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Influence of crude oil exposure on cardiac function and thermal tolerance of juvenile rainbow trout and European sea bass

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Abstract :

Oil spills pose a threat to aquatic organisms. However, the physiological effects of crude oil on cardiac function and on thermal tolerance of juvenile fish are still poorly understood. Consequently, in this paper, we will present results of two separate experiments where we exposed juvenile rainbow trout and European sea bass to crude oil and made cardiac thermal tolerances and maximum heart rate (fHmax) measurements after 1 week (rainbow trout) and 6-month recovery (sea bass). In both species, the fHmax was lower in crude oil-exposed fish than in the control ones at temperatures below the optimum but this difference disappeared at higher temperatures. More importantly, the oil-exposed fish had significantly higher Arrhenius break point temperature for fHmax, which gave an estimate for optimum temperature, than the control fish in both species even though the exposure conditions and recovery times differed between species. The results indicated that exposure of juvenile fish to crude oil did not have a significant negative impact upon their cardiac performance in high temperatures and upper thermal tolerance increased when the fish were tested 1 week or 6 months after the exposure. Our findings suggest that the cardiac function and thermal tolerance of juvenile fish are relatively resistant to a crude oil exposure.

Keywords : Arrhenius break point temperature, Critical thermal maximum, CTMAX, Fish, Heart rate, Oil spill, PAH

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40

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46 project FishHealth are also acknowledged.

47 **Introduction**

48

49 Accidental oil spills and crude oil exposure represent a threat to aquatic environments
50 worldwide, affecting not only aquatic animals but also human activities (e.g. fisheries,
51 aquaculture and tourism) (Endersen et al. 2003; Meski and Kaitaranta 2014). Previous studies
52 have shown that fish embryos and larvae are particularly sensitive to crude oil and its
53 components, especially polycyclic aromatic hydrocarbons (PAH). The heart is a particularly
54 sensitive organ, with numerous reports associating crude oil exposure with malformations
55 such as reduced cardiac looping and pericardial edema in developing fish (Thomaz et al.
56 2009; Incardona et al. 2009, 2012, 2014; Jung et al. 2013). Cardiac dysfunctions such as
57 reduced ventricular contractility (Jung et al. 2013), increased occurrence of arrhythmias and
58 variability of heart rate have also been reported in embryos (Incardona et al. 2009, 2012,
59 2014, Jung et al. 2013; Sørhus et al. 2016; Khursigara et al. 2017). Furthermore, it has been
60 shown that a year after embryonic stages have been exposed to petroleum hydrocarbon,
61 young fish still exhibit misshaped hearts and lower critical swimming velocities (U_{CRIT}) than
62 unexposed control fish (Hicken et al. 2011).

63

64 When comparing embryos to juveniles or adult fish, it seems that juveniles and adults might
65 be less dramatically affected by exposure to petroleum hydrocarbons than younger life stages,
66 although a large interspecific variability has been reported (e.g. Vosyliene et al. 2005;
67 Davoodi and Claireaux 2007; Claireaux and Davoodi 2010; Milinkovitch et al. 2012;
68 Claireaux et al. 2013). For example, common sole (*Solea solea*) and adult mahi-mahi
69 (*Coryphaena hippurus*) showed reduced cardiac output when measured shortly after an
70 exposure to crude oil (Davoodi and Claireaux 2007; Nelson et al. 2016). Johansen and
71 Esbaugh (2017) observed that a 24h acute exposure to $4.1 \mu\text{g L}^{-1}$ ΣPAH reduced U_{CRIT} and

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72 burst swimming capacity of adult red drum (*Sciaenops ocellatus*) while there was no change
73 in aerobic scope, cost of transport and in the capacity to repay oxygen debt following
74 exhaustive exercise. Brette et al. (2014) exposed isolated cardiomyocytes of juvenile bluefin
75 tuna (*Thunnus orientalis*) and yellowfin tuna (*Thunnus albacares*) to dilutions (20%, 10%
76 and 5%) of high-energy water-soluble fraction (WAF) of oil samples collected from the
77 *Deepwater Horizon* spill. These authors observed impaired cardiomyocyte function during
78 the exposure, with reduced amplitude and tail current of the delayed rectifier potassium
79 current, resulting in prolonged action potential duration (Brette et al. 2014). It must be noted,
80 however, that the lack of effects has also been reported in the literature. For instance, aerobic
81 scope, basal and active metabolic rates as well as U_{CRIT} were unchanged in juvenile golden
82 grey mullet (*Liza aurata*) 24h after oil exposure (Milinkovitch et al. 2012). Claireaux et al.
83 (2013) showed that 1 week after an exposure to crude oil or to chemically dispersed oil,
84 juvenile European seabass (*Dicentrarchus labrax*) displayed reduced hypoxia tolerance
85 (incipient lethal oxygen saturation) and thermal sensitivity (critical thermal maximum,
86 CT_{MAX}) compared to unexposed, control fish. These differences were, however, no longer
87 observed 4 weeks (Claireaux et al. 2013) or 10 months post-exposure (Mauduit et al. 2016).
88
89 Besides crude oil, fish are also exposed to other environmental changes in nature. One of
90 these is the ongoing climate change. Anthropogenic activities are changing the earth's
91 climate and water temperature has progressively increased around the world. This increase is
92 directly affecting the physiology of ectothermic fish and it is expected to have detrimental
93 effects if populations cannot migrate to new areas or increase their thermal tolerance via
94 phenotypic plasticity or adaptation through evolution by natural selection (Parmesan 2006;
95 BACC Author team 2008; Belkin 2009; Pörtner 2010; Marshall et al. 2014). The question is,
96 thus, to examine whether exposure to petroleum hydrocarbon compounds affects the capacity

1 97 of fish to face warming events, such as heat waves for instance, the occurrence of which is
2 98 predicted to increase with climate change (e.g. Teng et al. 2016). In this paper, we
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4 99 investigated the general nature of the consequences of crude oil-exposure on cardiac
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7 100 performance and thermal tolerance of juvenile fish. In these experiments, juveniles of two
8
9 101 ecologically different fish species, the freshwater rainbow trout (*Oncorhynchus mykiss*) and
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11 102 the seawater seabass were exposed to crude oil of different origin (trout: Russian export blend
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13 103 medium crude oil; seabass: Arabian light crude oil), with (seabass) or without (trout)
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15 104 treatment by dispersant. These species were chosen since they are important
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17 105 fisheries/aquaculture species and are living/reared in areas where the threat from oil accident
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19 106 is especially high (BACC Author team 2008; Marshall et al. 2014). Rainbow trout were
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21 107 studied 1 week post exposure while seabass were examined 6 months post exposure. The
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23 108 hypothesis was that oil exposure reduces the CT_{MAX} and cardiac function of both species and
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25 109 that the effects persist even after the fish have recovered in clean water.
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32 111 **Materials and Methods**

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34 113 This study contains two separate experiments that were done independently from each other,
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36 114 one in Finland with rainbow trout and one in France with seabass. In both experiments the
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38 115 endpoint measurements i.e. CT_{MAX} and maximum heart rate were done similarly and the
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40 116 results were remarkably consistent. Therefore, these separate experiments are shown together
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42 117 as they give a uniform general view of how the crude oil exposure influences cardiac
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44 118 performance and thermal tolerance in juvenile fish. The measuring techniques (i.e. CT_{MAX}
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46 119 and heart rate recordings) were used as screening tools and both techniques provided separate
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48 120 estimates of upper thermal tolerance of fish even though, in both cases, the rate of increase of
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50 121 temperature was higher than in natural situations.
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2 123 Since the experiments were done independently there were some differences between
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5 124 experiments that need to be noted. First, the origin of the crude oil used was different
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7 125 between experiments. Russian Export Blend medium crude oil was used in the rainbow trout
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9 126 study and Arabian light crude oil in the seabass study. Seabass were exposed to a ten times
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11 127 higher oil concentration than rainbow trout, since it had previously been shown that a lower
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13 128 concentration did not have a significant long-term effect on the thermal tolerance of seabass
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15 129 (Claireaux et al. 2013; Mauduit et al. 2016). Further, in seabass experiment chemical
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17 130 dispersant and weathering was used while in the rainbow trout study the crude oil was
18
19 131 weathered. Water Total Petroleum Hydrocarbons (TPH) was analyzed in both experiments
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21 132 whereas PAHs were analyzed in the water in the trout experiment and in the liver in the
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23 133 seabass experiment.
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29 134

