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1 **The effect of hypoxia and hydrocarbons on the anti-predator**
2 **performance of European sea bass (*Dicentrarchus labrax*)**

3
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13
14 **Abstract (294 words)**

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

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

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

39 **Capsule:**

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

42

43 **1. Introduction**

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

46 They also have been described as highly productive areas contributing to the economic value
47 of marine environments (Costanza et al., 1997). However, these ecosystems are under
48 multiple anthropogenic stressors. Among these stressors, hypoxia is in constant expansion
49 since the sixties and nowadays there are more than 400 hypoxic coastal ecosystems
50 distributed world-wide (Diaz and Rosenberg, 2008). Thus, a large body of literature has been
51 devoted to testing the effects of hypoxia on several organisms (e.g. Domenici et al., 2007;
52 Cheung et al., 2008; Kodama and Horiguchi, 2011; Cook et al., 2014; Rabalais and Turner,
53 2013).

54 Petrogenic hydrocarbons are also considered as some of the most important stressors
55 of coastal ecosystems and are present all over the world (GESAMP, 2007). The effects of
56 petroleum on marine flora and fauna have been extensively described at different levels of
57 biological organization (recently reviewed by Beyer et al., 2016 and Bejarano and Michel,
58 2016). Most of the current research has investigated the effects of hydrocarbons at the cellular
59 level through the assessment of biomarkers (eg: Milinkovitch et al., 2011, a, b, c, 2015;
60 Danion et al., 2014; Dussauze et al., 2015, a, b; Geraudie et al., 2016) and more recently,
61 studies were conducted at the individual and the population levels (e.g. Davoodi and
62 Claireaux, 2007; Milinkovitch et al., 2012 and Langangen et al., 2017, respectively).

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

71 Given the limited knowledge on the biological effects of hypoxia and petroleum and
72 considering the main goal of the Marine Directive (i.e. to achieve Good Environmental Status
73 of EU marine waters by 2020), this study aims at testing the biological effects of hypoxia in
74 fish contaminated by realistic exposure of petrogenic hydrocarbons. Specifically, this is the
75 first study to test the cumulative and separated effects of hypoxia and petroleum on behavioral
76 and kinematic parameters of the fish escape response. Fish escape responses have been
77 described as “high accelerations, often accompanied by a change in direction” (Domenici et
78 al., 2011) aimed at avoiding predators. This response is therefore fundamental for survival
79 and is consequently an important proxy of fitness (Walker et al., 2005). Thus, by measuring
80 alterations of behavioral and locomotor parameters at the individual level, this study also aims
81 at providing an insight of the impact of oil and hypoxia on fish fitness. In parallel, chemical
82 parameters - the polycyclic aromatic hydrocarbons (PAHs), the total petroleum hydrocarbons
83 (TPHs) and the PAHs biliary metabolites concentrations – will permit to link the biological
84 effects observed in fish with the exposure and the incorporation of contaminants. TPHs were
85 measured since they represent the whole hydrocarbons *i.e.* the polar compounds, the saturated
86 and the polycyclic hydrocarbons. PAHs were measured since they are considered to be the
87 most toxic compounds among hydrocarbons in accordance with the literature (review in Neff,
88 2002) and the United States Environmental Protection Agency (USEPA).

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

94

95 **2. Materials and Methods**

96 **2.1. Experimental animals**

97 Juvenile European seabass (*Dicentrarchus labrax*) were provided by Aquanord Ichthus
98 (Gravelines, France). During 3 weeks, 90 fish were kept in 6 tanks of 300 L (15 fish per tank;
99 tanks similar than those used for the experiment) under natural photoperiod, with open
100 circulation of sea water (free of nitrites and nitrates). Temperature, salinity, pH and dissolved
101 oxygen concentration were monitored every day and values were respectively 20.12 ± 0.19
102 $^{\circ}\text{C}$, 35 ± 0.1 ‰, 7.95 ± 0.01 and 93.59 ± 0.62 % of air saturation (mean \pm standard error of the
103 mean). Fish were fed daily with fish food (Neostart, Le Gouessant) and starved for 48 hours
104 before the experiment. Fish, in their second year, showed an average length and weight of
105 16.44 ± 0.08 cm and 49.79 ± 0.64 g, respectively. Fifty six fish were used for the experiment.

