

1 **Cardiac and behavioural responses to hypoxia and warming in free-swimming gilthead seabream**

2 ***Sparus aurata***

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16 **SUMMARY STATEMENT**

17 Implantable biologgers were used to provide the first measurements of cardiac responses to hypoxia  
18 and warming in a free-swimming fish, revealing that confinement in respirometer chambers raises  
19 heart rate, with consequences for estimates of metabolic rates.

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23 **ABSTRACT**

24 Cardiac and behavioural responses to hypoxia and warming were investigated in free-swimming  
25 gilthead seabream *Sparus aurata* equipped with biologging tags in the peritoneal cavity. After  
26 suitable recovery in a holding tank, heart rate ( $f_H$ ) and the variance of tri-axial body acceleration  
27 ( $VAR_m$ ) were logged during exposure to stepwise progressive hypoxia or warming, comparing when  
28 either swimming in a tank or confined to individual respirometer chambers. When undisturbed under  
29 control conditions (normoxia, 21 °C), mean  $f_H$  was significantly lower in tank than respirometers. In  
30 progressive hypoxia (100 - 15% oxygen saturation), mean  $f_H$  in the tank was significantly lower than  
31 respirometers at oxygen levels until 40%, with significant bradycardia in both holding conditions  
32 below this. Mean  $VAR_m$  was low and invariant in hypoxia. Warming (21 to 31 °C) caused progressive  
33 tachycardia with no differences in  $f_H$  between holding conditions. Mean  $VAR_m$  was, however,  
34 significantly higher in the tank during warming, with a positive relationship between  $VAR_m$  and  $f_H$   
35 across all temperatures. Therefore, spontaneous activity contributed to raising  $f_H$  of fish in the tank  
36 during warming. Mean  $f_H$  in respirometers had a highly significant linear relationship with mean rates  
37 of oxygen uptake, considering data from hypoxia and warming together. The high  $f_H$  of confined *S.*  
38 *aurata* indicates that static respirometry techniques may bias estimates of metabolic traits in some  
39 fish species. Biologging on free-swimming fish revealed novel information about cardiac responses to  
40 environmental stressors, which may be closer to responses exhibited by fish in their natural  
41 environment.

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43 KEY WORDS: Heart rate, acceleration, biologging, warming, hypoxia, respirometry, *Sparus aurata*,  
44 Star-Oddi

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46 *List of symbols and abbreviations:*

47 -  $f_H$ : heart rate frequency

48 -  $\dot{M}O_2$ : rate of oxygen uptake

49 - EA: external acceleration

50 -  $VAR_m$ : Variance of external acceleration

51

## 52 INTRODUCTION

53 In fishes the heart is a critical organ for survival, ensuring delivery of oxygen and nutrients to support  
54 metabolism, and the removal of metabolic wastes (reviewed by Farrell and Smith, 2017). Cardiac  
55 performance is, therefore, considered to be a core determinant of the ability of fishes to survive and  
56 thrive in their environment (Farrell and Portner, 2008; Farrell and Smith, 2017; Eliason and Anntilla,  
57 2017; Stecyk, 2017). This includes their ability to tolerate environmental conditions, especially when  
58 these become stressful. For instance hypoxia, a reduced availability of dissolved oxygen, is a common  
59 stressor in aquatic habitats (Diaz and Rosenberg, 2008) that challenges the ability of the heart to  
60 ensure tissue oxygen supply (Randall, 1982; Taylor, 1992). Most fishes are ectotherms, so increases  
61 in water temperature have direct thermodynamic effects on their metabolic rate and consequent  
62 oxygen demand, which the heart must be able to respond to (Cossins and Bowler, 1987; Schulte,  
63 2011; Rodgers et al., 2016). Investigating how the heart responds to hypoxia and warming is of  
64 increasing relevance, because of the hypoxic episodes and summer heatwaves that are occurring in  
65 many aquatic ecosystems due to global change (Eliason and Anntilla, 2017; Stecyk, 2017; Costa and  
66 Barletta, 2016; Altieri and Diaz, 2018; Stillman, 2019).

67 The primary cardiac response to hypoxia in fishes is a slowing of heart rate frequency ( $f_H$ ),  
68 known as hypoxic bradycardia (see Taylor, 1992; Farrell, 2007; Stecyk, 2017, for detailed reviews).  
69 Although bradycardia is known to be a chemoreflexive response, there is still debate about its actual  
70 functional significance for hypoxia tolerance (Farrell, 2007; McKenzie et al., 2009; Joyce et al., 2016;  
71 Stecyk, 2017). When warmed, fishes exhibit increased  $f_H$ , a tachycardia that may have multiple  
72 contributing mechanisms (Eliason and Anntilla 2017). It presumably serves to meet the increased  
73 oxygen demands caused by thermal acceleration of metabolism, such that intrinsic capacity to raise  
74  $f_H$  may be a determinant of a species' upper temperature tolerance (see Eliason and Anntilla, 2017,  
75 for a detailed review). Although these cardiac responses to hypoxia and warming have been  
76 described in multiple species, this has exclusively been from acute experiments under controlled  
77 conditions with animals confined in some way, almost always instrumented with wires connected to  
78 a measurement device (Stecyk 2017; Eliason and Anntilla, 2017). It is not known, therefore, whether  
79 such cardiac responses to hypoxia and warming would also be observed in fishes free to swim and  
80 exhibit behavioural responses to the stressors.

81 Small biologging tags that record  $f_H$  from the electrocardiogram (ECG) are now available,  
82 which can be implanted into fishes to measure their cardiac activity when they are recovered and  
83 free-swimming (Prystay et al. 2017, Brijs et al. 2018; Ekström et al. 2018, Bjarnason et al. 2019). Such  
84 tags can also log external tri-axial body acceleration (EA), such that it is possible to interpret changes  
85 in  $f_H$  against simultaneous measures of spontaneous behaviour (Clark et al. 2010). That is, such tags  
86 can measure cardiac responses to hypoxia and warming in fishes swimming freely, and provide

87 insight into how such responses might be affected by spontaneous behavioural reactions to the  
88 environmental stressors. This should show cardiac responses that are a more reliable reflection of  
89 response patterns by wild animals in their natural environment.

90 We implanted biologgers into gilthead seabream *Sparus aurata*, to compare cardiac and  
91 behavioural responses to progressive hypoxia and warming when the animals were either shoaling in  
92 a tank or confined in individual respirometer chambers. We expected that fish would exhibit hypoxic  
93 bradycardia and warming tachycardia in both tank and respirometers, but that patterns and  
94 thresholds of cardiac responses to the stressors could differ between the holding conditions. In  
95 particular, we expected that  $f_H$  of fish would be higher in the tank, due to spontaneous activity, with  
96 consequences for cardiac response patterns to the stressors that we were unable to predict with any  
97 confidence. Nonetheless, we expected to find an overall direct relationship between activity in the  
98 tank, logged as EA, and  $f_H$ . We also expected to confirm, using respirometry in the chambers, that  $f_H$   
99 is a predictor of metabolic rate in this species (Hachim et al., 2020).

## 100 MATERIAL AND METHODS

### 101 Ethical approval

102 Experimental procedures were approved by the ethics committee for animal experimentation n° 036  
103 of the French Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation, with  
104 reference number APAFIS#20294-2019040516446800 v3.

### 105 Animals

106 Experiments were performed on  $n = 12$  seabream with a mass of approximately 500 g and age of  
107 approximately 18 months. The seabream were obtained from the Ferme Marine du Douhet (La Brée  
108 les Bains, France) as post-larvae then reared at the Ifremer Aquaculture Research Station in Palavas-  
109 les-Flots, in indoor cylindrical tanks (vol 2 m<sup>3</sup>) under seasonal photoperiods, provided with a flow of  
110 biofiltered and UV-treated seawater at 21 °C. Fish were fed daily with commercial pellets but fasted  
111 for 24 h prior to surgery.

### 112 Surgery

113 Fish were anesthetized by immersion in 0.1 g l<sup>-1</sup> benzocaine in aerated seawater, until active  
114 ventilation ceased, then weighed and placed on an operating table with their gills irrigated with  
115 aerated seawater containing 0.05 g l<sup>-1</sup> benzocaine. Heart rate loggers (DST milli HRT-ACT, 13 mm ×  
116 39.5 mm, 12 g, Star-Oddi, Iceland, [www.star-oddi.com](http://www.star-oddi.com)) were implanted in the intraperitoneal space,  
117 via an incision in the midline below the pectoral fins. Loggers were advanced as close as possible to  
118 the pericardium and fixed with sutures (silk suture and non-absorbable monofilament) such that  
119 their ECG electrodes lay against the body wall, with the incision then closed with sutures (non-  
120 absorbable monofilament). Fish were recovered in a 1 m<sup>3</sup> cylindrical tank provided with a flow of  
121 aerated, biofiltered and UV-treated seawater at 21 °C, with minimal disturbance beyond visual

122 inspection in morning (08:30) and evening (17:00). Fish were not fed during recovery or subsequent  
123 experiments. The tank was in a separate room with a natural photoperiod through skylights, shielded  
124 behind black plastic curtains with all disturbance kept to an absolute minimum. The timing of  
125 recovery is shown in figure 1.

