Cardiac and behavioural responses to hypoxia and warming in free-swimming gilthead seabream, Sparus aurata

Alexandre Mignucci, Jérôme Bourjea, Fabien Forget, Hossein Allal, Gilbert Dutto, Eric Gasset, David J. Mckenzie

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

2 **(*Sparus aurata*)**

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38 **SUMMARY STATEMENT**

39 Biologging of cardiac responses to hypoxia and warming in a free-swimming fish revealed that

40 confinement in respirometer chambers raises heart rate, with consequences for estimates of

41 metabolic rates.

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46   **ABSTRACT**

47   Gilthead seabream were equipped with intraperitoneal biologging tags to investigate cardiac

48   responses to hypoxia and warming, comparing when fish were either swimming freely in a tank with

49   conspecifics or confined to individual respirometers. After tag implantation under anaesthesia, heart

50   rate (f_H) required 60 hours to recover to a stable value in a holding tank. Subsequently, when

51   undisturbed under control conditions (normoxia, 21°C), mean f_H was always significantly lower in the

52   tank than respirometers. In progressive hypoxia (100 - 15% oxygen saturation), mean f_H in the tank

53   was significantly lower than respirometers at oxygen levels until 40%, with significant bradycardia in

54   both holding conditions below this. Simultaneous logging of tri-axial body acceleration revealed that

55   spontaneous activity, inferred as the variance of external acceleration (VAR_m), was low and invariant

56   in hypoxia. Warming (21 to 31°C) caused progressive tachycardia with no differences in f_H between

57   holding conditions. Mean VAR_m was, however, significantly higher in the tank during warming, with a

58   positive relationship between VAR_m and f_H across all temperatures. Therefore, spontaneous activity

59   contributed to raising f_H of fish in the tank during warming. Mean f_H in respirometers had a highly

60   significant linear relationship with mean rates of oxygen uptake, considering data from hypoxia and

61   warming together. The high f_H of confined seabream indicates that respirometry techniques may bias

62   estimates of metabolic traits in some fishes, and that biologging on free-swimming fishes will provide

63   more reliable insight into cardiac and behavioural responses to environmental stressors by fishes in

64   their natural environment.

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68   *List of symbols and abbreviations:*

69   - f_H: heart rate frequency

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

71   - EA: external acceleration

72   - VAR_m: Variance of external acceleration

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74 **INTRODUCTION**

75 Cardiac performance is a core determinant of the ability of fishes to survive and thrive in their
76 environment, especially under challenging environmental conditions (Eliason and Anttila, 2017;
77 Farrell and Smith, 2017; Stecyk, 2017). For instance hypoxia, a reduced availability of dissolved
78 oxygen, is a common stressor in aquatic habitats (Diaz and Rosenberg, 2008) that challenges the
79 ability of the heart to ensure tissue oxygen supply (Randall, 1982; Taylor, 1992). Most fishes are
80 ectotherms, so increases in water temperature have direct thermodynamic effects on their
81 metabolic rate and consequent oxygen demand, which the heart must be able to respond to (Cossins
82 and Bowler, 1987; Rodgers, 2016; Schulte et al., 2011). Investigating how the fish heart responds to
83 hypoxia and warming is of increasing relevance, because of the hypoxic episodes and summer
84 heatwaves that are occurring in many aquatic ecosystems due to global change (Altieri and Diaz,
85 2019; Costa and Barletta, 2016; Eliason and Anttila, 2017; Stecyk, 2017; Stillman, 2019).

86 The primary cardiac response to progressive hypoxia in fishes is a slowing of heart rate (f_H),
87 known as hypoxic bradycardia (see Farrell, 2007; Stecyk, 2017; Taylor, 1992, for detailed review).
88 Although bradycardia is a chemoreflex response, there is still debate about its actual functional
89 significance for hypoxia tolerance (Farrell, 2007; Joyce et al., 2016; McKenzie et al., 2009; Stecyk,
90 2017). When progressively warmed, fishes exhibit increased f_H, a tachycardia that may have multiple
91 contributing mechanisms (Eliason and Anttila, 2017). It presumably serves to meet the increased
92 oxygen demands caused by thermal acceleration of metabolism, such that intrinsic capacity to raise
93 f_H may be a determinant of a species' upper temperature tolerance (see Eliason and Anttila, 2017, for
94 a detailed review). Although these cardiac responses to hypoxia and warming have been described in
95 multiple species, this has almost exclusively been from acute experiments under controlled
96 conditions with animals confined in some way and instrumented with wires connected to a
97 measurement device (Eliason and Anttila, 2017; Stecyk, 2017). Very little is known about cardiac
98 responses to hypoxia and temperature in free-swimming fishes (Claireaux et al., 1995a; Claireaux et
99 al., 1995b; Lefrançois et al., 1998; Prystay et al., 2017).