30 31 135 **Experiment 1. Rainbow trout**

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34 136 The experiments with rainbow trout (age 1+) were conducted at University of Turku, Finland
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36 137 during summer 2014. The experiments were approved by Finnish Animal Experiment Board
37
38 138 (ESAVI/4068/04.10.07/2013). The rainbow trout were obtained from a nearby commercial
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40 139 fish farm (The College of Fisheries and Environment, Kirjala, Finland) and the fish were
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42 140 transported to University of Turku 4 weeks before experiments. Fish were maintained under
43
44 141 natural photoperiod and temperature (16°C) conditions in their acclimation tanks (500 L).
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46 142 Fish were fed daily with commercial pellets (Raisio Group, Raisio, Finland) and the feeding
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48 143 was ceased 24h before any experiments/manipulations. The mean size of fish did not differ
49
50 144 significantly between experimental groups and the weights and lengths were 4.6 ± 0.5 g and
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52 145 8.2 ± 0.2 cm (fork length) for control rainbow trout, 4.4 ± 0.3 g and 8.1 ± 0.1 cm for oil-exposed
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54 146 rainbow trout.
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148 *Exposures*

149 Russian Export Blend medium crude oil was acquired from Neste Oil (Raisio, Finland) and
150 weathered at 22°C by bubbling air through the oil layer until approximately 10% of the oil
151 mass was lost. Such a treatment of the oil simulated a 12h aging of a slick released at sea
152 (Nordvik 1995). Fish exposures were conducted according to Milinkovitch et al. (2011). Fish
153 were allocated to two subgroups (oil-exposed and control, $n = 60$ fish per group) and
154 transferred from their acclimation tanks to identical, polyethylene tanks (185 L; 3 tanks per
155 treatment) 48h before exposures (biomass per tank 1.46 g L⁻¹). The exposure tanks were
156 equipped with air stones and custom-made mixing system, which allowed full
157 homogenization of water column and kept oxygen level above 80% of air saturation
158 throughout the exposures (see Milinkovitch et al. 2011 for further details about mixing
159 system). Temperature of exposure tanks was kept at fish acclimation temperature (16°C). The
160 exposure was started by pouring 12.5 g of weathered crude oil to the surface of the tanks (i.e.
161 the oil concentration was 0.07 g L⁻¹) and lasted 48h. One liter water samples were taken from
162 the middle of the water column of each tank, at the beginning and at the end of the exposure
163 period. The TPH and PAH concentrations were analyzed from the water samples by Novalab
164 Oy (Karkkila, Finland). Chemical analyses protocols are described in the supplementary
165 materials. The PAHs could not be measured from tissue samples in Novalab Oy. Following
166 exposure rainbow trout were briefly bathed in clean water containing 70 ppm buffered MS-
167 222 and the adipose fin cut to identify the fish later on. After bathing, fish were returned to
168 their initial acclimation tanks so that both control and exposed fish were under garden
169 conditions thereafter. Control fish followed the same protocol except that no chemicals were
170 added to their tank. No mortalities were observed during the exposure and the following
171 week.

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2 173 The pH of the water did not change during the exposure (7.5 ± 0.1) and nitrogen waste
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5 174 concentrations stayed below detection limits (nitrite $<0.25\text{ mg L}^{-1}$, nitrate $<10\text{ mg L}^{-1}$ and
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7 175 ammonium $<0.5\text{ mg L}^{-1}$). The oxygen concentration and water temperatures were $9.2\pm 0.1\text{ mg}$
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10 176 L^{-1} and $16.3\pm 0.05^\circ\text{C}$, respectively.

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14 178 *CT_{MAX} experiments*

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17 179 The first aim of our study was to examine whether a crude oil exposure, followed by a
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19 180 significant recovery period in a clean environment, influenced the upper critical thermal
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22 181 tolerance (CT_{MAX}) of fish. This was verified by exposing control and oil-treated fish to an
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24 182 acutely increasing temperature until the fish could no longer keep an upright position. The
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27 183 value of CT_{MAX} indicated the upper thermal tolerance of a fish beyond which survival was
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29 184 time-limited (Sunday et al. 2015).

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34 186 The CT_{MAX} experiments with rainbow trout were conducted 7 days after the oil exposure
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36 187 according to Anttila et al. (2013b). Briefly, both control and exposed rainbow trout ($n = 15$
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39 188 per group) were transferred to the experimental tank (100 L, 16°C) 1h before the experiment
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41 189 started. At the end of the acclimation period, the temperature of the water was increased at a
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44 190 constant rate of $0.3^\circ\text{C min}^{-1}$ up to 24°C and by $0.1^\circ\text{C min}^{-1}$ thereafter until the fish lost
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46 191 equilibrium. Water temperature was controlled with a circulating 2500 W heater (RC6,
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49 192 Lauda, Lauda-Königshofen, Germany). Water oxygenation and homogeneity were assured by
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51 193 bubbling air vigorously into the tanks and using aquarium pumps to circulate the water and
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54 194 keeping the oxygenation level above 80% of air saturation throughout the experiments. Once
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56 195 a fish lost equilibrium, the water temperature was recorded and the fish was quickly removed
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58 196 from the tank and placed in a recovery tank (all the fish had their own 10 L recovery tanks) at

197 the acclimation temperature. After the experiment, the rainbow trout were euthanized with
198 200 ppm MS-222 buffered with sodium bicarbonate. The mass and length of the fish were
199 recorded.

200

201 *Heart rate measurements*

202 The second aim of the study was to measure whether a crude oil exposure influenced the
203 maximum heart rate (f_{Hmax}) of the fish at different temperatures. With this technique we were
204 also able to estimate the upper thermal tolerance of cardiac function. The temperature where
205 f_{Hmax} stops increasing exponentially with increasing temperature (Arrhenius break point
206 temperature, T_{AB}) has been connected to optimum temperature of aerobic scope and growth
207 in several species (rainbow trout: Anttila et al. 2013a; coho salmon, *Oncorhynchus kisutch*:
208 Casselman et al. 2012; Atlantic salmon, *Salmo salar*: Anttila et al. 2014; sockeye salmon, *O.*
209 *nerka*: Chen et al. 2013; chinook salmon, *O. tshawytscha*: Muñoz et al. 2014; Arctic cod,
210 *Boreogadus saida*: Drost et al. 2014 and goldfish, *Carassius auratus*: Ferreira et al. 2014).
211 Thus, T_{AB} was calculated and used as a proxy for the optimum temperature of fish (T_{OPT}). We
212 also measured the temperature (T_{PEAK}) where the highest heart rate (f_{Hpeak}) was found and the
213 temperature where cardiac arrhythmias were observed (T_{ARR}). These temperature values were
214 indicative the upper thermal limit for cardiac function (Casselman et al. 2012; Anttila et al.
215 2013a).