### 126 **Hypoxia and temperature challenges**

127 Fish were instrumented and studied in two groups of six individuals, with each individual tested  
128 when swimming freely in the tank and also when confined in a respirometer. On the fourth day after  
129 surgery (Fig 1), three of the six fish were netted from the 1 m<sup>3</sup> tank and placed in individual  
130 rectangular respirometer chambers (vol. 9 l) which were submerged in a small raceway in the same  
131 room and provided with the same water as the tank. After 24h acclimation to the respirometers  
132 (hence at 5 days after surgery for all animals, Fig 1), experiments were conducted over five days. For  
133 the first set of six individuals, these had the following sequence: on the first day fish were exposed to  
134 a warming challenge, the second to a hypoxia challenge. At the end of the second day, the three fish  
135 were exchanged between tank and respirometers and allowed 24 h to recover and acclimate to their  
136 new holding condition. On the fourth day they were given a hypoxia challenge, on the fifth, a  
137 warming challenge. For the second set of six, the order of exposure was changed to offset any effect  
138 of experimental sequence on responses. The order was hypoxia, warming, exchange fish, warming,  
139 hypoxia. Black plastic curtains were used to screen tank and respirometers from visual disturbance,  
140 fish were observed through small holes in the curtain. Care was taken to reduce all disturbance to a  
141 minimum during experiments, experimenters entered at 08:20 to set up the trials then gave fish 30  
142 min to recover from any possible disturbance before commencement.

143 For progressive hypoxia, oxygen partial pressure in the tank and raceway was decreased  
144 simultaneously by bubbling with 100% nitrogen, from 100 % (normoxia) to 80 %, then in steps of 10  
145 % from 80 % to 20 %, then finally 15%. Each step had a duration of 30 min, water oxygen levels were  
146 recorded using optical oxygen probes (Firesting sturdy dipping probes, Pyroscience,  
147 [www.pyroscience.com](http://www.pyroscience.com)) and meter (FireSting FSO2-4), with data displayed and recorded in the Pyro  
148 Oxygen Logger software, with nitrogen flow and setpoints controlled manually. For acute warming,  
149 temperature was raised simultaneously in the tank and respirometers, in steps of 1 °C every 30 min,  
150 from 21 to 31 °C, using automated temperature control systems (AquaMedic T controller twin,  
151 [www.aqua-medic.de](http://www.aqua-medic.de)) that reached incremental setpoints precisely by controlling activity of a  
152 submerged pump, in tank or raceway, that generated a flow of water through heat exchange coils  
153 immersed in a reservoir (1 m<sup>3</sup>) of tapwater held at 40 °C. All fish were exposed to these levels of  
154 hypoxia and warming; the limits of 15 % saturation in hypoxia and 31 °C in warming were chosen to  
155 avoid major stress, which might affect subsequent responses during the 5-day exposure protocol. At

156 the end of the trials fish were euthanized by a lethal dose of benzocaine ( $1 \text{ g l}^{-1}$ ) to retrieve the logger  
157 and data.

158 Rates of oxygen uptake ( $\dot{M}O_2$ ,  $\text{mmol kg}^{-1} \text{ h}^{-1}$ ) were measured on the fish in the respirometers,  
159 using automated intermittent stopped-flow respirometry (Steffensen, 1989) over a 15 min cycle,  
160 providing two measures of  $\dot{M}O_2$  for each 30 min step of hypoxia or warming. Water oxygen  
161 concentrations were recorded continuously in each respirometer using Firesting sturdy dipping  
162 probes and meter, with data displayed and recorded in Pyro Oxygen Logger. Each fish's  $\dot{M}O_2$  was  
163 then calculated considering rate of decline in oxygen concentration in the chamber, chamber volume  
164 and the mass of the fish (McKenzie et al., 1995; 2007). Background measurements, on empty  
165 chambers, were made prior to placing the fish and at the end of each series. These were always  
166 negligible and so no corrections were applied.

### 167 **Logger programming and data processing**

168 The loggers were programmed with Mercury software (Star-Oddi) according to the manufacturer's  
169 instructions. For  $f_H$ , the ECG data were sampled at 200 Hz for 4 s, once every 5 min from 07:00 to  
170 18:00 during experiments. During recovery from surgery and at night during experiments,  $f_H$  was  
171 measured only once every two hours. An ECG trace was saved with each measure of  $f_H$ , for visual  
172 confirmation of data quality (Fig. S1). For EA, data were collected at 10 Hz for 1 min, once every 5  
173 min from 07:00 to 18:00 during experiments for the first series, but only from 09:00 to 17:00 for the  
174 second series, to ensure sufficient battery life. Water temperature, date and time were also recorded  
175 with each  $f_H$  and EA measurement.

176 Heart rate was returned in  $\text{beats min}^{-1}$ , calculated by the manufacturer's Patternfinder  
177 v.1.16.0 software from R-R intervals in the QRST wave of the ECG (Altimiras et al. 1997). Each  
178 measure was confirmed by visual inspection of the ECG trace and manual calculation of R-R interval  
179 within Patternfinder. The variance of EA ( $\text{VAR}_m$ )  $\text{VAR}_m$  was used to identify periods where variation in  
180 acceleration indicated bouts of activity or agitation. It was calculated as the variance of the 600 EA  
181 measurements per minute, which indicated when the sensor was measuring acceleration above 1 g,  
182 in units of mg ( $1000 \text{ mg} = 1 \text{ g}$ ), where EA = 0 is equal to 1 g and EA = 1000 is equal to 2 g. Each  
183 measure of  $f_H$  or  $\text{VAR}_m$  was associated with temperature, date and time recorded on the logger. Date  
184 and time information on the logger were used to establish the associated oxygen levels for the  
185 hypoxia trials, based on the oxygen probe recordings.

### 186 **Statistical analysis**

187 Statistics were performed with R version 3.5.3 (R Core Team 2019), with  $p = 0.05$  taken as the fiducial  
188 level for statistical significance. A one way analysis of variance ANOVA with repeated measures (stats  
189 package, aov function) was used to compare  $f_H$  across recovery days following surgery. Holm-  
190 Bonferroni post-hoc tests were used to identify any significant differences among days of recovery.

191 Paired Student t-test was used to compare discrete mean  $f_H$  values between tank and respirometer,  
192 namely undisturbed values in the morning prior to trials, plus the maximum and minimum  $f_H$  during  
193 trials and the oxygen partial pressure or temperatures at which these occurred. For these tests,  
194 normality, homoscedasticity and independence of the residuals were verified visually.

195 Effects of stressors on  $f_H$  were evaluated and compared between holding conditions by two-  
196 way ANOVA with repeated measures (afex package, aov\_car function), where one factor was tank  
197 versus respirometer and the repeated factor was either oxygen level or temperature. Undisturbed  
198 values (21 °C, normoxia) were included in the ANOVA. Normality of the data was verified with a  
199 Shapiro-Wilk test. Sphericity of the data was not met, therefore a Greenhouse-Geisser correction  
200 was applied. Homoscedasticity of the residuals was verified visually by plotting them as a function of  
201 their fitted values. Holm-Bonferroni post-hoc tests were used to identify where significant  
202 differences occurred. As a two-way ANOVA with repeated measures does not tolerate missing data,  
203 17  $f_H$  measures were imputed using either nearest neighbours' method (kNN function from DMwR  
204 package) or linear regression method (see results). These missing and imputed data represented  
205 1.7% of total data. Linear regression models were always significant and calculated  $f_H$  was always  
206 plausible.

207 As  $VAR_m$  data were not normal, effects of the stressors and comparison between holding  
208 conditions were evaluated with a generalized mixed linear model, with fixed factors being holding  
209 condition and either oxygen partial pressure or temperature, and individuals as a random effect.  
210 Tukey post-hoc tests were used to identify where any significant differences lay.

211 A linear relationship between  $VAR_m$  and  $f_H$  was established for all individuals using a  
212 generalized mixed linear model, and between  $f_H$  and  $\dot{M}O_2$  using a mixed linear model, with  
213 individuals as a random effect. Regression slopes between temperature and hypoxia trials were  
214 compared using the function `lstrends` from the `lsmeans` R package. A linear relationship was also  
215 established between  $f_H$  and  $\dot{M}O_2$  for each individual, using a linear model. Homoscedasticity and  
216 independence of the residuals were verified visually.