106 **2.2. Exposure to contaminants**

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

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

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

120 concentration measured at three different depths within the experimental tanks). After this
121 period, every day, two juvenile fish were introduced in the oil treatment tank and two juvenile
122 fish were introduced in the control treatment tank, for an 18 hours exposure. The operation
123 was repeated 14 times (7 times with the oil treatment and 7 times with control treatment with
124 randomized order) so that 28 fish were exposed to the oil treatment and 28 fish to the control
125 treatment. A 300 watts HYDOR™ resistance was used to maintain the temperature at $19.89 \pm$
126 0.13 °C. In parallel, pH and dissolved oxygen were maintained at 8.09 ± 0.01 and 99.9 ± 0.47
127 % of air saturation, respectively.

128 **2.3. Total petroleum hydrocarbons (TPHs) measurement**

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

139

140 **2.4. Escape response under hypoxia or normoxia**

141 **2.4.1. Experimental set-up and protocol**

142 After the contamination period (described in 2.2), oil-contaminated and uncontaminated fish
143 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 (t_1) with the first detectable movement of the head. It
195 ends with the beginning of stage 2 (t_2), 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 (t_3) (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 t_1 and t_3 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 (t_0) and the first
205 detectable movement of the head of the fish (t_1)

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

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

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

227 -Escape trajectory is expressed in degree and ranged between 0 and 360° (Domenici et al.
228 2011a, 2011b). This circular data is measured as the angle between the line passing through
229 the stimulus position and CM at t0 and the anterior body midline at t3. By convention,
230 stimulus position was always considered to be on the right side of the fish at the onset of the
231 response (Domenici and Blake 1993).

232

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

239

240 **2.5. Fixed wavelength fluorescence analysis of bile**

241 Bile samples were diluted (1:500) in absolute ethanol (Sigma Aldrich, France) and assays, in
242 quartz cuvettes (Sigma Aldrich, USA) on a spectrofluorimeter (SAFAS Flx-Xenius, Monaco),
243 were conducted for five fixed wavelength fluorescence (FF). According to Aas et al. (2000),
244 naphthalene-derived metabolites were detected for the emission:excitation wavelength pairs
245 290:335 , pyrene-derived metabolites for 341:383 and benzo[a] pyrene-derived metabolites
246 for 380:430. Moreover, fluorene type derived metabolites were detected at 275:328 and
247 phenanthrene type derived metabolites at 250:370, according to Kopecka-Pilarczyk and
248 Correia (2009) and Szlinder-Richert et al. (2014), respectively. Obtained values were
249 expressed as arbitrary units of fluorescence.

250 **2.6. Statistical analysis**

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

263 1981) were employed to highlight significant non-random circular distribution of trajectories
264 for each group of fish. Subsequently, a two-way ANOVA for circular data (based on Harrison
265 and Kanji, 1988; Berens, 2009) were conducted in order to compare the different angles of
266 escape trajectories for the different group of fish. A Mann-Whitney U test was used to
267 highlight statistical differences between the TPH concentrations at T=0h and at T=18h.
268 Statistica™ software was used for all analysis except for circular statistics (escape
269 trajectories) for which Oriana™ software was used. Significance was accepted at P<0.05.
270 Results are expressed as mean ± standard error of the mean, except for escape trajectories
271 (expressed as mean ± 95% confidence interval).

272

273 **3. Results**

274 **3.1. Hydrocarbon exposure and organism incorporation**

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

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

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

288 **3.2. Fast start performance**

289 **3.2.1. Escape response duration, responsiveness, latency and distance-time performances**

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

295 **3.2.2. Maneuverability, directionality and escape trajectories**

296 Maneuverability parameters were measured for S1 and S2. S1 turning angles (**Figure 2A**)
297 ranged between 58.6 ± 5.4 degrees in Ctrl hyp and 76.6 ± 3.7 degrees in Oil hyp. S1 average
298 turning rates (**Figure 2B**) ranged between 2408.2 ± 75.4 in Oil hyp and 2013.0 ± 186.4
299 $\text{degrees}\cdot\text{s}^{-1}$ in Oil norx group. S1 maximum turning rate (**Figure 2C**) was 2944.4 ± 133.5
300 $\text{degrees}\cdot\text{s}^{-1}$ for the control group. For these parameters, no effects of stressors was observed
301 ($P>0.05$).