216

217 f_{Hmax} of rainbow trout was measured a week after the exposure ($n = 12$ per group, different
218 fish from those in CT_{MAX} measurements). The measurements were done according to
219 Casselman et al. (2012) with slight adjustments in drug concentrations. Briefly, the fish were
220 anesthetized in buffered 80 ppm MS-222, weighted and placed in the experimental setup. The
221 set-up consisted of 4 chambers made of a PVC-pipe with sealed ends and top removed so that

222 fish were completely submerged. A water flow to these chambers was achieved via a
223 chilling/heating unit (RC6, Lauda, Lauda-Königshofen, Germany). Note that a portion of that
224 water flow was diverted, via pipe-mouthpieces, through fish mouth and gills. This water
225 contained a maintenance dose of anesthetics (70 ppm of buffered MS-222). To detect fish
226 electrocardiogram (ECG), two silver electrodes were placed on the bottom of each chambers
227 underneath the fish. The signal from these electrodes was amplified and filtered using a
228 Grass P122 AC/DC Strain Gage Amplifier (Grass Technologies, Warwick, U.S.A.). ECG
229 recording and analysis were performed with BIOPAC data acquisition unit (MP100) and
230 Acknowledge software (ver. 3.9.1).

231
232 Following their transfer into the experimental chambers, fish were equilibrated at the initial
233 temperature (12°C) for 1h, after which they were given an intraperitoneal injection of
234 atropine sulphate (1.8 mg kg⁻¹, Sigma-Aldrich Chemie GmbH, Munich, Germany) to
235 completely block the vagal tone. Fifteen minutes later fish were given an isoproterenol
236 injection (4 µg kg⁻¹, Sigma-Aldrich Chemie GmbH, Munich, Germany) to maximally
237 stimulate cardiac β₁-adrenoceptors. Both drugs were dissolved in saline (0.9% NaCl). These
238 drug concentrations had been tested in preliminary experiments to ensure that maximum heart
239 rate was recorded. Once heart rate had stabilized (15 min after the isoproterenol injection),
240 water temperature was increased in 1°C increments at a rate of 10°C h⁻¹. After every
241 temperature increment, heart rate was allowed to stabilize before recording a value. Warming
242 continued until cardiac arrhythmias developed, after which fish were rapidly removed from
243 the apparatus, euthanized by cranial percussion and their weight and length measured.

244
245 For each individual fish temperatures corresponding to T_{ARR} and T_{PEAKT} were recorded and
246 the T_{AB} was calculated with Arrhenius plots according to Yeager and Ultsch (1989).

247

248 **Experiment 2. Seabass**

249 The seabass (age 1+; 11.3 ±1.1 cm; 17.7 ±5.0 g) were obtained from Aquastream Lorient,
250 France and transferred to a 2000-L rearing tank at Unité de Physiologie Fonctionnelle des
251 Organismes Marins, Ifremer, France. They were maintained under natural photoperiod and
252 temperature conditions (10°C at the time of arrival) and fed daily with commercial pellets (Le
253 Gouessant, Saint-Martin de Valgagues, France). Fish were acclimated 8 weeks to the
254 laboratory conditions before experiments began and feeding was ceased 24h before any
255 experiments/manipulations. Two weeks before experiments started, seabass were
256 anaesthetized (MS-222; 100 mg L⁻¹, Sigma-Aldrich Chimie GmbH, Munich, Germany) and
257 individually implanted subcutaneously with an identification tag (RFID; Biolog-id, Bernay,
258 France). All the experiments were approved by Ministère Délégué à l'Enseignement
259 Supérieur et à la Recherche.

260

261 *Exposures*

262 The exposures were conducted as in experiment 1, apart from a different crude oil (Arabian
263 light crude oil) and concentration (0.8 g L⁻¹). A higher concentration was used because it has
264 been previously shown that a lower concentration (0.07 g L⁻¹) did not influence the thermal
265 tolerance of seabass (Claireaux et al. 2013; Mauduit et al. 2016). Furthermore, in the
266 experiment with seabass, crude oil was treated with dispersant (Finasol OSR 52, 0.017 g L⁻¹)
267 while in the experiment with rainbow trout weathered oil was only mechanically dispersed in
268 water. In seabass experiment 250g of crude oil and 10g of dispersant was mixed vigorously in
269 three separate bottles. The oil-dispersant mixture was directly poured in the three 300 L
270 experimental tanks and then bubbled with air overnight to mimic a 12-h aging of an oil slick
271 at sea (Nordvik 1995). The number of exposed seabass per treatment was 180. Exposure

1 272 started when sixty fish were transferred for 48h into triplicate exposure tanks that contained
2 273 only sea water (control group) or dispersant-treated oil (oil group; total biomass per tank was
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4 274 4.2 g L⁻¹). The pH of the water did not change during the exposure (8.1±0.1) and the oxygen
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7 275 concentration was maintained above 80% of air saturation. Water samples were taken from
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10 276 each tank shortly after the exposure started and at the end of the exposure to analyze the TPH.
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12 277 The water samples were taken from the middle of the water column using a bottle and the
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14 278 TPH in water samples were analyzed at CEDRE (Brest, France). Nine seabass were
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17 279 sacrificed 24h after the exposure in order to measure liver PAH concentrations.
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19 280 Measurements were conducted in CEDRE (see Supplementary material for the methods of
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22 281 chemical analyses).

23 24 282 25 26 283 *CT_{MAX} experiments*

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29 284 Experiments were conducted 4 weeks post oil-exposure. The CT_{MAX} protocol (*n* = 20 per
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31 285 group) differed slightly from the protocol for rainbow trout in that the thermal challenge test
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34 286 was conducted directly in the fish rearing tank. Moreover, temperature was increased from
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36 287 acclimation temperature to 27°C in 2.5 hours and thereafter the heating rate was lowered to
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39 288 0.5°C h⁻¹ until the last fish lost its equilibrium and was removed from the tank (Claireaux et
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41 289 al. 2013). The water temperature was controlled with JULABO F10, 2500 W heater
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44 290 (Seelbach, Germany). Each time a fish lost its ability to maintain an upright position, the
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46 291 water temperature was recorded, the fish PIT tag number was read and it was transferred to a
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49 292 recovery tank for 2h before being returned to its original rearing tank. The percentage of
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51 293 mortality after the CT_{MAX} experiment was below 1%. Fish were then reared under natural
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54 294 photoperiod and temperature conditions until heart rate measurements (6 months later). There
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56 295 were no differences in mortality between groups.

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297 *Heart rate measurements*

298 Maximum heart rate of seabass ($n = 18$ per group, the same fish as in CT_{MAX} measurements)
299 was measured 6 months after oil exposure (water temperature at that time was $17^{\circ}C$). At the
300 time of heart rate measurements, the mean size of fish did not differ significantly between
301 groups (66.4 ± 4.1 g and 17.2 ± 0.3 cm for control seabass and 71 ± 3.7 g and 17.5 ± 0.3 cm for
302 oil-exposed seabass, respectively). The experimental protocol was exactly the same as with
303 rainbow trout except that anesthesia solution did not contain bicarbonate because of the
304 buffering capacity of seawater. Salinity level of the water was 32-33 ppt. The water
305 temperature was controlled with a recirculating chiller/heater (F10, JULABO, Seelbach,
306 Germany) and the ECG was recorded and analyzed with BioPac MP36R (BIOPAC Systems
307 Inc, Essen, Germany) which had an in-built amplification system. The starting temperature of
308 the measurements for seabass was $17^{\circ}C$. The concentration of atropine sulphate was 3 mg kg^{-1}
309 ¹ (Sigma-Aldrich Chemie GmbH, Munich, Germany) and the concentration of isoproterenol
310 was $3.2 \text{ } \mu\text{g kg}^{-1}$ (Sigma-Aldrich Chemie GmbH, Munich, Germany).