## 217 RESULTS

218 A complete dataset was collected for  $n = 10$  seabream (four from first and six from second series),  
219 with a mean ( $\pm$  S.D.) mass of  $534 \pm 86$  g, ranging from 363 to 801 g.

### 220 Routine $f_H$

221 Over three days of recovery with absolutely minimal disturbance (Fig 1), overall daily mean ( $\pm$  S.D.)  $f_H$   
222 showed a progressive and significant decrease ( $p < 0.001$ ) after which some fish were netted and  
223 transferred to chambers, to start experiments. For the ensuing five days, fish were necessarily  
224 subjected to daily disturbance from presence of experimenters, plus the exposures to hypoxia and  
225 warming. Nonetheless, when relatively undisturbed between 07:00 and 08:00 in the morning prior to

226 starting exposures, the mean  $f_H$  of fish was significantly lower in the tank than the respirometers  
227 (Tab. 1, Figs 2 and 3).

### 228 **Responses to hypoxia**

229 During progressive hypoxia, *S. aurata* displayed bradycardia in both tank and respirometers (Fig. 2).  
230 Within each condition,  $f_H$  did not vary significantly from 100 % down to 40 %, but then  $f_H$  decreased  
231 significantly at 30 %, 20 % and 15 %. There was, however, a significant difference in  $f_H$  between tank  
232 and respirometer during hypoxia trials ( $p < 0.01$ ) and a significant interaction between holding  
233 condition and oxygen level ( $p < 0.01$ ). That is, mean  $f_H$  was significantly lower in the tank than in the  
234 respirometers at all oxygen steps between 80 % and 40 %. Although undisturbed normoxic  $f_H$  differed  
235 significantly (Table 1, Fig 2), this was not true of the measures taken in normoxia as the first step of  
236 the exposure trial, notably because of increased individual variation in  $f_H$  in the respirometers (Fig 2).  
237 This was presumably because the fish had been disturbed by presence of experimenters. Once  
238 bradycardia occurred, namely at 30 %, 20 % and 15 %, there were no significant differences in  $f_H$   
239 between tank and respirometers (Fig. 2). These different patterns of  $f_H$  during hypoxia, between the  
240 two holding conditions, were reflected in the fact that mean maximum  $f_H$ , whenever this might have  
241 occurred during hypoxia trials, was significantly lower ( $p < 0.01$ ) in the tank than in the respirometers  
242 (Table 1). On the other hand, the mean minimum  $f_H$  was similar and occurred at a similar very low  
243 oxygen saturation (Table 1). Activity was generally low in hypoxia, with no significant differences in  
244 mean  $VAR_m$  at any level of hypoxia, or between tank and respirometer (Fig. S2). Note that  
245 undisturbed values of EA were not collected for all fish but, for four animals,  $VAR_m$  was low in both  
246 conditions, especially so in the tank (data not shown). Visual inspection of the tank revealed that the  
247 seabream were moving slowly around the perimeter in hypoxia and tended to stop swimming  
248 entirely and rest on the bottom of the tank at hypoxic levels that caused bradycardia.

### 249 **Responses to warming**

250 During progressive warming, *S. aurata* displayed tachycardia in both respirometers and tank (Fig. 3).  
251 Mean  $f_H$  was statistically similar between holding conditions at all temperatures, despite having been  
252 different when undisturbed at 21 °C (Table 1, Fig. 3). Once again,  $f_H$  at the initial 21 °C step of the  
253 exposure protocol was variable among individuals, presumably due to mild disturbance. There was,  
254 however, a significant interaction between holding condition and temperature ( $p < 0.01$ ). That is,  $f_H$   
255 increased significantly from 21 up until 27 °C in the tank, but only increased from 21 up until 25 °C in  
256 respirometers (Fig 3). Furthermore, the mean temperature at which maximum  $f_H$  occurred was  
257 significantly higher in the tank than in the respirometers, being closer to the maximum temperature  
258 tested (31 °C) in the tank (Table 1). During the warming trials,  $VAR_m$  was highly variable in the tank  
259 and, at 31 °C, was higher than at all other temperatures (Fig. 4). As would be expected on confined  
260 fish,  $VAR_m$  was relatively low in the respirometers and did not vary significantly with temperature



261 (Fig. 4). As a result, mean  $VAR_m$  in the tank was significantly higher than in the respirometer at many  
262 temperatures (Fig. 4). Visual inspection of the tank showed that the fish were swimming actively  
263 around the perimeter at high temperatures, with occasional bursts of speed, especially at 31 °C.

#### 264 **Relationships between $VAR_m$ and $f_H$ , and $f_H$ and $\dot{M}O_2$**

265 There was a significant linear relationship between  $VAR_m$  and  $f_H$  in the tank during the warming trials  
266 ( $p < 0.001$ ), the only condition where animals showed significant activity (Fig. 5). There was no relation  
267 of  $f_H$  to  $VAR_m$  under any other condition.

268 There was a significant positive linear relationship between  $f_H$  and  $\dot{M}O_2$  in the respirometers  
269 during both hypoxia ( $\dot{M}O_2 = f_H (0.034) + 0.4$ ;  $R^2 = 0.65$ ;  $p < 0.001$ ) and warming trials ( $\dot{M}O_2 = f_H (0.02)$   
270  $+ 3.37$ ;  $R^2 = 0.72$ ;  $p < 0.001$ ). There was no significant difference between these two slopes, so a  
271 single linear relationship between  $f_H$  and  $\dot{M}O_2$  was fitted for all hypoxia and warming values plotted  
272 together, which was highly significant ( $p < 0.001$ ; Fig. 6). Heart rate was also a predictor of metabolic  
273 rate for each individual fish (Table S1).

#### 274 **DISCUSSION**

275 This study is the first report of cardiac responses to hypoxia and warming in a free-swimming fish,  
276 although these cardiac loggers have been used on several species (e.g. Norling, 2017; Davidsen et al.,  
277 2019; Prystay et al., 2017, 2019; Brijs et al., 2018, 2019; Skeeles et al. 2020). The results supported  
278 our expectations, in that fish exhibited hypoxic bradycardia and warming tachycardia in both tank  
279 and respirometer. For warming tachycardia, there was clear evidence that increased spontaneous  
280 activity levels in the tank could contribute to increased  $f_H$  and affect response pattern. A major  
281 unexpected result, however, was that routine  $f_H$  of undisturbed seabream was higher when they  
282 were confined compared to free-swimming.

#### 283 **Routine heart rates and the effects of confinement**

284 The relatively high  $f_H$  measured over a day after surgery presumably indicates an acute stress  
285 response, which may have included a release of circulating catecholamines (Reid et al., 1998, Gallo  
286 and Civinini 2003) and/or removal of inhibitory cholinergic neural control (Randall 1982, reviewed in  
287 Farrell et al. 1984). The progressive decline in  $f_H$  during ensuing recovery presumably indicates an  
288 associated decline in stress and recovery of autonomic control (Campbell et al. 2004, 2007; Taylor et  
289 al. 2010, reviewed in Sandblom et al. 2011). In free-swimming Atlantic salmon *Salmo salar*, Føre et al.  
290 (2020) found that an average of 4 days was required for to recover a stable  $f_H$  after implantation of  
291 these loggers. The routine  $f_H$  of the seabream in the third day of recovery, a mean of 59 beats  $min^{-1}$   
292 at 21 °C, are amongst the lowest resting values reported for this species (Aissaoui et al. 2000, 2005;  
293 Altimiras et al. 1997; Hachim et al., 2020). Comparisons are confounded by differences in size and  
294 water temperature, and by our finding that confinement raises  $f_H$  in this species, since all previous  
295 studies were on confined fish.

296 The most obvious explanation for the fact that, when undisturbed, seabream had higher  $f_H$  in  
297 the respirometers would be a stress response to confinement, as stress is known to increase heart  
298 rate in fishes (Farrell et al. 1991, Sopinka et al. 2016, Rabben and Furevik 1993, Claireaux et al. 1995,  
299 Lefrancois et al. 1998, Svendsen et al. 2021). The proximate mechanism for the high  $f_H$  in seabream  
300 confined in chambers requires further investigation. This implies, nonetheless, that allowing the  
301 seabream to shoal with conspecifics was less stressful than being confined alone. This finding  
302 indicates that confinement may introduce bias into studies of physiological responses by fishes to  
303 environmental stressors, in a manner that may differ among species. Notably, it could bias measures  
304 of metabolic rate by static respirometry, given that  $f_H$  can be a predictor of  $\dot{M}O_2$ , which is the case for  
305 *S. aurata* (Hachim et al., 2020). The experiments also revealed how sensitive seabream were to  
306 disturbance as, despite taking great care, our simple presence was enough to obscure differences in  
307  $f_H$  between tank and respirometer at trial commencement in normoxia at 21 °C.