100 Small biologging tags that record f_H from the electrocardiogram (ECG) are now available,
101 which can be implanted into fishes to measure their cardiac activity when they are recovered and
102 free-swimming (Bjarnason et al., 2019; Brijs et al., 2018; Ekström et al., 2018; Prystay et al., 2017). It
103 is not known whether cardiac responses to progressive hypoxia and warming will differ markedly in
104 fishes swimming freely compared to when they are confined, but responses in free-swimming
105 animals should be a more reliable reflection of responses by wild animals in their natural
106 environment (Claireaux et al., 1995 a,b; Lefrançois et al., 1998). The tags can also log external tri-axial
107 body acceleration (EA), such that it is possible to interpret cardiac responses of free-swimming
108 animals against simultaneous measures of spontaneous behaviour (Clark et al., 2010).

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109   We implanted biologgers into gilthead seabream *(Sparus aurata)* to compare cardiac

110 responses to progressive hypoxia and warming when each animal was either shoaling in a tank with

111 conspecifics, or confined in an individual respirometer chamber. We expected that, although fish

112 would exhibit hypoxic bradycardia and warming tachycardia when swimming freely in the tank

113 (Claireaux et al., 1995a,b; Lefrançois et al., 1998), the ability to express spontaneous activity would

114 alter responses compared to when confined in a respirometer. We expected that f_H of undisturbed

115 animals would be higher in the tank in normoxia at acclimation temperatures, due to spontaneous

116 swimming activity. Progressive hypoxia might cause progressive declines in fH in the tank, which

117 initially and prior to the chemoreflexive hypoxic bradycardia, were due to reduced spontaneous

118 swimming activity (Remen et al., 2015). In the respirometer, by contrast, fH would be low and

119 relatively stable until onset of chemoreflexive bradycardia (Perry et al., 2009). We expected f_H to

120 increase more rapidly in the tank with warming, due to stimulation of activity (Claireaux et al.,

121 1995a), with a maximum f_H reached at a lower temperature. We expected to demonstrate that

122 swimming activity was a determinant of cardiac activity in the tank, by revealing a direct relationship

123 between logged EA and f_H. Finally, the respirometers allowed us to investigate whether f_H was a

124 predictor of metabolic rate in *S. aurata* during exposure to hypoxia and warming, as it is during

125 aerobic exercise (Hachim et al., 2020).

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127 **MATERIAL AND METHODS**

128 **Ethical approval**

129 Experimental procedures were approved by the ethics committee for animal experimentation n° 036

130 of the French Ministère de l'Enseignement Superieur, de la Recherche et de l'Innovation, with

131 reference number APAFIS #20294.

132 **Animals**

133 Experiments were performed on n = 12 seabream with a mass of approximately 500 g and age of

134 approximately 18 months. Sex was not verified, but fish were most probably male, as *S. aurata* is a

135 protandrous hermaphrodite species. The seabream were obtained from the Ferme Marine du

136 Douhet (La Brée les Bains, France) as post-larvae then reared at the Ifremer Aquaculture Research

137 Station in Palavas-les-Flots, in indoor cylindrical tanks (vol 2 m^3) under seasonal photoperiods,

138 provided with a flow of biofiltered and UV-treated seawater at 21°C. Fish were fed daily with

139 commercial pellets (B-Grower Marin, Le Gouessant, www.legouessant.com) but fasted for 24 h prior

140 to surgery.

141 **Surgery**

142 Fish were anesthetized by immersion in 0.1 g l^{-1} benzocaine (Benzocaine ethyl 4-Aminobenzoate,

143 VWR, www.vwr.com) in aerated seawater, until active ventilation ceased, then weighed and placed

144   on an operating table with their gills irrigated with aerated seawater containing 0.05 g l^{-1} benzocaine.