311

312 **Statistical analyses of both experiments**

313 Data normality and homogeneity were tested with Kolmogorov-Smirnov and Levene tests,
314 respectively. Two-way repeated measures ANOVA was used to calculate the differences in
315 f_{Hmax} values between oil-exposed and control fish during warming (temperature and treatment
316 as factors) and was followed by Holm-Sidak *post hoc* test. Both species were tested
317 separately because of differences in starting temperatures and treatments. Two-way repeated
318 measures ANOVAs were also used to analyze 1) the differences in f_{Hpeak} and scope for heart
319 rate (difference between lowest f_{Hmax} and f_{Hpeak}) between exposed and control animals and 2)
320 the differences in T_{AB} , T_{PEAK} , T_{ARR} , CT_{MAX} between exposed and control animals (exposure
321 and measurement as factors). However, since the individuals for CT_{MAX} experiment were

322 different from individuals used in heart rate experiment for rainbow trout, the differences in
323 CT_{MAX} between exposed and control rainbow trout were analyzed with t-test. All the
324 statistical analyses were performed with SigmaPlot 13 (Systat Software Inc., San Jose, CA,
325 USA). Statistical significance for comparisons of mean values was set at $\alpha=0.05$. The values
326 are presented as means \pm SEM.

328 **Results**

330 **Experiment 1. Rainbow trout**

331 Rainbow trout were exposed to 0.07 g L^{-1} of weathered Russian export blend medium crude
332 oil. The mean \sum hydrocarbons in water samples from the three exposure tanks at the end of
333 the exposure period was $3.8 \pm 1.1 \text{ mg L}^{-1}$ while the sum of 16 US-EPA PAHs was $4.5 \pm 0.14 \mu\text{g}$
334 L^{-1} . However, it needs to be noted that only naphthalene, acenaphthalene, fluorene and
335 phenanthrene concentrations were above detection limits. The list of assayed PAH compounds
336 is shown in a Supplementary Table. There were no mortalities after exposures.

338 *Heart rate*

339 Exposure to crude oil reduced the heart rate at temperatures below and near optimum
340 temperature ($12\text{-}18^\circ\text{C}$, optimum around 17°C , Anttila et al. 2013a) ($F_{1,6}=6.3$, $P=0.013$, Fig.
341 1a) while there were no differences between oil-exposed and control fish at temperatures
342 $>18^\circ\text{C}$. Oil exposure did not affect the highest f_{Hmax} recorded (f_{Hpeak}) ($108 \pm 5.1 \text{ bpm}$ and 116
343 $\pm 6.7 \text{ bpm}$ for control and oil-exposed rainbow trout, respectively; $P=0.29$). The difference
344 between the lowest recorded f_{Hmax} and the highest recorded f_{Hmax} (scope for heart rate) were
345 50.2 ± 4.1 and $64.5 \pm 6.5 \text{ bpm}$ in control and oil-exposed rainbow trout, respectively, and were
346 not statistically different from each other ($P=0.088$).

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2 348 *Indices of upper thermal tolerance*

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5 349 Oil exposure had a significant influence on rainbow trout thermal tolerance (Fig. 2a). One
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7 350 week post exposure, T_{AB} was significantly higher in oil-exposed ($16.3 \pm 0.4^\circ\text{C}$) than in control
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9 351 ($15.1 \pm 0.4^\circ\text{C}$) fish ($P=0.036$, Fig. 1a, 2a). Similarly, all the indices of upper thermal tolerances
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11 352 (i.e. T_{PEAK} , T_{ARR} and CT_{MAX}) were higher in oil-exposed than in control rainbow trout: the
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13 353 differences between oil-exposed and control fish were 1.8°C , 2.3°C and 0.8°C , for T_{PEAK} ,
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15 354 T_{ARR} and CT_{MAX} , respectively (Fig. 2a) (T_{PEAK} : $P=0.002$; T_{ARR} : $P=0.033$; CT_{MAX} : $P=0.005$).
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17 355 CT_{MAX} , T_{ARR} , T_{PEAK} and T_{AB} differed significantly from each other ($P<0.001$).
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24 357 **Experiment 2. Seabass**

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26 358 The Σ hydrocarbons experienced by seabass at the end of the exposure to 0.8 g L^{-1} of
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28 359 weathered Arabian light crude oil mixed with 0.017 g L^{-1} of dispersant (Finasol OSR 52) was
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30 360 $141 \pm 7.8 \text{ mg L}^{-1}$. Twenty four hours post-exposure, the sum of 16 US-EPA PAHs and 5
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32 361 supplementary PAHs (benzo[b]thiophene, biphenyl, dibenzothiophene, benzo[e]pyrene and
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34 362 perylene) in seabass liver ($n = 9$) was $34\,299 \pm 8\,926 \text{ ng g}^{-1}$. The concentrations of PAHs are
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38 363 detailed in Supplementary table.
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43 365 *Heart rate*

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45 366 The exposure to crude oil caused similar effects in seabass as observed in rainbow trout
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47 367 although experimental designs and post-exposure recovery times were different. For example,
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49 368 in seabass, as in rainbow trout, the oil-exposed fish had lower f_{Hmax} values ($F_{1,5}=4.8$, $P=0.03$)
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51 369 than the control fish when measured below the optimum temperature ($17\text{-}22^\circ\text{C}$, optimum
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53 370 around $22\text{-}24^\circ\text{C}$, Claireaux and Lagardère 1999), (Fig. 1b). However, there were no
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55 371 differences between oil-exposed and control seabass at temperature $> 24^\circ\text{C}$. As in rainbow
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372 trout the oil exposure did not influence the f_{Hpeak} values ($P=0.91$). The values for f_{Hpeak} were
373 125 ± 6.7 bpm and 126 ± 4.9 bpm for control and oil-exposed seabass, respectively. The scopes
374 for f_{Hmax} were 34.0 ± 5.0 and 35.7 ± 5.8 for control and oil-exposed seabass, respectively, and
375 the groups did not differ from each other ($P=0.83$).

376

377 *Indices of upper thermal tolerance*

378 Again, as in rainbow trout, the oil-exposed seabass had higher T_{AB} ($1.0^{\circ}C$ difference)
379 compared to control seabass ($P=0.05$). The T_{AB} values were $19.3\pm 0.3^{\circ}C$ and $20.3\pm 0.3^{\circ}C$ for
380 control and oil-exposed seabass, respectively (Fig. 1d, 2b). Furthermore, the T_{PEAK} was
381 higher ($P=0.03$, difference $1.3^{\circ}C$, Fig. 2b) in oil-exposed seabass than in the control. The
382 T_{PEAK} values were $21.8\pm 0.4^{\circ}C$ and $23.1\pm 0.4^{\circ}C$ for control and oil-exposed seabass,
383 respectively. However, in seabass the T_{ARR} and CT_{MAX} did not differ statistically between oil-
384 exposed and control fish after a 6-month recovery (T_{ARR} : $P=0.62$; CT_{MAX} : $P=0.94$, Fig. 2b).

385

386 **Discussion**

387

388 Recent studies have demonstrated that embryonic fish exposed to hydrocarbon compounds
389 can display various cardiac malformations and functional disorders (e.g. Thomaz et al. 2009;
390 Incardona et al. 2009, 2012, 2014; Jung et al. 2013; Sørhus et al. 2016; Khursigara et al.
391 2017). However, there is very limited literature available about the influence of petroleum
392 hydrocarbon exposure on later life stages. Moreover, when available, reports about disturbed
393 homeostasis or functional disorders generally concern a very short period post exposure,
394 preventing attempts to draw the distinction between normal regulatory processes, a stress
395 response or a true, potentially long-lasting, functional disorders. The aim of the current study
396 was, therefore, to measure, in experiments involving two different fish species, how exposure

397 to crude oil, followed by a significant recovery period in a clean environment, influenced the
398 upper critical thermal tolerance and cardiac function of juvenile fish. We found that a week
399 (trout) to months (seabass) post exposure, crude oil-exposed fish displayed higher (trout) or
400 unchanged (seabass) upper critical thermal tolerance than control, unexposed fish. We also
401 found that crude oil did not have a significant chronic residual influence on the cardiac
402 performance of our juvenile fish at high temperatures. In contrast to what was expected, our
403 estimate of optimal temperature for cardiac function even increased after exposure in both
404 species.