### 308 **Responses to hypoxia**

309 The data demonstrate that hypoxic bradycardia is observed in free-swimming fish, with a general  
310 pattern of response very similar to that observed when confined in a respirometer. Hypoxic  
311 bradycardia is a reflex response in teleosts, the sensory arm being chemoreceptor nerve cells in and  
312 around the gills that sense oxygen levels in ventilatory water and blood streams and transmit this  
313 information to the brainstem. The reflex response occurs via cholinergic fibres in the cardiac branch  
314 of the vagus nerve, which slow the heart (reviewed by Taylor 1992; Farrell and Smith 2017; Stecyk,  
315 2017). The functional significance of hypoxic bradycardia is still debated but it may protect function  
316 of the cardiac pump, a purely aerobic organ, by conserving contractility and reducing myocardial  
317 energy requirements when oxygen supply in the blood is below a critical level (Farrell, 2007;  
318 McKenzie et al., 2009; Iversen et al., 2009; Joyce et al., 2016).

319 It is noteworthy that, although  $f_H$  was significantly lower in the tank compared to the  
320 respirometer at oxygen levels above the threshold for hypoxic bradycardia, this threshold did not  
321 differ, being between 40 and 30 % in both conditions. The higher  $f_H$  in seabream confined in a  
322 respirometer should, presumably, have been accompanied by a higher  $\dot{M}O_2$  than when swimming  
323 freely in the tank, given the direct relationship between these two variables. It might be expected,  
324 therefore, that the threshold for bradycardia would be higher in respirometer than in the tank. The  
325 fact that the threshold was the same and that, once bradycardia did occur,  $f_H$  was similar between  
326 tank and respirometer, requires further investigation.

327 The low  $VAR_m$  during progressive hypoxia, and absence of differences between tank and  
328 respirometers, presumably indicates that movements in the tank did involve changes in speed, which  
329 are necessary to engender variation in acceleration (Tanaka et al. 2001; Hinch et al. 2002; Kawabe et

330 al. 2003). That is, the gentle movements observed during hypoxia in the tank were clearly below the  
331 sensitivity of the accelerometer in the tag.

### 332 **Responses to warming**

333 Although these tags have been used to study cardiac responses to acute warming in an anaesthetised  
334 fish (Skeeles et al. 2020), this is the first report of responses by a fully-recovered free-swimming  
335 animal. As for hypoxia, cardiac responses were generally similar between tank and respirometer,  
336 with a pronounced tachycardia in both cases. Warming tachycardia in fishes presumably represents a  
337 response to increased oxygen demand when metabolism is accelerated by warming, as  
338 demonstrated by the linear relationship of  $f_H$  and  $\dot{M}O_2$  during warming in the respirometer. In terms  
339 of the heart itself, this response may reflect both direct effects of temperature on pacemaker  
340 function and modulation of autonomic control (see Eliason and Anntilla, 2017, for a detailed review).  
341 In the seabream, the maximum  $f_H$  observed during warming, 205 beats  $\text{min}^{-1}$  at a temperature of 31  
342 °C, was about double the maximum achieved during forced exercise in a swim-tunnel at 16 °C in this  
343 species (Hachim et al., 2020). It was also considerably higher than the maximum  $f_H$  typically reported  
344 for temperate fishes, of around 140 beats  $\text{min}^{-1}$  (Casselmann et al. 2012, Farrell et al. 2009). These  
345 very high  $f_H$  in the seabream were all confirmed by visual inspection of the traces, with clear ECG  
346 waveforms (Fig. S1).

347 It is interesting that, unlike in hypoxia, the accelerometer detected activity in the tank during  
348 warming, especially at the higher temperatures. The consistently higher  $\text{VAR}_m$  in the tank relative to  
349 the respirometer, for most warming steps, would explain why  $f_H$  did not differ significantly between  
350 the holding conditions. That is, the  $\text{VAR}_m$  data confirm that, in free-swimming individuals, some of  
351 the tachycardia was due to behavioural responses to warming. The fact that activity contributed to  
352 the cardiac response in the tank may explain why the mean temperature for maximum  $f_H$  differed  
353 between holding conditions, being higher in the tank and close to the maximum temperature tested,  
354 31 °C. The data revealed a threshold for a behavioural response, with the significant increase in  $\text{VAR}_m$   
355 relative to acclimation temperature, when fish had reached 31 °C. The activity observed in the tank,  
356 especially the bursts of speed at high temperatures, may have reflected attempts to escape the  
357 conditions, although fish did not become agitated at the same temperature when confined in the  
358 respirometer. Such behavioural thresholds may be useful indicators of tolerance that are more  
359 sensitive than, for example, loss of equilibrium at a critical thermal maximum ( $\text{CT}_{\text{max}}$ ). In *S. aurata*,  
360  $\text{CT}_{\text{max}}$  ranges from about 34.3 °C to 36.6 °C, depending on acclimation temperature (Kir et al. 2020,  
361 Madeira et al., 2014, 2016)

### 362 **Relationships of heart rate to acceleration and metabolic rate**

363 The significant dependence of  $f_H$  on  $\text{VAR}_m$  during warming in the tank is further proof that activity  
364 was responsible for raising  $f_H$  of the free-swimming fish. The relationship was, nonetheless, rather

365 noisy with low predictive power. This may be because increases in  $VAR_m$ , especially at high  
366 temperatures, reflect agitation and burst swimming movements powered by fast-twitch glycolytic  
367 muscle (Webb, 1998). The metabolic costs of such movements are paid during recovery, rather than  
368 during the activity itself (Webb, 1998; Kieffer, 2000), so changes in  $f_H$  may have been out of phase  
369 with changes in  $VAR_m$ . Although measures of body acceleration have also been used to predict  
370 metabolic rate in fishes (Gleiss et al., 2010; Wilson et al., 2013; Wright et al., 2014; Metcalfe et al.,  
371 2016; Bouyoucos et al., 2019), it seems unlikely they will ever have the same predictive power as  $f_H$ ,  
372 not least because movement is only one component of metabolic activity in fishes.

373         The data confirm that heart rate can be used to predict metabolic rate in *S. aurata* (Hachim  
374 et al., 2020). The fact that a single linear relationship between mean  $f_H$  and mean  $\dot{M}O_2$  could be  
375 described, irrespective of whether data derived from exposure to hypoxia or warming, demonstrates  
376 a tight coupling of cardiac pumping activity to metabolic oxygen demand under diverse  
377 environmental conditions in this species. The relationships for individual animals were highly  
378 significant but their predictive power differed markedly among fish, with variation in  $f_H$  explaining  
379 less than 70 % of variation in  $\dot{M}O_2$  in six of the ten seabream. For this reason, we did not perform the  
380 exercise of predicting individual  $\dot{M}O_2$  from their  $f_H$  when in the tank. Further research is required to  
381 establish the extent to which this variation among individuals is methodological, for example because  
382  $f_H$  and  $\dot{M}O_2$  were measured over different time scales, or is physiological. Nonetheless, the results are  
383 promising in terms of calibrating the relationship of  $f_H$  to  $\dot{M}O_2$  using respirometry and then using  
384 logged  $f_H$  data to estimate patterns of energy use by free-swimming seabream (Lucas et al., 1993;  
385 Clark et al., 2010; Cooke et al., 2016; Treberg et al., 2016). The need to retrieve the tag is still a  
386 limitation on performing such studies on fish released into their natural environment (Prystay et al.,  
387 2017, 2019).

## 388 **Conclusions**

389 The results demonstrate that hypoxic bradycardia and warming tachycardia are observed in fish  
390 whether they are free to shoal in a tank or confined in a respirometer. The fact, however, that  
391 confining *S. aurata* in a respirometer raised their routine  $f_H$ , presumably due to stress, and that  $f_H$  is a  
392 predictor of metabolic rate, has clear implications for estimating metabolic traits by static  
393 respirometry in some fish species. Tachycardia in free-swimming fish during warming was due, to  
394 some degree, to increased spontaneous activity. That is, the combined measures of  $f_H$  and  $VAR_m$  in  
395 free-swimming fish provided novel insight into drivers of cardiac responses to temperature, and  
396 revealed a threshold for a behavioural response to warming. Overall, the results demonstrate that  
397 biologging of physiological and behavioural responses to hypoxia and warming, in free-swimming  
398 fish, can provide more valid and reliable data than on confined fish, and has potential to reveal  
399 sensitive sub-lethal thresholds for impacts of these stressors.