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

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

311 S2 average and maximum turning rates for Ctrl norx groups were 1623.6 ± 121.7 and 2229.2
312 ± 162.6 degrees.s⁻¹, respectively and two-way ANOVA revealed an effect of oil ($P<0.05$).
313 More specifically, a similar pattern of results was observed for S2 average and maximum
314 turning rates (**Figure 2E and 2F**) as for S2 angle. Indeed, post-hoc tests showed that turning
315 rates were significantly lower for contaminated groups, when compared to Ctrl norx group
316 ($P<0.05$) while no significance was observed when comparing contaminated fish under
317 normoxia with contaminated fish under hypoxia ($P>0.05$).

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

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

326 The mean escape trajectories of Ctrl norx and Ctrl hyp groups were 104.1 ± 31.2 (95%
327 confidence interval) and 97.8 ± 45.4 (95% confidence interval) degrees, respectively.
328 Contaminated fish (*i.e.* Oil norx and Oil hyp) showed average values of $179.3 \pm$ degrees and
329 56.8 degrees, respectively. Two-way ANOVA for circular data did not show significant
330 difference between fish groups.

331 **3.2.3. Distancing (Figure 5)**

332 Uncontaminated fish under normoxia (Ctrl norx) distance themselves from the stimulus by
333 26.8 ± 8.11 mm. Distancing for uncontaminated fish under hypoxia (Ctrl hyp) and for
334 contaminated fish under normoxia (Oil norx) were respectively 6.1 ± 10.0 mm and $21.4 \pm$
335 10.8 mm. Oil hyp fish get closer to the stimulus during escape response with a negative value
336 of distancing (-14.4 ± 12.6 mm). Statistics revealed no effect of the contamination nor the
337 hypoxia but an effect of the interaction of stressors with a significance between Oil hyp and
338 Ctrl groups ($P < 0.05$).

339

340 **4. Discussion**

341 **4.1. Exposure to hydrocarbons in normoxic or hypoxic conditions and metabolites**

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

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

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

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

381 **4.2. Effects of hypoxic conditions and hydrocarbons on responsiveness and response**
382 **latency**

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

397 Our study showed no effect of hydrocarbons (interacting or not with hypoxia) on latency and
398 responsiveness. Comparison with previous studies is limited since little is known on the effect
399 of hydrocarbons on fish behaviour. Gonçalves et al. (2008) and Johansen et al. (2017)
400 revealed an effect of hydrocarbons upon spontaneous locomotor activity in juvenile sea bream
401 (*Sparus aurata*) and upon sheltering and shoaling in coral reef fishes, respectively, however
402 no study has yet investigated the effect of hydrocarbons on escape response. Other pollutants
403 such as metallic compounds have been shown to affect responsiveness and latency in seabass
404 (Faucher et al., 2006, 2008) and in zebrafish (Weber, 2006). In seabass, these authors linked
405 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

