and challenged in hypoxic condition (Ctrl hyp); fish exposed to oil and challenged in normoxic condition (Oil norx); fish exposed to oil and challenged in hypoxic condition (Oil hyp) (A) FF 290:335 (naphthalene type derived metabolites); (B) FF 275:328 (fluorene type derived metabolites); (C) FF 250:370 (phenanthrene type derived metabolites); (D) FF 341:383 (pyrene type derived metabolites); (E) FF 380:430 (benzo[a]pyrene derived type of metabolites). Levels are expressed as fluorescence intensity. Values represent means ± standard errors. Different letters above bars indicate a significant difference, where $P < 0.01$.

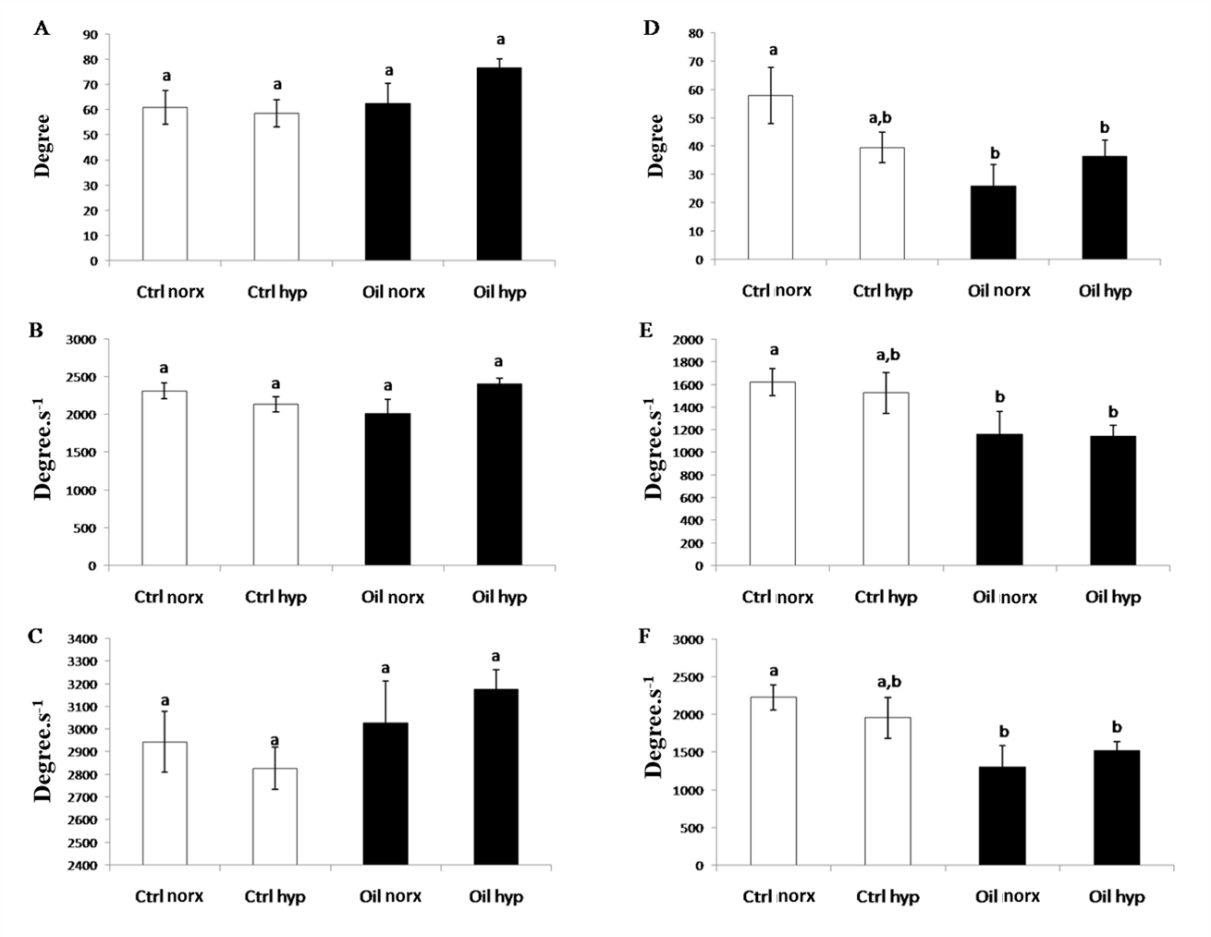
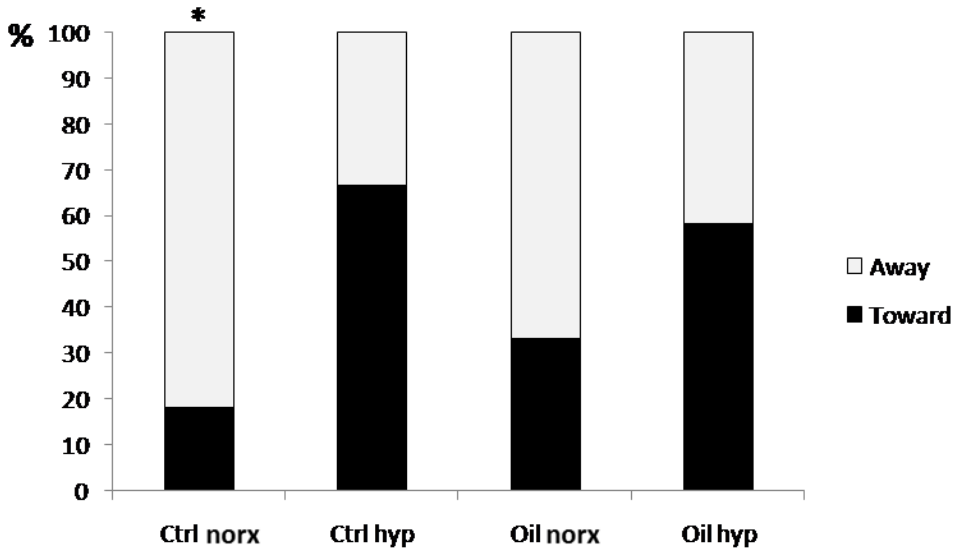