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- 567

568 Table 1. Elements of mean ( $\pm$  S.D.) heart rate ( $f_H$ , beats  $\text{min}^{-1}$ ) of *Sparus aurata* fitted with biologging  
 569 tags and exposed to hypoxia or warming, comparing when swimming as groups of three in a tank or  
 570 confined individually in a respirometer chamber. Undisturbed  $f_H$  indicates as measured between  
 571 07:00 and 08:00 in normoxia at 21°C, prior to the respective trial. Maximum refers to the mean of  
 572 the highest, and minimum to mean of the lowest,  $f_H$  observed in each fish in each trial. For hypoxia,  
 573  $\text{PO}_2$  at max or min refers to the mean oxygen partial pressure at which maximum or minimum  
 574 measures occurred, respectively. For warming, T at max or min refers to the mean temperature at  
 575 which these measures occurred. Asterisks indicate difference between holding conditions for that  
 576 variable, \* < 0.05, \*\*\* < 0.001

Stressor	Holding condition	
	Tank	Respirometer
Hypoxia		
Undisturbed	72 $\pm$ 12***	105 $\pm$ 13
Maximum	103 $\pm$ 10**	118 $\pm$ 13
$\text{PO}_2$ at max	71 $\pm$ 24	65 $\pm$ 18
Minimum	37 $\pm$ 6.7	39 $\pm$ 6
$\text{PO}_2$ at min	17 $\pm$ 4	19 $\pm$ 6
Warming		
Undisturbed	72 $\pm$ 15 ***	103 $\pm$ 11
Maximum	172 $\pm$ 22	166 $\pm$ 17
T at max	30.3 $\pm$ 1.2*	28.6 $\pm$ 2.2
Minimum	71.5 $\pm$ 22	81 $\pm$ 25
T at min	21.4 $\pm$ 1.8	22.8 $\pm$ 3.6

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580 **Figure captions**

581 Fig. 1. Timeline of fish recovery after surgery, with mean ( $\pm$  S.D.) heart rates ( $f_H$ ) for specific intervals.  
582 Each arrow represents an entire day. The undisturbed value corresponds to the mean  $f_H$  of all fish  
583 between 07:00 and 08:00, prior to experiments.

584 Fig. 1. Mean heart rate ( $f_H$ ) of *S. aurata* during progressive hypoxia while free-swimming in a tank or  
585 confined in a chamber. Central bars denote mean values and boxes denote standard deviation, with  
586 each point being the mean value of a single fish ( $n = 10$  in all cases). “U100” refers to  $f_H$  of fish when  
587 undisturbed in normoxia between 07:00 and 08:00, before hypoxia trials. The vertical dashed line  
588 indicates the beginning of the hypoxia trial. Asterisks show oxygen levels where there was a  
589 significant difference between tank and chamber; \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

590 Fig. 2. Mean heart rate ( $f_H$ ) of *S. aurata* during acute warming while free-swimming in a tank or  
591 confined in a chamber. Central bars denote mean values and boxes denote standard deviation, with  
592 each point being the mean value of a single fish ( $n = 10$  in all cases). “U21” refers to  $f_H$  of fish when  
593 undisturbed at 21 °C between 07:00 and 08:00, before temperature trials. The vertical dashed line  
594 indicates the beginning of the temperature trial. Asterisks show temperature levels where there was  
595 a significant difference between tank and chamber \*\*  $p < 0.01$ .

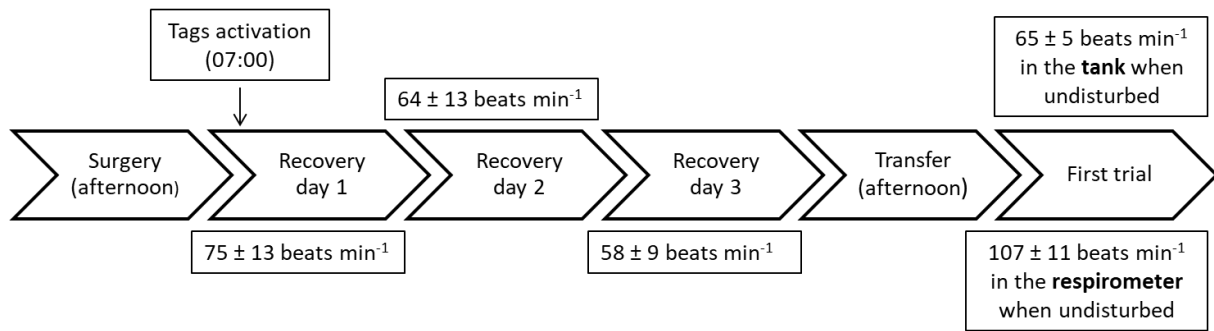
596 Fig. 3. Mean variance of acceleration ( $VAR_m$ ) of *S. aurata* during acute warming. Central bars are  
597 mean values, each point is the mean value of one fish, and boxes represent standard deviation at  
598 each temperature step ( $n = 10$  in all cases). Asterisks show temperature levels where there was a  
599 significant difference between tank and chamber \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ .

600 Fig. 4. The relationship of variance of acceleration ( $VAR_m$ ) to heart rate ( $f_H$ ) in *S. aurata* during  
601 warming trials in the tank. Points are  $VAR_m$  calculated over 60 s after the corresponding  $f_H$  value for  
602 every fish ( $n = 10$  in all cases). The relationship is described by  $VAR_m = f_H (0.87) + 41.2$  ( $R^2 = 0.36$ )

603 Fig. 5. The relationship of oxygen consumption ( $\dot{M}O_2$ ) to heart rate ( $f_H$ ) in *S. aurata* confined in  
604 respirometers, during exposure to hypoxia and warming. Each point represents the mean oxygen  
605 consumption as a function of mean  $f_H$  for a single fish at a given oxygen partial pressure or  
606 temperature ( $n = 10$  in all cases). The relationship is described by  $\dot{M}O_2 = f_H (0.045) - 0.23$  (conditional  
607  $R^2 = 0.76$ ) for all trials combined, represented by the black dashed line.

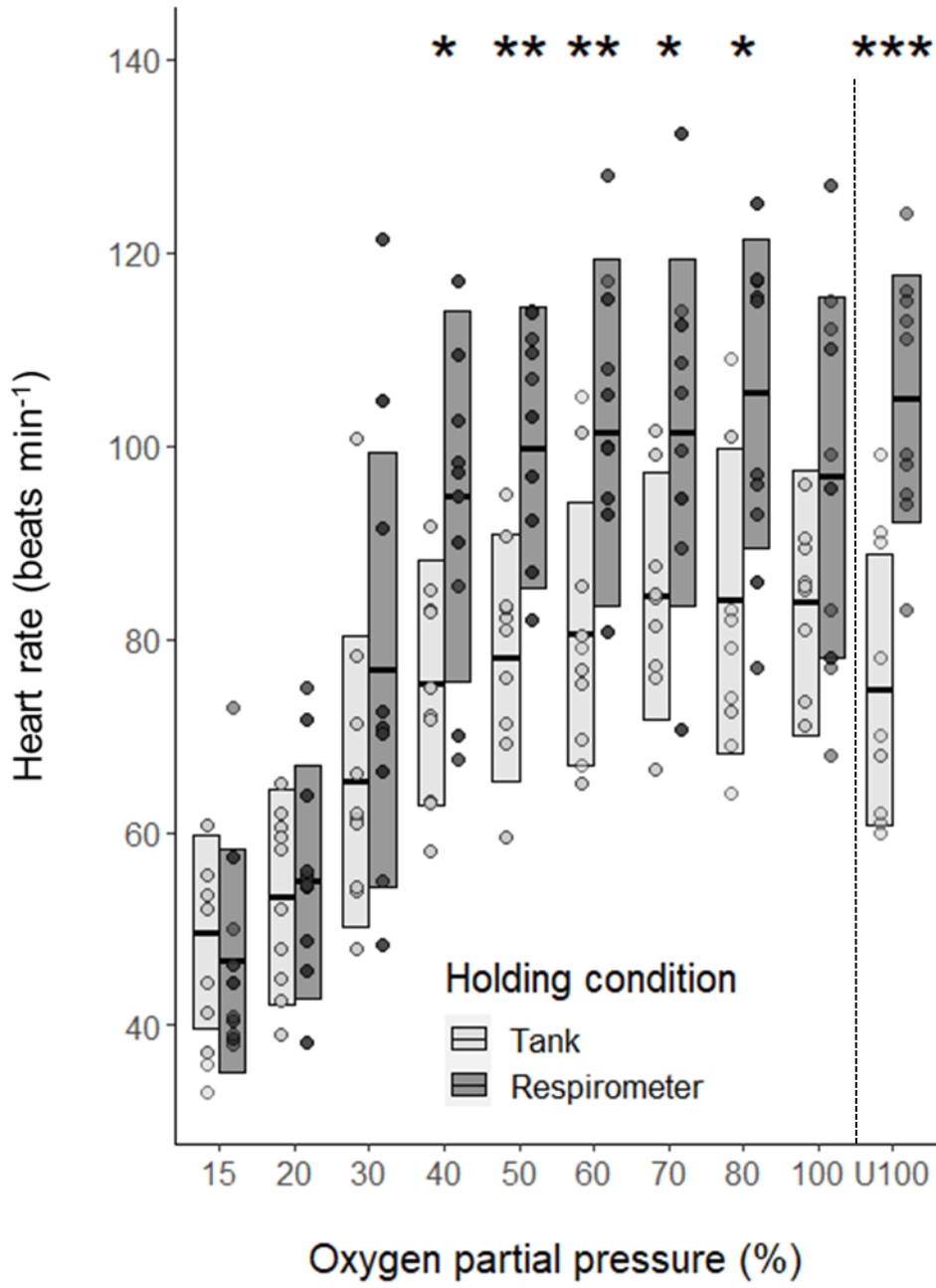
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609 Figure 1