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406 The upper critical temperature (CT_{MAX}) corresponds to temperature at which a fish exposed
407 to a progressive and controlled increase in water temperature is no longer able to maintain an
408 upright position. When the CT_{MAX} is reached survival is time-limited and, thus, CT_{MAX} is
409 used as a proxy for upper critical thermal tolerance of fish (Sunday et al. 2015). In the current
410 study, we observed that the oil-exposed rainbow trout had a significantly higher CT_{MAX} than
411 control fish. In seabass, on the other hand, no difference in CT_{MAX} was observed between
412 control and oil-exposed fish. These values ($29.0 \pm 0.2^\circ\text{C}$ for rainbow trout acclimated at 16°C
413 and $31.3 \pm 0.3^\circ\text{C}$ for seabass acclimated to 17°C) were similar to those reported earlier
414 (29.4°C and $28\text{-}35^\circ\text{C}$, Beitinger et al. 2000; Claireaux et al. 2013). It has previously been
415 found in juvenile seabass that exposure to crude oil (0.07 g L^{-1}) did not affect their upper
416 critical thermal tolerance (CT_{MAX}) when the measurements were done ten months after
417 exposure (Mauduit et al. 2016). The present study, using 12.5 times higher oil concentration,
418 confirms that exposure to petroleum hydrocarbons has no significant, long-term effect on
419 upper critical thermal tolerance in this species. Since we did not measure CT_{MAX} immediately
420 after oil exposure it is not certain whether this lack of effect results from a recovery process
421 or that oil-exposure has simply no effect on thermal tolerance in juvenile fish. However,

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422 Claireaux et al. (2013) have shown that shortly after oil exposure juvenile seabass do display
423 a reduction in CT_{MAX} . This therefore suggests that juvenile fish have the capacity to recover
424 from the initial impact of oil exposure on their upper critical thermal tolerance.

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426 Optimal temperatures (T_{OPT}) of rainbow trout and seabass are 16.5-17°C and 22-24°C,
427 respectively (Jobling 1981; Claireaux and Lagardère, 1999). In both species we observed that
428 at suboptimal temperatures, maximum heart rate (f_{Hmax}) of oil-exposed individuals was lower
429 than in control specimens. Previously, Milinkovitch et al. (2013) have found in juvenile
430 golden grey mullet exposed to crude oil (0.07 g L⁻¹, Σ PAH 3.3-60.1 μ g L⁻¹) that the capacity
431 of ventricular muscle strips to generate force at suboptimal temperature (14.5°C) and high
432 stimulation frequency (1.2 Hz) was not affected when measured directly after exposure.

433 Furthermore, these authors reported that oil exposure did not significantly affect the force-
434 frequency relationship established in control, unexposed fish. Similarly, Nelson et al. (2016)
435 found that a 24h exposure to PAHs (9.6 μ g L⁻¹) did not influence the routine heart rate of
436 adult mahi-mahi when measured directly after exposure. However, a significant decrease in
437 routine cardiac output was reported by these authors, probably resulting from a decreased
438 stroke volume. Claireaux and Davoodi (2010) found that juvenile common sole had reduced
439 cardiac output, as well as impaired cardio-respiratory responses to an acute warming, 24h
440 after exposure to crude oil (Σ PAH 39 ng L⁻¹ of water and 15–71 ng g⁻¹ dry liver tissues).

441 These authors found that 24h post-exposure there was no statistically significant difference in
442 routine heart rates between oil-exposed and control sole at temperatures between 15-20°C
443 (optimal temperature in sole is 19°C; Lefrançois and Claireaux 2003). However, a difference
444 progressively appeared when temperature increased above 20°C with the cardiac output of
445 the oil-exposed common soles gradually losing its capacity to match the temperature driven
446 increase in metabolic oxygen demand (Claireaux and Davoodi 2010). There are no other

447 studies about the effect of crude oil or PAHs on heart rate in non-embryonic fish. However,
448 work on embryonic fish indicates that exposure conditions affect the cardiac response to oil
449 compounds. For example, developing zebrafish (*Danio rerio*) exposed to oiled gravel effluent
450 displayed reduced routine heart rate, while exposure to water-accommodated fractions
451 containing dispersed oil microdroplets had no effect (Jung et al. 2013). In Pacific herring
452 (*Clupea pallasii*), oiled-gravel exposure increased the variability of routine heart rate and
453 generally reduced it (Incardona et al. 2009). A similar response was observed in herring
454 embryos exposed to bunker fuel after *Cosco Busan* collision in San Francisco Bay (Incardona
455 et al. 2012), as well as in haddock (*Melanogrammus aeglefinus*) embryos exposed 0.7–7 μg
456 L^{-1} ΣPAH (Sørhus et al. 2016) and in rainbow trout larvae exposed to 1.6 g L^{-1} of crude oil
457 (Vosyliene et al. 2005). An exposure to $\Sigma\text{PAH} \sim 2\text{--}5 \mu\text{g L}^{-1}$ (which is similar to the PAH
458 concentration in our experiment with rainbow trout) reduced routine heart rate in embryos of
459 bluefin and yellowfin tuna (Incardona et al. 2014). In larval red drum (*Sciaenops ocellatus*)
460 the exposure to crude oil (2.6 $\mu\text{g L}^{-1}$ ΣPAH) reduced the routine stroke volume but did not
461 affect heart rate (Khursigara et al. 2016). In the current study, the reduction of f_{Hmax} at
462 suboptimal temperatures in the oil-exposed fish was rather small (~ 4 bpm). Thus, it seems
463 that the juvenile fish might be more resistant against oil-exposure than earlier life stages. In
464 embryonic and larval fish significant reductions in heart rate and arrhythmias are seen already
465 after nominal exposure to PAHs (e.g. Thomaz et al. 2009; Incardona et al. 2009, 2012, 2014;
466 Jung et al. 2013; Sørhus et al. 2016; Khursigara et al. 2017). Furthermore, exposure in
467 embryonic stages may have extremely long lasting effects e.g. on swimming capacity
468 (Hicken et al. 2011; Mager et al. 2014) which could have consequences on the ecology of
469 whole species.

471 In the current study despite the fact that f_{Hmax} was lower in exposed fish than in control fish at
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2 472 temperatures below the optimal temperature (T_{OPT}) of the species considered, this difference
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4 473 disappeared at temperature above T_{OPT} . As a result, the highest f_{Hmax} values were similar in
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7 474 exposed and control fish. Thermal tolerance of cardiac function i.e., T_{PEAK} (temperature
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10 475 where highest f_{Hmax} was observed) and T_{ARR} (temperature where arrhythmias were observed)
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12 476 were also higher in exposed rainbow trout than in control trout. In seabass, T_{PEAK} was also
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14 477 higher in exposed than in control seabass. Furthermore, in both species Arrhenius break point
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17 478 temperature (T_{AB}), which is indicative of the species' optimal temperature (e.g. Casselman et
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19 479 al. 2012; Anttila et al. 2013a; Ferreira et al. 2014), was higher in oil-exposed animals than in
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22 480 control ones. In general, these findings show that oil-exposure does not have significant
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24 481 negative impact either on cardiac function at high temperatures or the thermal tolerance of
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27 482 cardiac function. These are important findings, as in several species at high temperatures the
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29 483 maximum cardiac function is critical for fish survival through preserving aerobic capacity
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32 484 (Steinhausen et al. 2008; Farrell 2009; Eliason et al. 2013). A 4-month field experiment
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34 485 recently illustrated the ecological significance of these results as the survival and growth of
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36 486 juvenile oil-exposed seabass (0.07 g L^{-1}) was shown to be similar to that of control fish
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39 487 although they were exposed to naturally high summer temperatures (Mauduit et al. 2016).
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41 488 Taken together, our heart rate results indicate that oil exposure does not have significant
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44 489 lasting negative impact on the f_{Hmax} on juvenile fish when fish are tested after sufficient
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46 490 recovery time following exposure. Again, we cannot be sure if this is because of the recovery
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49 491 capacity or because used crude-oil concentrations do not have impact on maximum heart rate
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51 492 in the first place. Previously, Johansen and Esbaugh (2017) have shown in adult red drum
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53 493 (*Sciaenops ocellatus*) that acute oil exposure ($12.1 \mu\text{gL}^{-1} \Sigma\text{PAH}_{50}$) reduced the swimming
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56 494 capacity as well as aerobic scope and that these effects were still seen 6 weeks post exposure.
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58 495 We can therefore assume that oil-exposure in the current study did not have significant effect
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5 496 on f_{Hmax} in the first place. However, it cannot be ruled out that other aspects of cardiac
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7 497 function, like stroke volume, would have been influenced as seen in mahi-mahi (Nelson et al.
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9 498 2016).