145   Heart rate loggers (DST milli HRT-ACT, 13 mm × 39.5 mm, 12 g, Star-Oddi, Iceland, www.star-oddi.

146   com) were implanted in the intraperitoneal space, via an a ~2 cm longitudinal incision along the

147   ventral midline, ~0.5 cm posterior to the pectoral fins. Loggers were advanced as close as possible to

148   the pericardium and fixed with sutures (silk suture and non-absorbable monofilament) such that

149   their ECG electrodes lay against the body wall, with the incision then closed with sutures (non-

150   absorbable monofilament). Fish were left to recover in a 1 m^3 cylindrical tank provided with a flow of

151   aerated, biofiltered and UV-treated seawater at 21°C. The tank was isolated in a room dedicated

152   exclusively to the study, with a natural photoperiod through skylights. The tank was shielded behind

153   an opaque black plastic curtain. During recovery fish were checked by visual observation through

154   small holes in the curtain (McKenzie et al., 2007) in the morning (08:30) and evening (17:00). Fish

155   were not fed during recovery or subsequent experiments.

156   **Hypoxia and temperature challenges**

157   Fish were instrumented and studied in two groups of six individuals. To allow us to compare each

158   individual's responses to the challenges when either swimming freely in the tank or confined in a

159   respirometer, we used a protocol where they were sequentially exposed in one holding condition

160   and then in the other.

161   At 90h after surgery (Fig 1), three of the six fish were netted from the 1 m^3 tank and placed in

162   individual rectangular clear plastic respirometer chambers (vol. 9 l) which were submerged in a small

163   raceway in the same room, shielded behind an opaque black curtain and provided with the same

164   water as the tank. The other three fish were left in the tank without any handling. After 24h recovery

165   from this disturbance (hence at 5 days after surgery for all animals, Fig 1), experiments were

166   conducted over five days. Over the first two days, the fish were exposed to warming and hypoxia

167   challenges, with overnight recovery between the stressors. After this, fish were exchanged into their

168   reciprocal holding condition and allowed to recover for 24h. Then, they were once again exposed to

169   the two challenges over two successive days. The exact order in which each fish experienced

170   warming and hypoxia, in the tank or in a respirometer, is provided in Tab S1. Fish were observed

171   throughout all exposure protocols, at all exposure levels, through small holes in the curtains

172   (McKenzie et al., 2007b). Care was taken to reduce all disturbance to a minimum during experiments,

173   therefore experimenters entered at 08:20 to set up the trials then gave fish 30 min to recover from

174   any disturbance before commencement.

175   For progressive hypoxia, oxygen partial pressure in the tank and raceway was decreased

176   simultaneously by bubbling with 100% nitrogen, from 100% (normoxia) to 80%, then in steps of 10%

177   from 80% to 20%, then finally 15%. Each step had a duration of 30 min, water oxygen levels were

178   recorded using optical oxygen probes (Firesting sturdy dipping probes, Pyroscience,

179 www.pyroscience.com) and meter (FireSting FSO2-4), with data displayed and recorded in the Pyro

180 Oxygen Logger software, with nitrogen flow and setpoints controlled manually. For acute warming,

181 temperature was raised simultaneously in the tank and respirometers, in steps of 1°C every 30 min,

182 from 21 to 31°C, using automated temperature control systems (AquaMedic T controller twin,

183 www.aqua-medic.de) that reached incremental setpoints precisely by controlling activity of a

184 submerged pump, in the tank or the raceway, that generated a flow of water through heat exchange

185 coils immersed in a reservoir (1 m^3) of tapwater held at 40°C. All fish were exposed to these levels of

186 hypoxia and warming. The limit of 15% saturation in hypoxia was chosen because seabream of a

187 similar size have been reported to be able to tolerate exposure to below 10% at 21°C (Remen et al.,

188 2015). A limit of 31°C in warming is some degrees below CT_{max} for *S. aurata*, which is at least 34°C (Kır

189 et al., 2020; Madeira et al., 2014; Madeira et al., 2016). Thus, these exposures were sublethal but

190 should nonetheless have engendered significant cardiac and behavioural responses (Claireaux et al.,

191 1995a,b. Remen et al., 2015).

192 Rates of oxygen uptake ($\dot{M}O_2$, mmol kg^{-1} h^{-1}) were measured on the fish in the respirometers,

193 using automated intermittent stopped-flow respirometry (Steffensen, 1989), over a 15 min cycle

194 with a 6 min closure and 9 min flush period, providing two measures of $\dot{M}O_2$ for each 30 min step of

195 hypoxia or warming. Water oxygen concentrations were recorded continuously in each respirometer

196 using Firesting sturdy dipping probes and meter, with data displayed and recorded in Pyro Oxygen

197 Logger. Each fish's $\dot{M}O_2$ was then calculated considering rate of decline in oxygen concentration in

198 the chamber, chamber volume and the mass of the fish (McKenzie et al., 1995; McKenzie et al.,

199 2007a). Background measurements, on empty chambers, were made prior to placing the fish and at

200 the end of each series. These were always negligible and so no corrections were applied.