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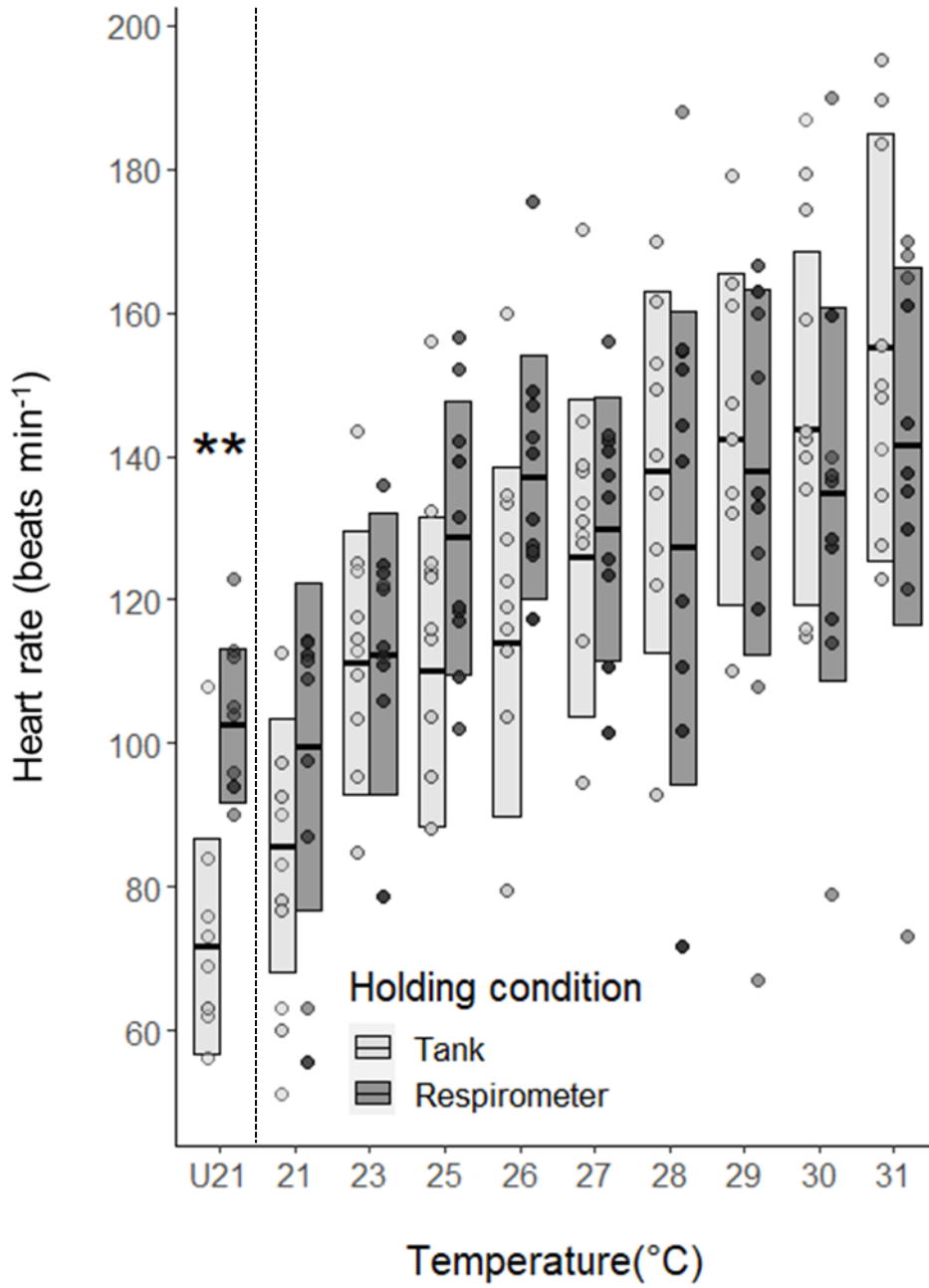
637 Figure 2