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

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

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

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

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

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

460 **4.4 Effects on distance, time and distance-time variables**

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

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

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

487 **4.5. Distancing**

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

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

506 **Conclusion**

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

526

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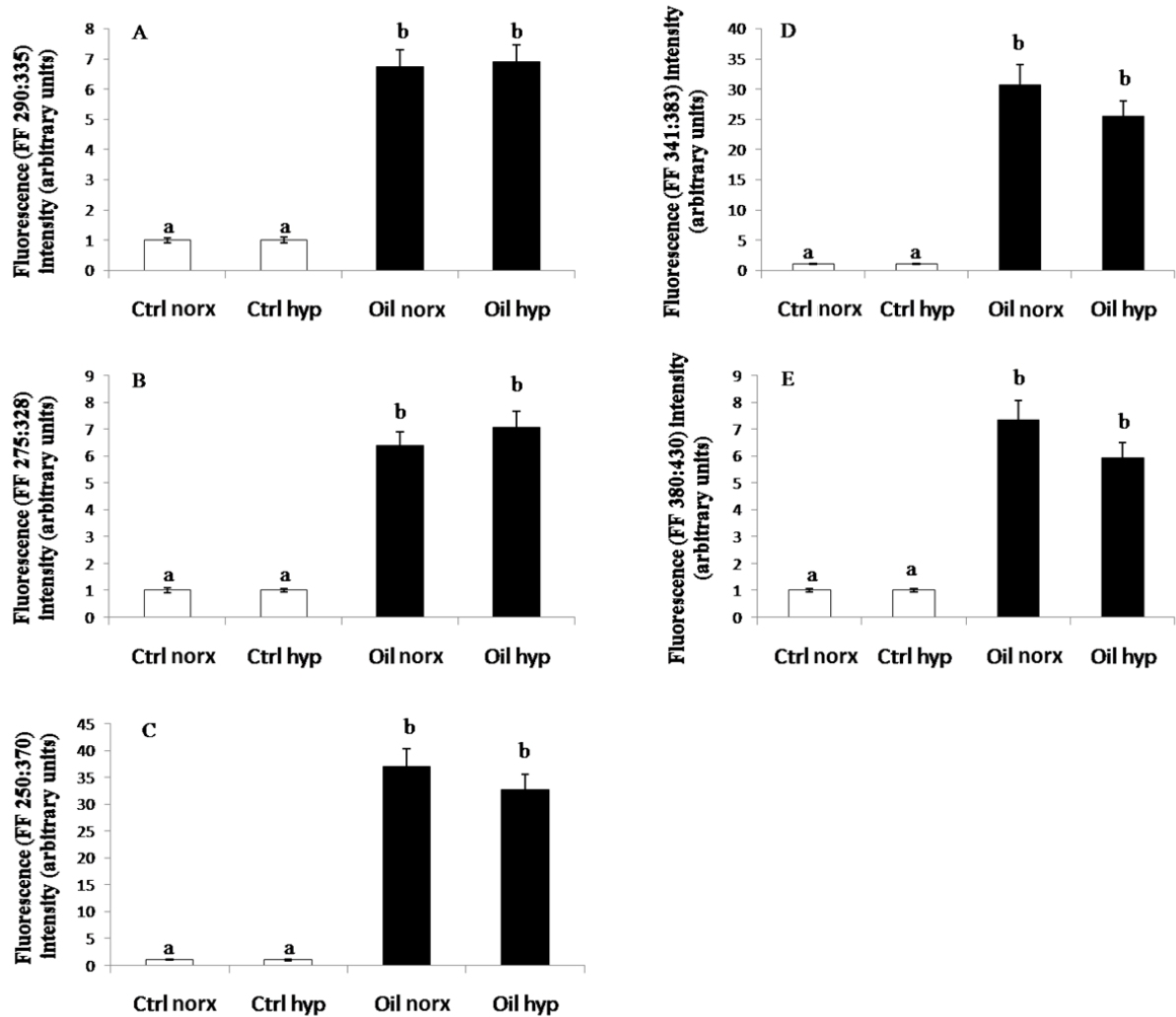
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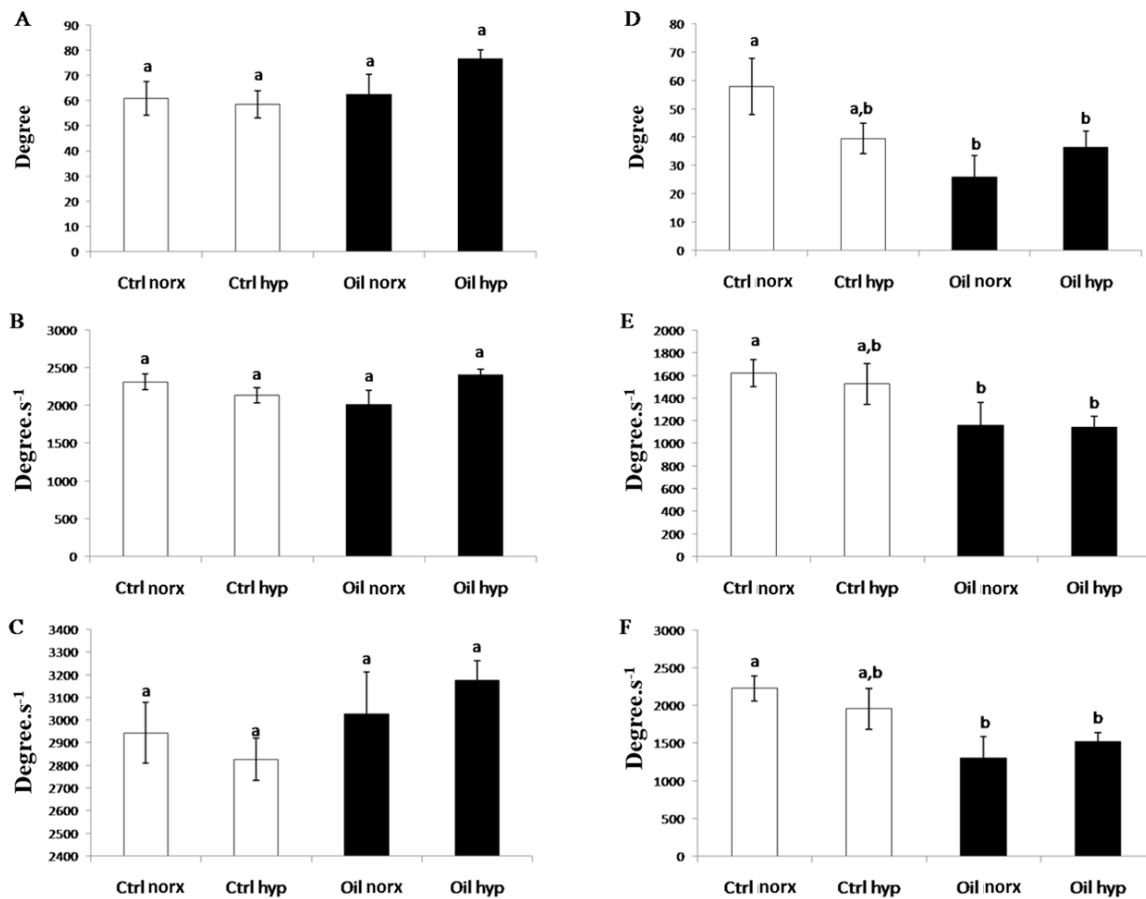
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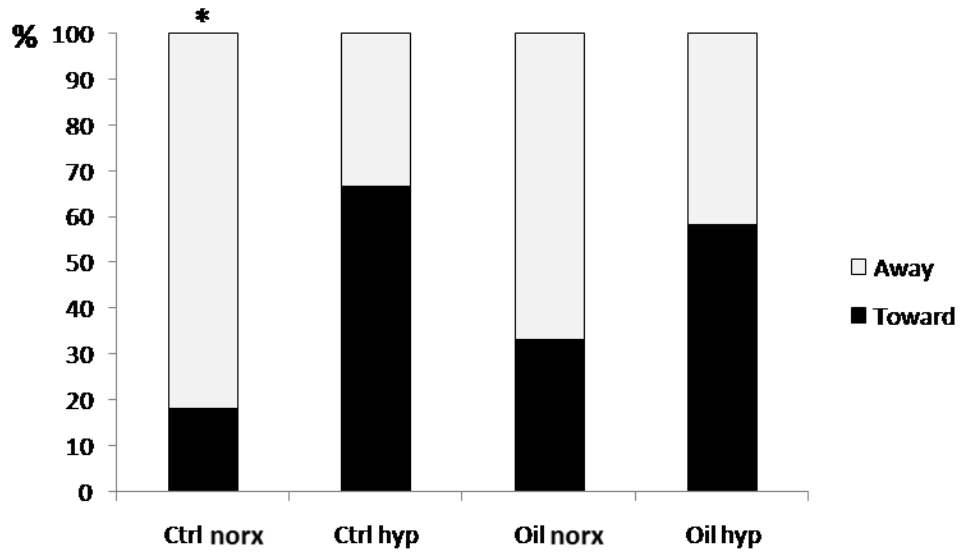