201 **Logger programming and data processing**

202 The loggers were programmed with Mercury software (Star-Oddi) according to the manufacturer's

203 instructions. Fish were all operated in the morning, with tags activated at 07:00 the following day.

204 For f_H, the ECG data were sampled at 200 Hz for 4 s. During recovery from surgery and at night during

205 experiments, f_H was measured once every two hours. It was measured once every 5 min from 07:00

206 to 18:00 during the exposure trials. An ECG trace was saved with each measure of f_H, for visual

207 confirmation of data quality. For EA, data were sampled at 10 Hz for 1 min, only during experiments,

208 once every 5 min from 07:00 to 16:00 for the first series, and from 09:00 to 17:00 for the second

209 series. Water temperature, date and time were also recorded with each f_H and EA measurement.

210 Heart rate was returned in beats min^{-1}, calculated by the manufacturer's Patternfinder

211 v.1.16.0 software from R-R intervals in the QRST wave of the ECG (Altimiras et al., 1997). Each

212 measure was confirmed by visual inspection of the ECG trace and manual calculation of R-R interval

213 within Patternfinder. The variance of EA (VAR_m) was used to identify periods where variation in

214   acceleration indicated bouts of activity or agitation. It was calculated as the variance of the 600 EA

215   measurements per minute, which indicated when the sensor was measuring acceleration above 1 g,

216   in units of mg (1000 mg = 1 g), where EA = 0 is equal to 1 g and EA = 1000 is equal to 2 g. Each

217   measure of f_H or VAR_m was associated with temperature, date and time recorded on the logger. Date

218   and time information on the logger were used to establish the associated oxygen levels for the

219   hypoxia trials, based on the oxygen probe recordings.

220   **Data and statistical analysis**

221   Statistics were performed with R version 3.5.3 (R Core Team 2019) with p = 0.05 taken as the fiducial

222   level for statistical significance. To evaluate f_H across recovery days following surgery, data for each

223   individual were averaged into 6h bins (3 2-hourly measures), starting at tag activation until 84h post-

224   surgery, when fish were handled for experimentation. A one-way analysis of variance ANOVA with

225   repeated measures was used to compare the sequential 6h values, using the aov function from the

226   stats package (R Core Team 2019). Holm-Bonferroni post-hoc tests were used to identify where

227   significant differences lay. For the five-day experimental protocol, 'undisturbed' f_H at 21°C in

228   normoxia was evaluated from a single measure at 07:00 for each fish each morning. A two-way

229   ANOVA was used to compare the undisturbed values with one factor being holding condition and the

230   second being the sequential days of the protocol. The overall mean for each individual was compared

231   between the tank and the respirometers by paired t-test. For these tests, normality,

232   homoscedasticity and independence of residuals were verified visually.

233   Effects of progressive hypoxia or warming on f_H were evaluated and compared between

234   holding conditions by two-way ANOVA with repeated measures using the aov_car function from the

235   afex package (Singmann et al., 2021) where one factor was holding condition and the other was

236   either oxygen level or temperature. Since measures were repeated, both factors were treated as

237   within subject factors. Undisturbed values at 07:00 prior to each trial were included in the ANOVA. As

238   a two-way ANOVA with repeated measures does not tolerate missing data, 1.7% of the f_H measures

239   were imputed using either nearest neighbours' or linear regression method, using the method kNN

240   function from DMwR package (Torgo 2010). Linear regression models were always significant and

241   calculated f_H was always plausible. Normality of the data was verified with a Shapiro-Wilk test.

242   Sphericity of the data was not met, therefore a Greenhouse-Geisser correction was applied.

243   Homoscedasticity of the residuals was verified visually by plotting them as a function of their fitted

244   values. Holm-Bonferroni post-hoc tests were used to identify where significant differences occurred.

245   A paired t-test was also used to compare maximum and minimum f_H during trials and the oxygen

246   partial pressure or temperatures at which these occurred, with normality, homoscedasticity and

247   independence of the residuals verified visually.