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640 Figure 3

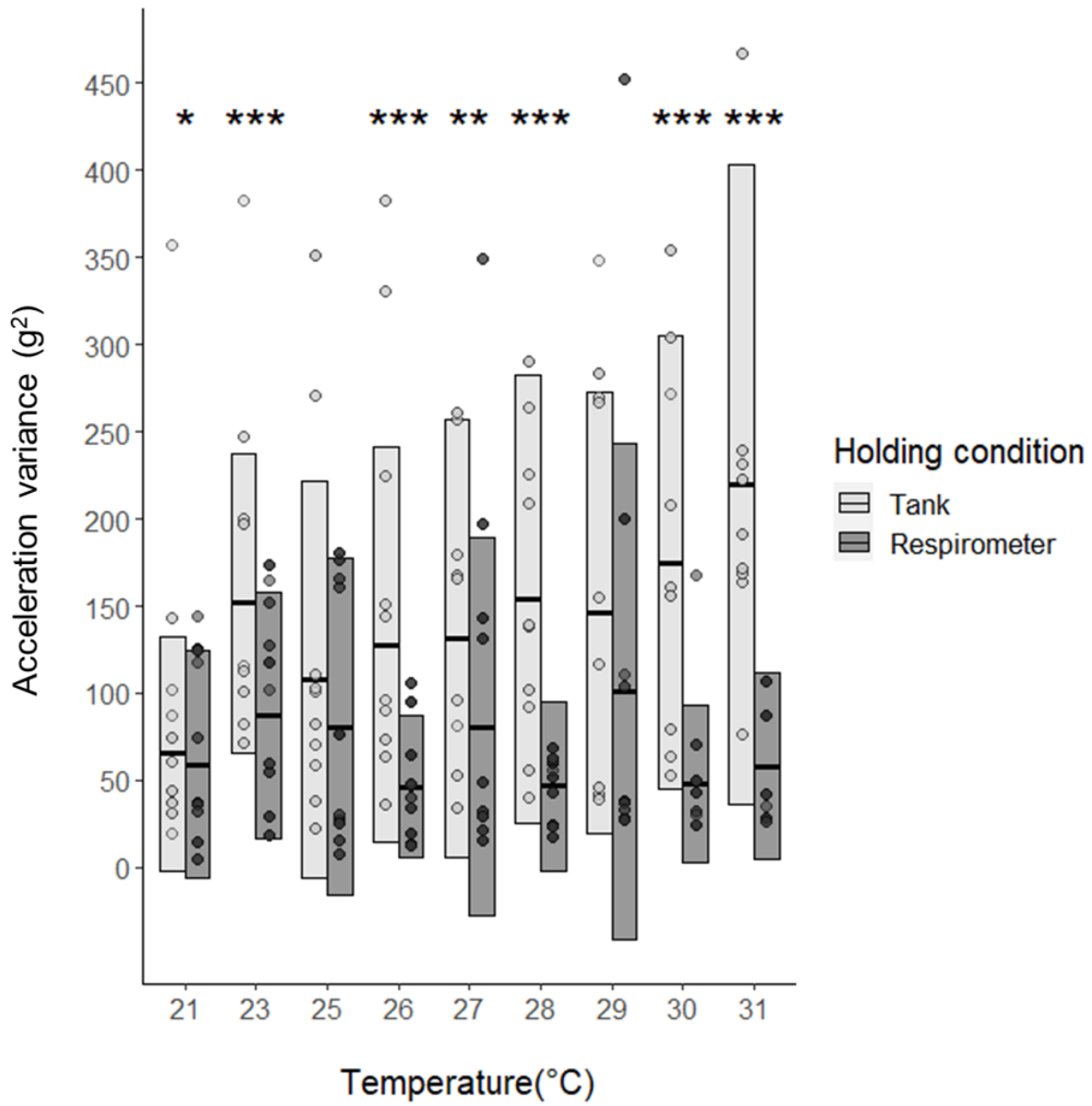


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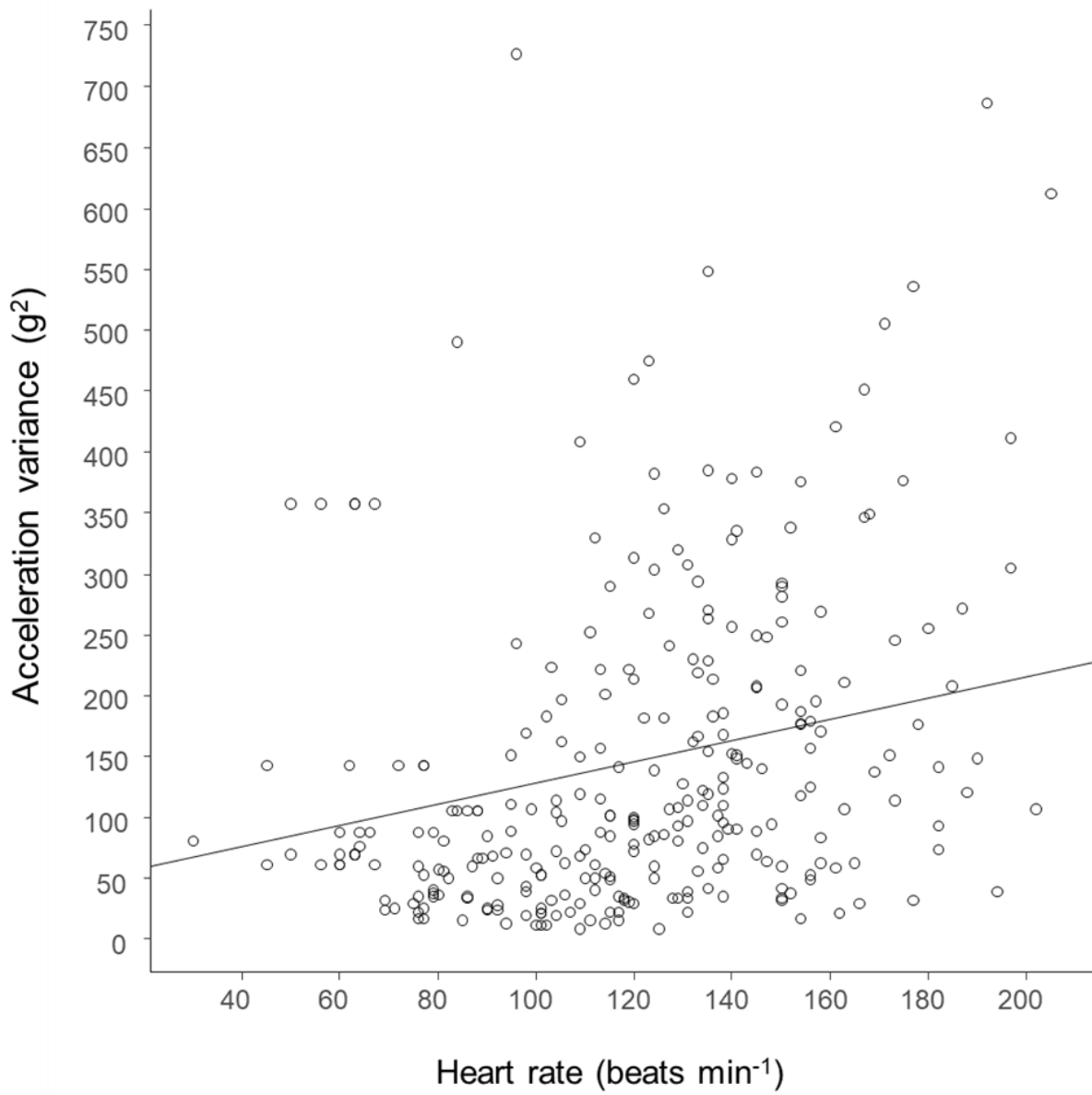
644 Figure 4