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11 500 Our values for heart rate and upper thermal tolerance lie within the range of published values
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13 501 from these animals. Rainbow trout swimming at maximum sustainable velocity at 15°C has a
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15 502 heart rate of 96 bpm after vagotomy (Priede 1974). This value compares nicely with the
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17 503 values of 80 bpm observed in the present study (see also Mercier et al. 2000). The maximum
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19 504 heart rate of exercising seabass has been measured at around 90 bpm at 20°C (Chatelier et al.
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21 505 2005) which agrees relatively well with the current finding of 90 bpm at 17°C. The T_{AB}
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23 506 values ($15.1 \pm 0.4^\circ\text{C}$ and $19.3 \pm 0.3^\circ\text{C}$ for rainbow trout and seabass, respectively) are
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25 507 somewhat lower, but near the optimum growth temperatures of these species (16.5-17°C and
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27 508 ~22-24°C, Jobling 1981; Claireaux and Lagardère 1999).

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34 510 The two experiments that are reported here were conducted separately and differences in the
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36 511 experimental conditions and design must be considered, i.e., PAH composition of the oil
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38 512 tested, duration of the post-exposure recovery, concentration of crude oil tested, usage of
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40 513 dispersant and availability of tissue PAH concentrations.

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45 515 Crude oil used in the seabass and trout experiments was of different origin and this was
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47 516 particularly reflected in their composition of PAHs, the most potent oil compounds from the
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49 517 cardiac standpoint. In embryonic fish it has been shown that PAHs are particularly
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51 518 detrimental to the cardiac function although reported effects are compound-specific (see
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53 519 review by Incardona and Scholz 2016). For instance naphthalene, chrysene, anthracene and
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55 520 benzo[k]fluoranthene, abundant PAHs in both tested oils, are known for having only small
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1 521 effects on the physiology (conduction properties) and anatomy/pathology (pericardial edema)
2 522 of the heart (Incardona et al. 2004; 2011). On the other hand, dibenzothiophene and
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4 523 phenanthrene have been shown to cause pericardial edema and to reduce heart rate by
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7 524 blocking atrioventricular conduction in a concentration-dependent manner (Incardona et al.
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9 525 2004). Furthermore, benzo[e]pyrene and benzo[a]pyrene have also been shown to induce
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11 526 pericardial edema (Incardona et al. 2011). In the current experiments phenanthrene was
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13 527 present in the water and fish tissues and dibenzothiophene was even the most abundant PAH
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15 528 in the seabass experiment. Thus, from this stand point, both oils contained PAH compounds
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17 529 that should have had influence on cardiac function.
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24 531 Another difference between the two experiments is the duration of the post-exposure
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26 532 recovery period. The rainbow trout measurements were done one week after the exposure
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28 533 while seabass were measured 6 months post-exposure. The short-term effects of oil exposure
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30 534 upon the cardiac function are well documented (e.g. Incardona et al. 2009, 2012, 2014;
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32 535 Claireaux and Davoodi 2010; Jung et al. 2013; Milinkovitch et al. 2013; Brette et al. 2014;
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34 536 Nelson et al. 2016; Sørhus et al. 2016; Khursigara et al. 2017). A review of the literature
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36 537 shows, however, that published studies of the toxicological impacts of oil exposure on fish
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38 538 predominantly investigated acute exposure, short exposure duration and immediate
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40 539 assessment, mostly at low biological organization levels. While these studies contribute to
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42 540 increase our mechanistic understanding of contamination and decontamination processes (e.g.
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44 541 Ramachandran et al. 2004; Milinkovitch et al. 2013; Danion et al. 2014; Dussauze et al.
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46 542 2014; Sadauskas-Henrique et al. 2016; Sandrini-Neto et al. 2016; Sanni et al. 2016), they are
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48 543 of limited help when addressing issues such as, for instance, impact on the resilience, health,
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50 544 production and recruitment of the affected populations (Forbes et al. 2006). From this
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52 545 perspective, the present experiments are remarkable as, although providing little additional
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1 546 information about the well documented patterns of PAH bioaccumulation and metabolization
2 547 in fish (Claireaux and Davoodi 2010; Milinkovitch et al. 2013; Nelson et al. 2016), they
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4 548 provide evidence in favor of the absence of chronic latent effects of oil exposure on fish
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7 549 thermal tolerance and cardiac performance. These results substantiate the recovery process
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10 550 reported by Claireaux et al. (2013) and Mauduit et al. (2016) regarding swimming capacity,
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12 551 tolerance to heat and to hypoxia. Interestingly, although the two sets of experiments involved
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14 552 two different fish species with quite different exposures and at two different times post-
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17 553 exposure, very similar response patterns were observed. This is an important result, because it
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19 554 shows that cardiac recovery could be a relatively general phenomenon in juvenile fish.
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22 555 However, in order to evaluate the recovery process more thoroughly, more time points should
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24 556 be examined in future.

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29 558 One other difference between the two studies was that we didn't use dispersant in the trout
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31 559 study and that tissue PAH concentrations were not measured in that species. Nevertheless,
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34 560 nominal concentrations tested in present studies are representative of the concentrations
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36 561 measured following an accidental spill (Boehm and Fiest 1982; Milinkovitch et al. 2011),
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39 562 which have been shown to result in detrimental effects on cardiac function when measured
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41 563 within days-weeks post-exposure (e.g. Claireaux and Davoodi 2010; Incardona et al. 2014;
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43 564 Sørhus et al. 2016; Khursigara et al. 2017).

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48 566 In conclusion, exposure to crude oil did not have a significant impact on maximum heart rate
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51 567 and cardiac function of juvenile fish at temperatures above their optimum when the
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53 568 measurements were done 1 week (trout) to 6 months (seabass) after the exposure. We
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56 569 observed that exposure may even improve the upper thermal tolerance of fish. The reason for
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58 570 this unexpected result is unknown, thus, future studies should focus on this issue. Our
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571 findings also suggest that recovery processes exist that can reverse the functional
572 impairments observed shortly following direct exposure in previous studies. This is also an
573 important finding for the fisheries as both of these species are important human food species
574 and are living/reared at areas where the threat for both oil accidents and warming surface
575 waters is particularly high.