715
 716 **Figure 1:** Relative concentration of biliary PAH metabolites measured by fixed wavelength
 717 fluorescence (FF) levels for each category of fish: fish exposed to seawater and challenged in
 718 normoxic condition (Ctrl norx); fish exposed to seawater and challenged in hypoxic condition
 719 (Ctrl hyp); fish exposed to oil and challenged in normoxic condition (Oil norx); fish exposed
 720 to oil and challenged in hypoxic condition (Oil hyp) (A) FF 290:335 (naphthalene type
 721 derived metabolites); (B) FF 275:328 (fluorene type derived metabolites); (C) FF 250:370
 722 (phenanthrene type derived metabolites); (D) FF 341:383 (pyrene type derived metabolites);
 723 (E) FF 380:430 (benzo[a]pyrene derived type of metabolites). Levels are expressed as
 724 fluorescence intensity. Values represent means \pm standard errors. Different letters above bars
 725 indicate a significant difference, where $P < 0.01$.

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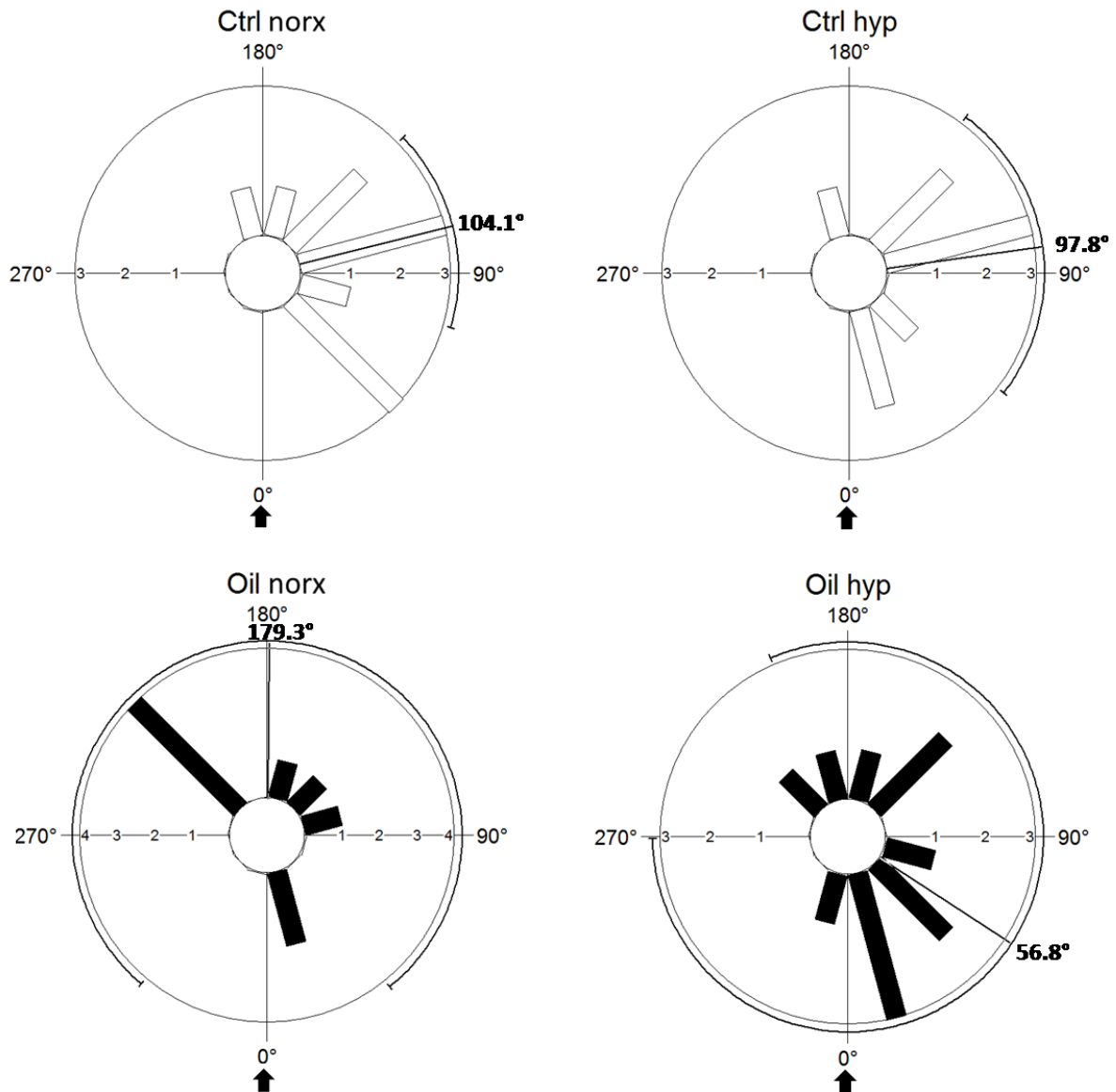
727
 728 **Figure 2:** Maneuverability variables for each category of fish during S1 and S2 stages: (A) S1
 729 angle expressed in degree; (B) S1 average turning rate expressed in degree per second; (C) S1
 730 maximum turning rate expressed in degree per second; (D) S2 angle expressed in degree; (E)
 731 S2 average turning rate expressed in degree per second; (F) S2 maximum turning rate
 732 expressed in degree per second. Parameters are evaluated during the mean time for tested fish
 733 to complete the escape response (55.4 ms). Values represent mean \pm standard error. Different
 734 letters above bars indicate a significant difference, where $P < 0.05$.