248   As VAR_m data were not normal, effects of the stressors and comparison between holding

249   conditions were evaluated with a generalized mixed linear model, with fixed factors being holding

250   condition and either oxygen partial pressure or temperature, and individuals as a random effect.

251   Tukey post-hoc tests were used to identify where any significant differences lay. Models were

252   calculated with the function glmer from lme4 package (Bates et al., 2020) and post-hoc analysis was

253   performed with emmeans package (Lenth et al., 2021).

254   A linear relationship between VAR_m and f_H was established for all individuals using a

255   generalized mixed linear model, and between f_H and $\dot{M}O_2$ using a mixed linear model, with

256   individuals as a random effect. Regression slopes between temperature and hypoxia trials were

257   compared using the function lstrends from the lsmeans R package (Lenth, 2018). A linear relationship

258   was also established between f_H and $\dot{M}O_2$ for each individual, using a linear model. Homoscedasticity

259   and independence of the residuals were verified visually.

260

261   **RESULTS**

262   A complete dataset was collected for n = 10 seabream (four from first and six from second series),

263   with a mean (± S.D.) mass of 534 ± 86 g, ranging from 363 to 801 g.

264   **Undisturbed f_H**

265   Over 72h of recovery with absolutely minimal disturbance (Fig 1), all individuals showed a

266   progressive decline in f_H (Fig 1). The mean (± S.E.) f_H declined significantly until 60h, after which it

267   showed no further significant change (Fig 1). At 90h of recovery, corresponding to 15:00 in the

268   afternoon, fish were disturbed by netting and transfer of individuals to chambers, to start

269   experiments. Over the ensuing five-day protocol, the mean undisturbed fH (i.e. the mean fH at 07:00

270   each day) was significantly lower in the tank than in the respirometers. This was irrespective of the

271   days elapsed or individual exposure history during the protocol (Tab 1, Figs 2 and 3, Fig S1).

272   **Responses to hypoxia**

273   During progressive hypoxia, *S. aurata* displayed bradycardia in both the tank and respirometers (Fig

274   2). Within each condition, f_H did not vary significantly from undisturbed normoxia down to 40%, but

275   then f_H decreased significantly at 30%, 20% and 15%. There was, however, a significant difference in

276   f_H between the tank and the respirometers during hypoxia trials ($p < 0.01$) and a significant

277   interaction between holding condition and oxygen level ($p < 0.01$). Thus, mean f_H was significantly

278   lower in the tank than in the respirometers at all oxygen steps between 80% and 40%. Although

279   undisturbed normoxic fH differed significantly (Tab 1, Fig 2), this was not true of the measures taken

280   in normoxia at the first step of the exposure trial, likely because of increased individual variation in fH

281   in the respirometers (Fig 2). This was presumably because the fish had been slightly disturbed by

282   presence of experimenters. Once bradycardia occurred, namely at 30%, 20% and 15%, there were no

283 significant differences in f_H between the tank and the respirometers (Fig 2). These different patterns

284 of f_H during hypoxia, between the two holding conditions, were reflected in the fact that mean

285 maximum f_H, whenever this might have occurred during hypoxia trials, was significantly lower (p <

286 0.01) in the tank than in the respirometers (Tab 1). On the other hand, the mean minimum f_H was

287 similar and occurred at a similar very low oxygen saturation (Tab 1).

288   The VAR$_m$ of all individuals was generally low in hypoxia, with no significant differences

289 between the tank and the respirometers at any level of hypoxia. (Fig S2). Visual inspection of the

290 tank revealed that the seabream were moving slowly around the perimeter in hypoxia and tended to

291 stop swimming entirely and rest on the bottom of the tank at hypoxic levels that caused bradycardia.

292 Inspection of the respirometers showed no signs of agitation at any of the hypoxic levels.

293   Oxygen uptake in hypoxia showed a typical teleost response, where it was regulated at levels

294 similar to normoxia until 40%, from where there was a significant and progressive decline in mean

295 $\dot{M}O_2$ (Fig S3).