Figure 2: Maneuverability variables for each category of fish during S1 and S2 stages: (A) S1 angle expressed in degree; (B) S1 average turning rate expressed in degree per second; (C) S1 maximum turning rate expressed in degree per second; (D) S2 angle expressed in degree; (E) S2 average turning rate expressed in degree per second; (F) S2 maximum turning rate expressed in degree per second. Parameters are evaluated during the mean time for tested fish to complete the escape response (55.4 ms). Values represent mean ± standard error. Different letters above bars indicate a significant difference, where $P < 0.05$.



735

736 **Figure 3:** Escape response directionality (black bars represent toward responses; white bars
737 represent away responses) expressed in percentage for each category of fish. *indicates a
738 toward/away ratio significantly different from 50:50.

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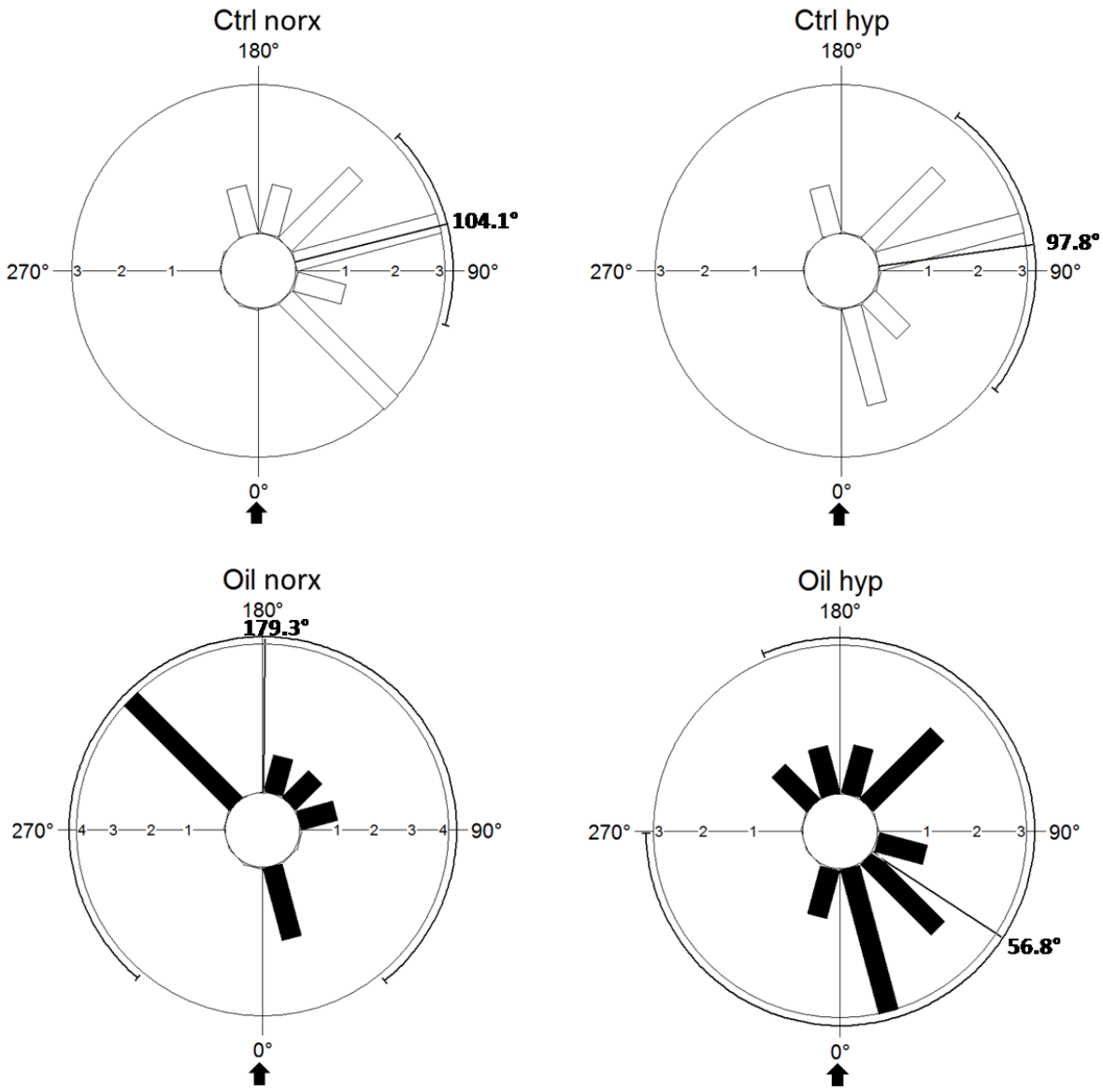
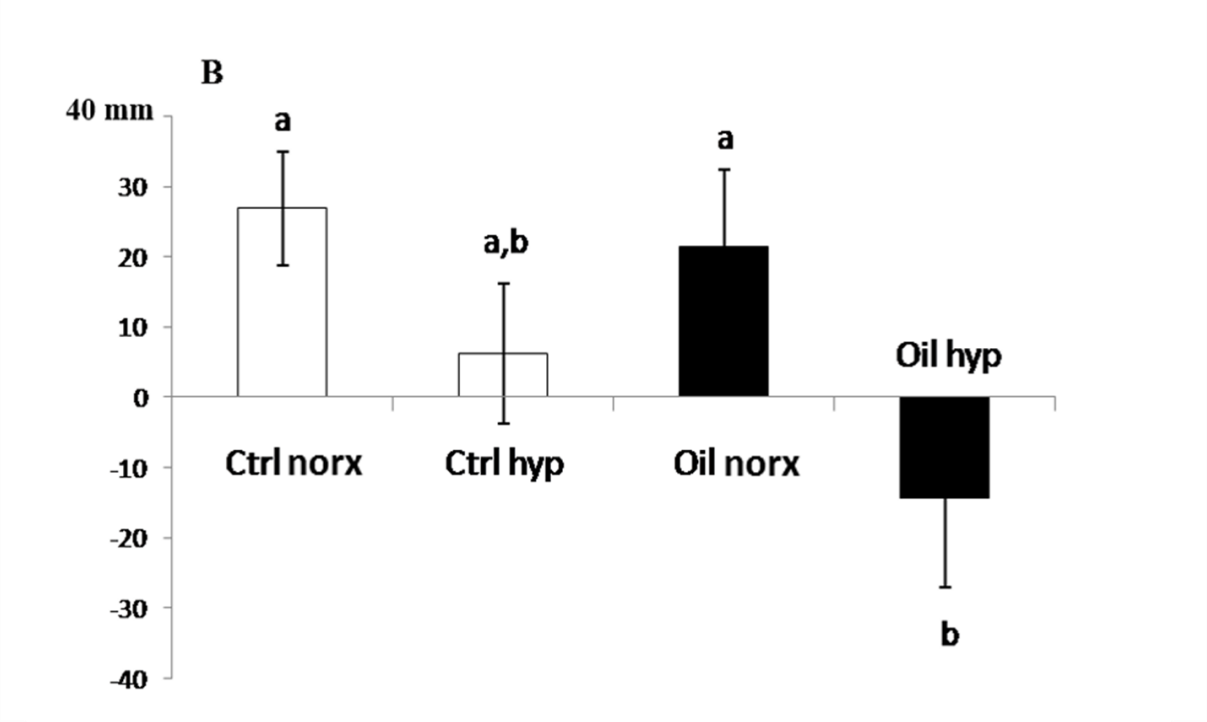


Figure 4: Frequency distribution of escape trajectory plotted on 360° (divided in 12 section of 30°). The black radius represents the mean of the values in degree (also written) and the circular black error bars represent 95 % confidence intervals.



744
745 **Figure 5:** Distancing. Parameters are evaluated during the mean time for tested fish to
746 complete the escape response (55.4 ms). Values are expressed in mm and represent mean ±
747 standard error. Different letters above bars indicate a significant difference, where $P < 0.05$.

748