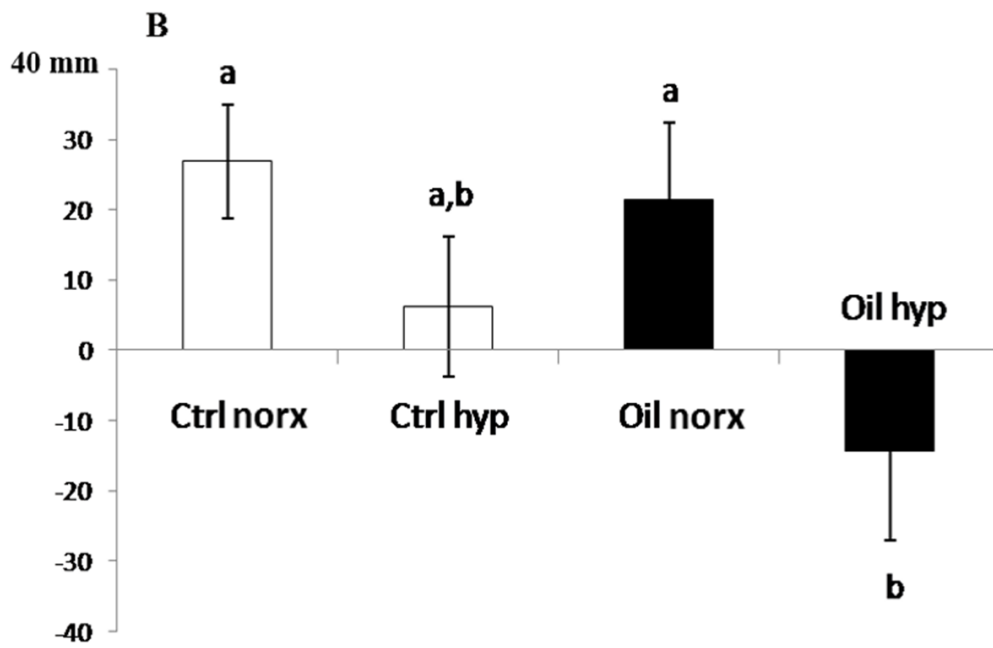
735

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.

739



740
 741 **Figure 4:** Frequency distribution of escape trajectory plotted on 360° (divided in 12 section of
 742 30°). The black radius represents the mean of the values in degree (also written) and the
 743 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 \pm
 747 standard error. Different letters above bars indicate a significant difference, where $P < 0.05$.

748