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647 Figure 5

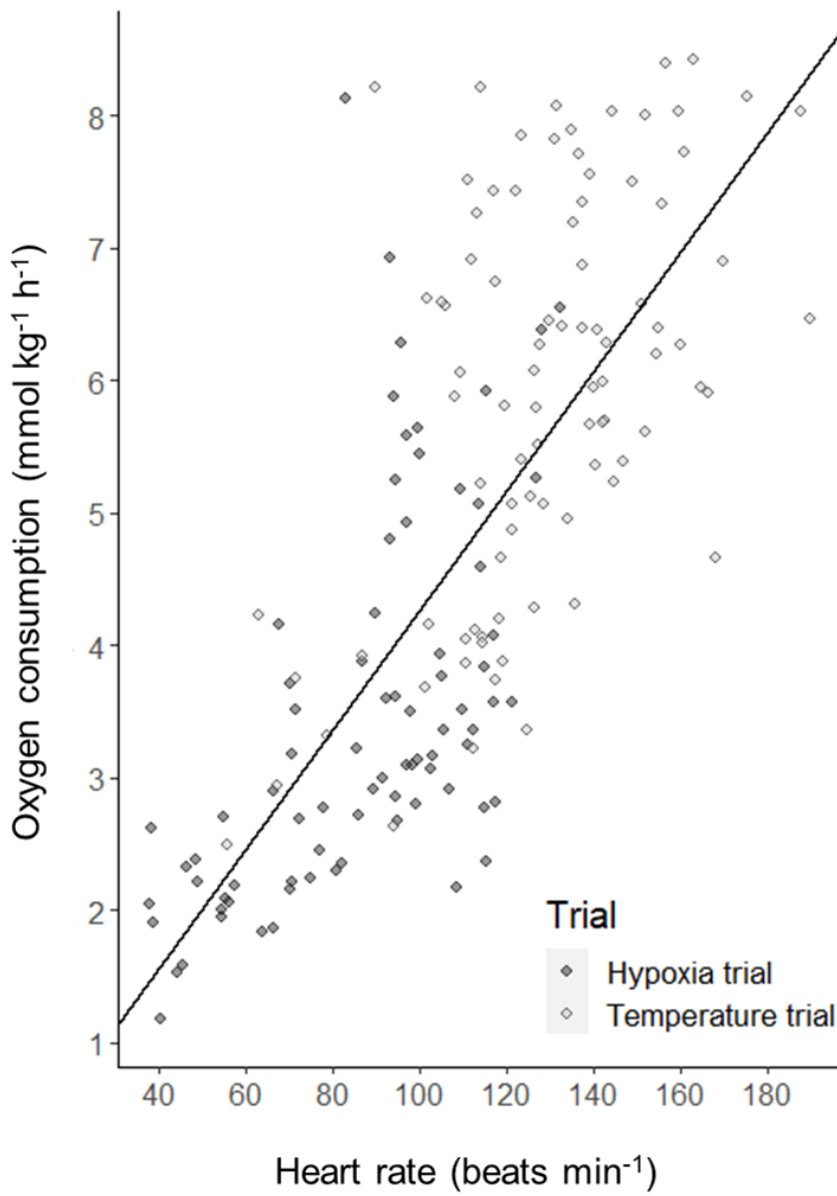


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650 Figure 6



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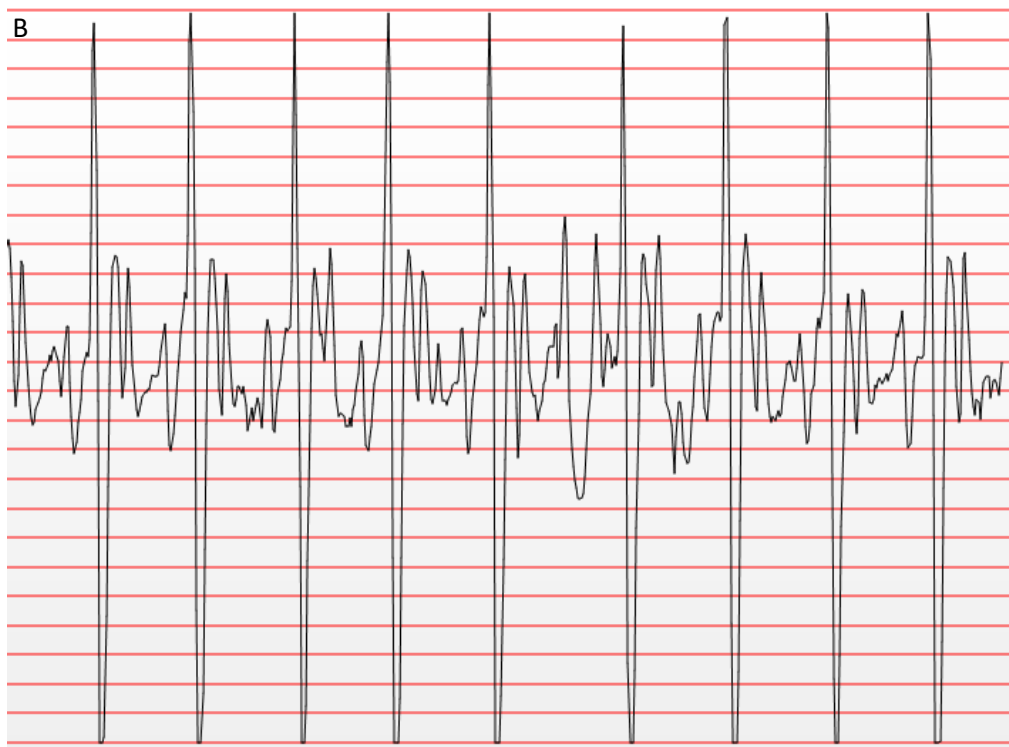
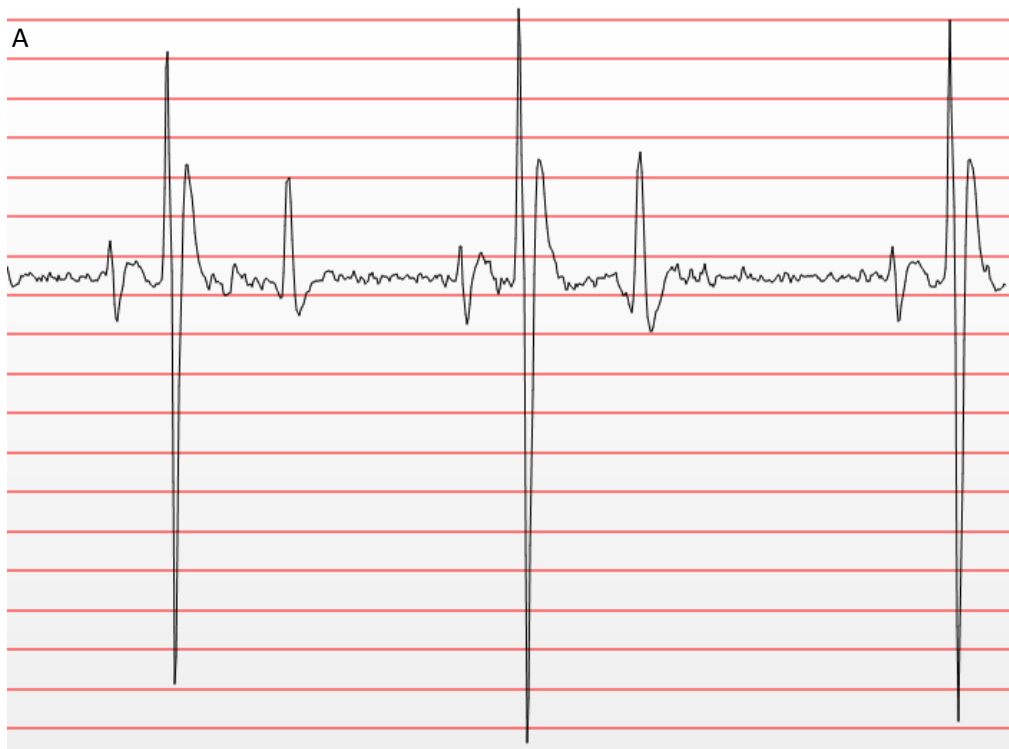
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654 Table S1: Slope, intercept p-value and  $R^2$  associated to the linear regression between  $f_H$  and  $MO_2$  for  
655 each fish.

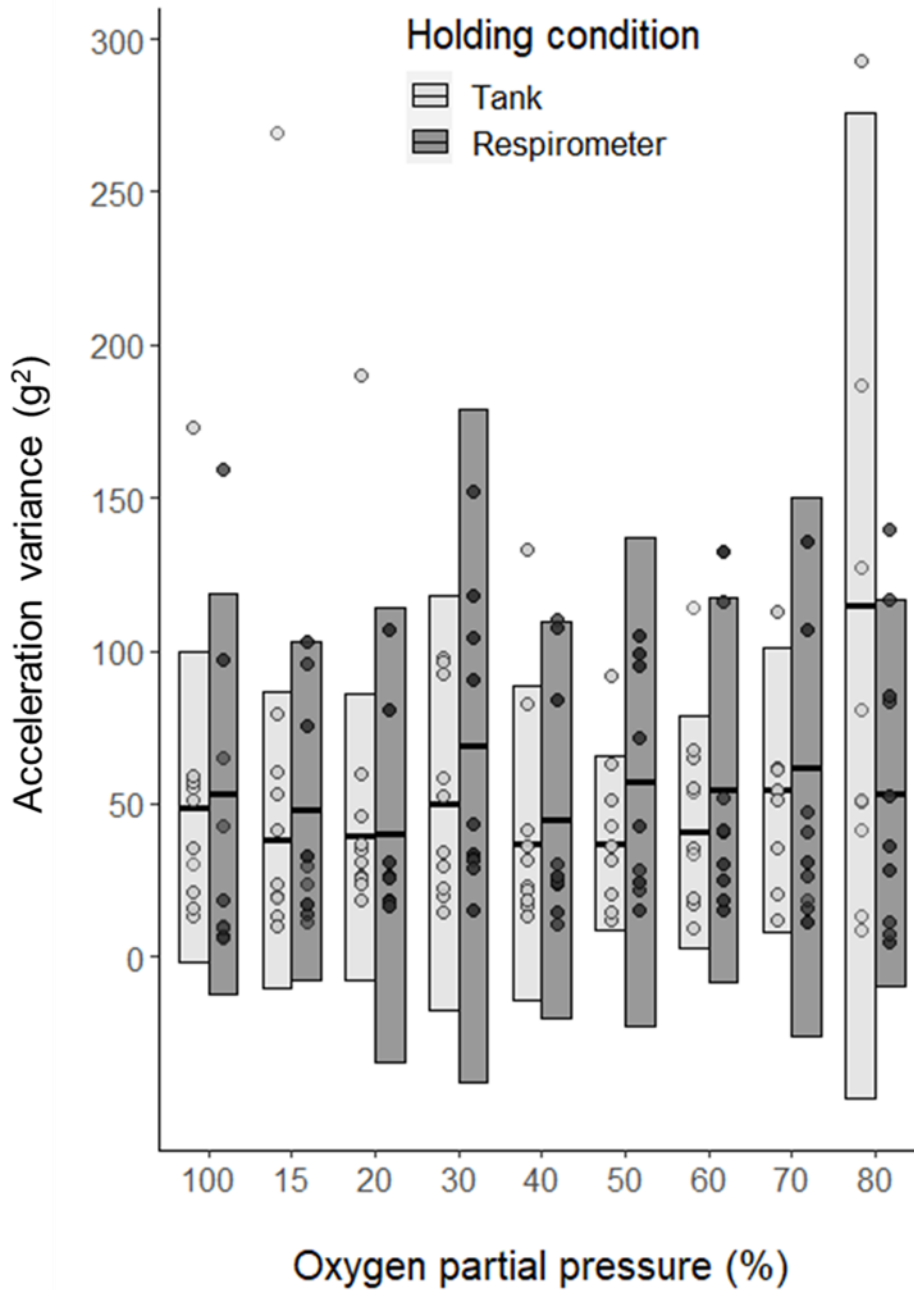
Fish number	1	2	3	4	5	6	7	8	9	10
p_value	$3e^{-7}$	0.00044	$5.6e^{-5}$	0.00019	$7.8e^{-5}$	$3.3e^{-9}$	0.0004	$5.6e^{-7}$	$6.4e^{-5}$	$5.3e^{-4}$
$R^2$	0.84	0.63	0.73	0.68	0.61	0.92	0.58	0.91	0.64	0.51
Slope	0.035	0.045	0.047	0.03	0.052	0.058	0.05	0.058	0.048	0.038
Intercept	0.67	-0.94	-1.4	0.48	-0.14	-0.098	-0.68	-1.27	-0.77	1

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657 Figure S1: Representative traces of ECG of seabream N°8, mass 500 g, recorded over a 4 s interval by  
658 the Star-Oddi 0086 tag, and displayed in PatternFinder® software. Panel A waveform is 51 beats min<sup>-1</sup>  
659 at 21 °C, panel B waveform is 194 beats min<sup>-1</sup> at 31 °C.



663 Figure S2: Mean variance of acceleration ( $VAR_m$ ) of *S. aurata* during acute hypoxia. Central bars are  
664 mean values, each point is the mean value of one fish, and boxes represent standard deviation at  
665 each oxygen partial pressure step ( $n = 10$  in all cases).  
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