576 **Figure legends**

577

578 **Fig. 1** The cardiac responses to increasing temperature in control and oil-exposed fish after
579 one-week (rainbow trout) and six-month (seabass) recovery. The effect of increasing
580 temperature on the maximum heart rate (f_{Hmax}) of **a** juvenile rainbow trout and **b** European
581 seabass. The grey area indicates the temperature window where there are significant
582 differences between control and oil-exposed fish. The dotted lines indicate temperatures
583 where some of the fish already had arrhythmias. The Arrhenius plots of f_{Hmax} responses to
584 increasing temperature for rainbow trout are given in **c** and for seabass in **d**. The vertical lines
585 (dotted line for oil-exposed fish, solid line for control fish) indicate an Arrhenius break point
586 temperature (T_{AB}). $n = 12$ for rainbow trout and $n = 18$ for seabass

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588 **Fig. 2** The temperature tolerance values of control and oil-exposed fish. Arrhenius break
589 point temperature (T_{AB}), temperature for peak maximum heart rate (T_{PEAK}), temperature
590 where cardiac arrhythmias are observed (T_{ARR}) and critical upper temperature tolerance
591 (CT_{MAX}) of **a** rainbow trout and **b** European seabass. * indicates statistically significant
592 differences between oil-exposed and control fish. $n = 12$ for rainbow trout (except for CT_{MAX}
593 measurements $n = 15$) and $n = 18$ for seabass (except for CT_{MAX} measurements $n = 20$)

594 **Compliance with ethical standards**

595

596 **Conflict of Interest**

597 The authors declare that they have no conflict of interest.

598

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Figure 1

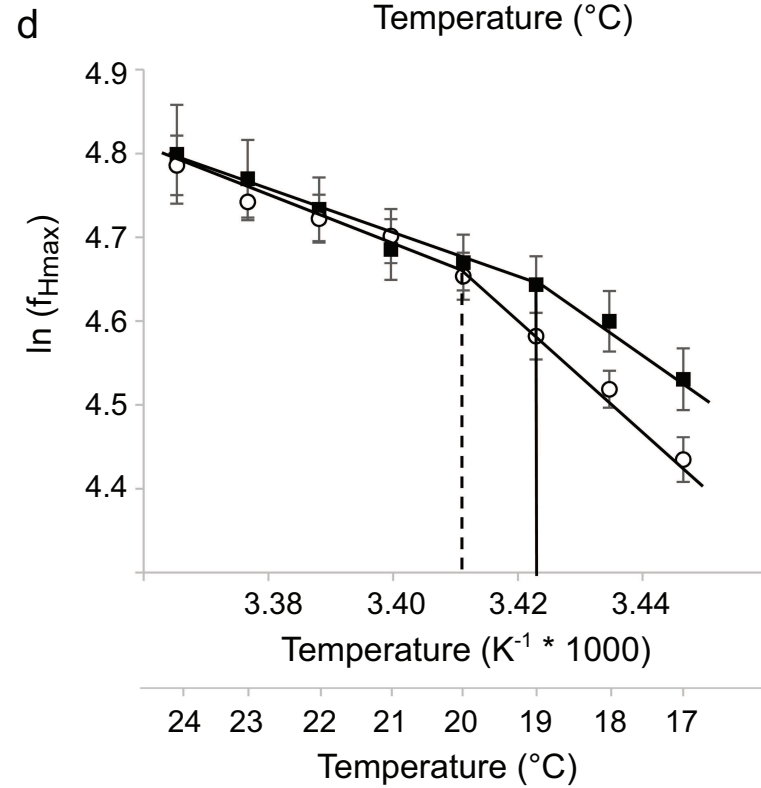
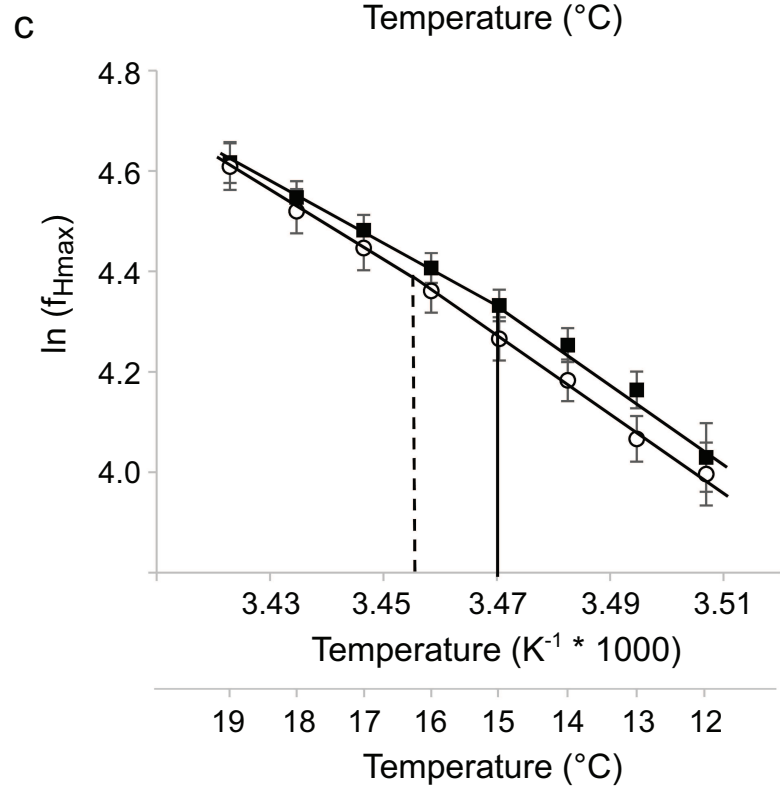
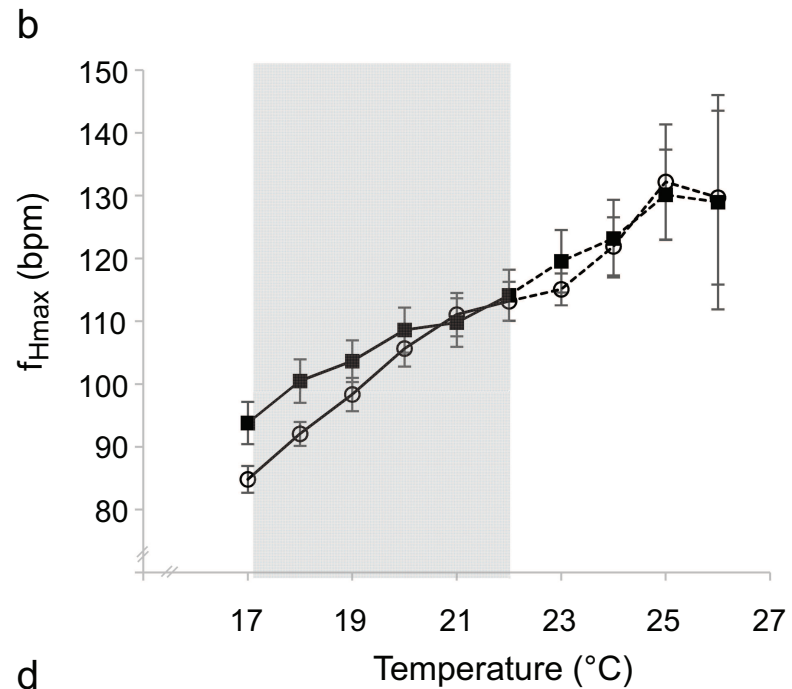
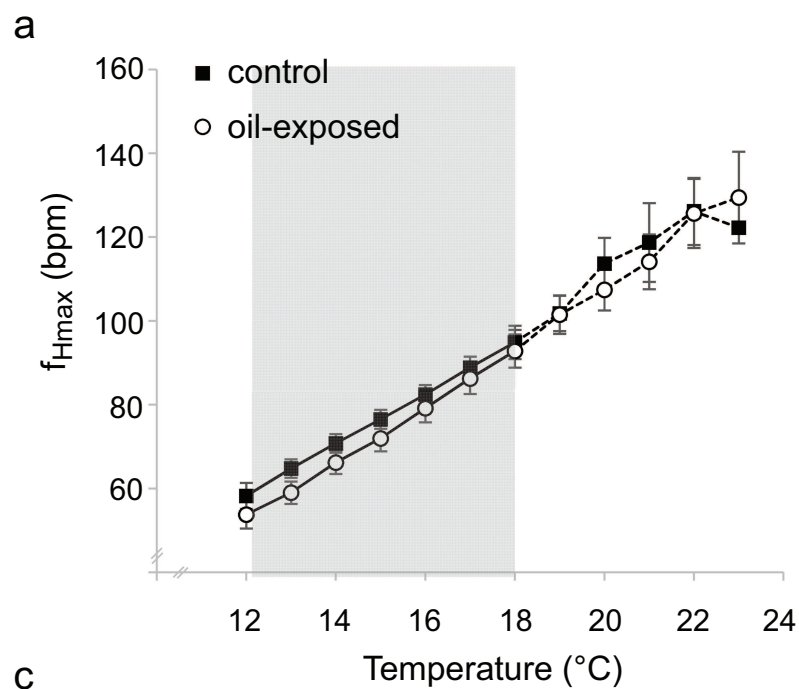
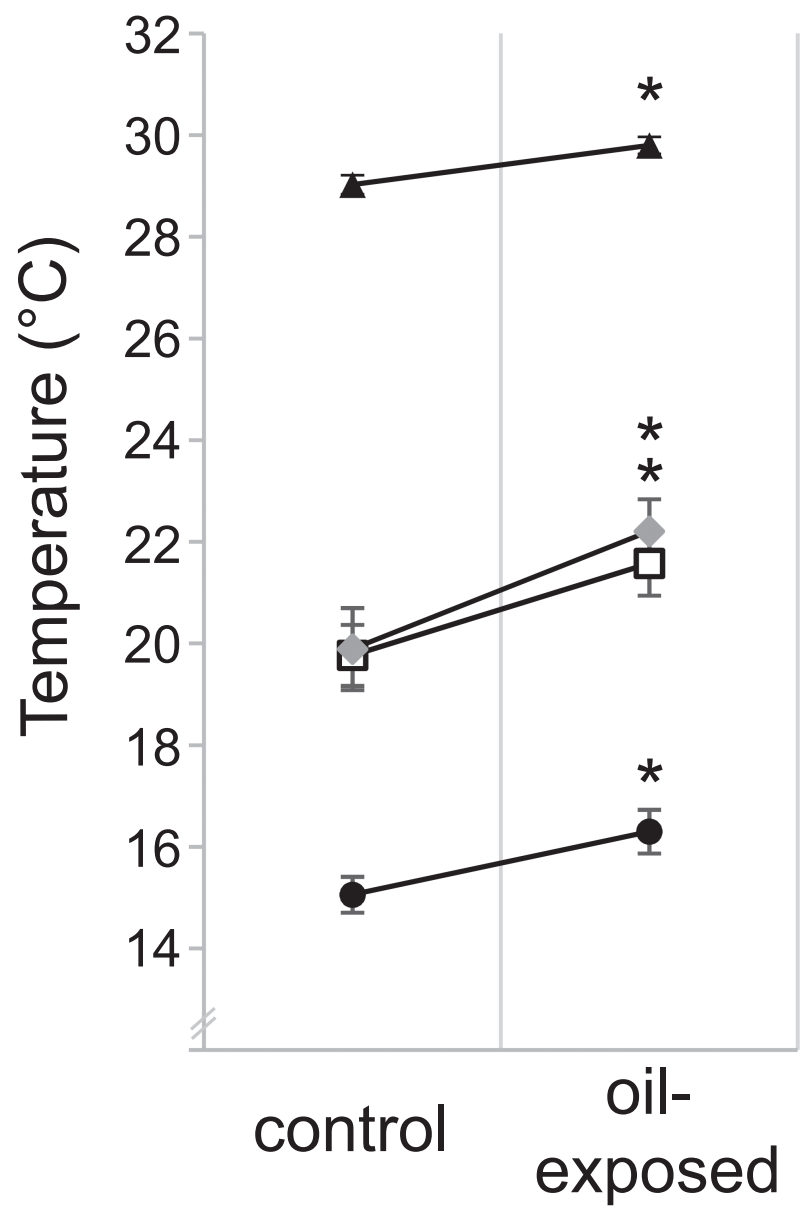
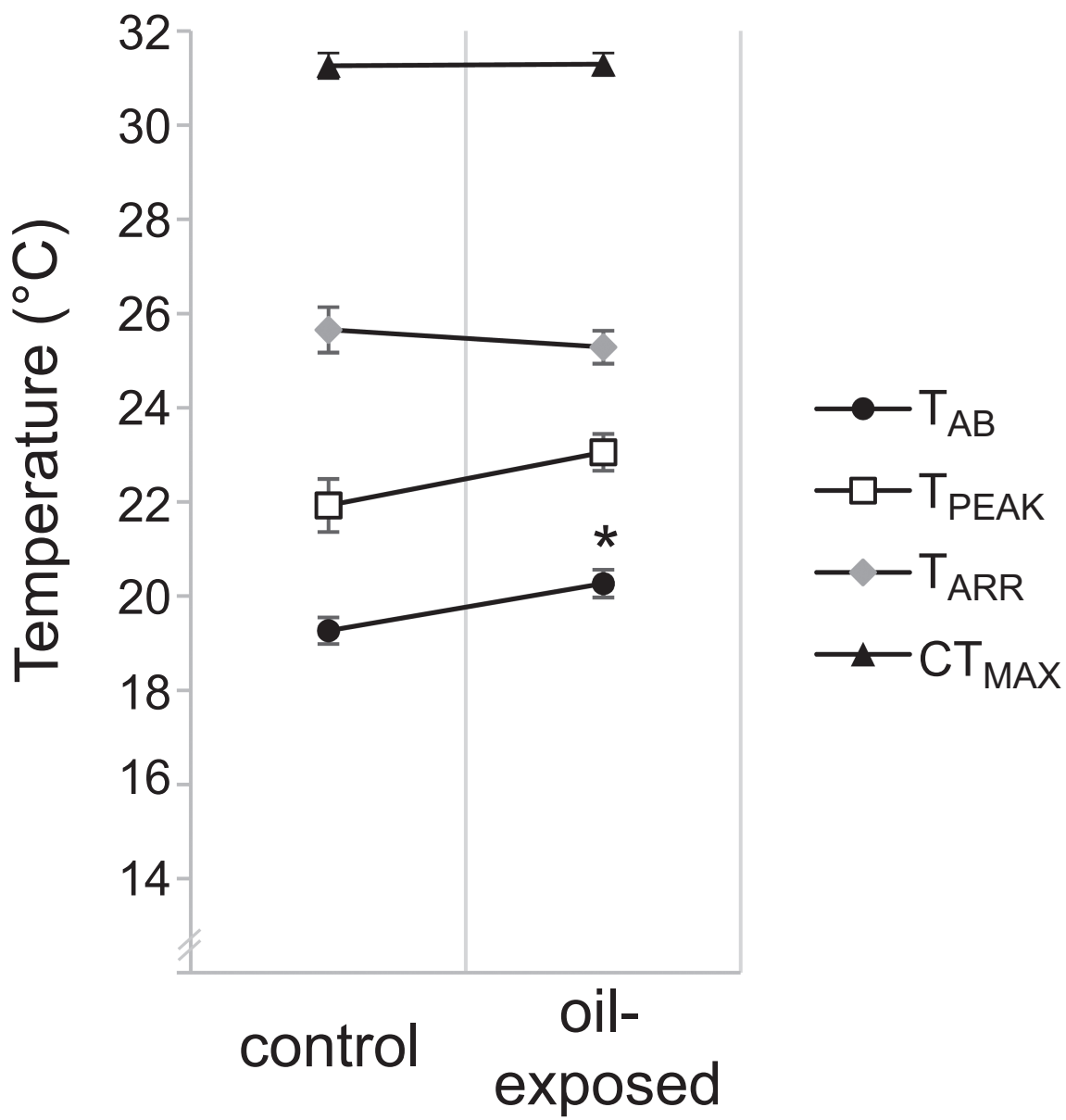


Figure 2

a



b





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