296 **Responses to warming**

297 During progressive warming, *S. aurata* displayed tachycardia in both the respirometers and in the

298 tank (Fig 3). Mean f_H was statistically similar between holding conditions at all temperatures, despite

299 having been different when undisturbed at 21°C (Tab 1, Fig 3). Once again, f_H at the initial 21°C step

300 of the exposure protocol was variable among individuals, presumably due to mild disturbance. There

301 was, however, a significant interaction between holding condition and temperature (p < 0.01). Thus,

302 f_H was similar between undisturbed and 21°C steps, but then increased significantly from 21 up until

303 27°C in the tank, but only increased from 21 up until 23°C in respirometers (Fig 3). Furthermore, the

304 mean temperature at which maximum f_H occurred was significantly higher in the tank (30.3 ± 0.34°C)

305 than in the respirometers (28.6 ± 0.68°C), being closer to the maximum temperature tested (31°C) in

306 the tank (Tab 1). One fish in a respirometer (Fish n. 4, Tab S1) showed an aberrant cardiac response

307 to temperature, with a drastic drop in f_H at 28°C, which then remained low until 31°C (Fig 3), which

308 probably contributed to the interaction between holding condition and temperature. Visual

309 inspection revealed that the fish was very dark coloured and striped at 28°C, and then showed signs

310 of losing equilibrium at the end of trial. It was immediately removed and transferred to the tank. It

311 then performed normally in subsequent trials, so we had no reason to exclude this animal from

312 analyses.

313   During the warming trials, VAR$_m$ was highly variable in the tank but generally showed a

314 progressive increase (Fig 4). It increased significantly from 21 to 23°C (p <0.01), the first warming

315 step, and then showed various further significant changes, being highest overall at 31°C, the highest

316 temperature (p <0.001) compared to 21°C (Fig 4). As would be expected in confined fish, VAR$_m$ was

317 usually low in the respirometers and did not vary significantly with temperature (Fig 4). As a result,

318   mean VAR_m in the tank was significantly higher than in the respirometer at many temperatures (Fig

319   4). Visual inspection of the tank showed that the fish were swimming actively around the perimeter

320   at high temperatures, with occasional bursts of speed, especially at 31°C. Inspection of the

321   respirometers only revealed signs of activity in one individual, starting at 26°C, which was reflected in

322   a high VAR_m at 26 and 28°C (Fig 4). Unfortunately, VAR_m data were not collected on this animal at

323   higher temperatures due to an error in our programming of the Star-Oddi tag.

324   During warming, animals in respirometers generally showed an increase in $\dot{M}O_2$ with

325   warming that was significant at temperatures above 23°C (Fig S4). The individual that showed the

326   aberrant cardiac response also showed an aberrant metabolic response, with a decline in $\dot{M}O_2$ from

327   28°C (Fig S4).

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

329   There was a significant linear relationship between VAR_m and f_H in the tank during the warming trials

330   (p<0.001), which was the only condition where animals showed significant activity (Fig 5). There was

331   no relation of f_H to VAR_m under any other condition.

332   There was a significant positive linear relationship between f_H and $\dot{M}O_2$ in the respirometers

333   during both hypoxia ($\dot{M}O_2 = f_H$ (0.034) + 0.4; marginal R^2 = 0.31; conditional R^2 = 0.65; p < 0.001) and

334   warming trials ($\dot{M}O_2 = f_H$ (0.02) + 3.37; marginal R^2 = 0.085; conditional R^2 = 0.72; p < 0.001). There

335   was no significant difference between these two slopes, so a single linear relationship between f_H

336   and $\dot{M}O_2$ was fitted for all hypoxia and warming values plotted together, which was highly significant

337   (p < 0.001; Fig 6). Heart rate was also a predictor of metabolic rate for each individual fish (Tab S2).

338

339   **DISCUSSION**

340   This study provides the first explicit demonstration of cardiac responses to progressive hypoxia and

341   progressive warming in a free-swimming fish, and comparison to responses by the same individuals

342   confined in a respirometer. The cardiac loggers have, however, already been used on several species

343   (Arvén Norling, 2017; Brijs et al., 2018; Brijs et al., 2019a; Brijs et al., 2019b; Davidsen et al., 2020;

344   Prystay et al., 2017; Skeeles et al., 2020), including to demonstrate that f_H is significantly affected by

345   water temperature in sockeye salmon (*Oncorhynchus nerka*) migrating in the wild (Prystay et al.,

346   2017). A major unexpected result was that the f_H of undisturbed seabream was higher when they

347   were confined compared to free-swimming. As a consequence, cardiac responses to hypoxia and

348   warming in the free-swimming animals did not follow our predictions, although they did exhibit some

349   interesting differences when compared to the fish when confined.

350   **Undisturbed heart rates and the effects of confinement**

351   The relatively high f_H after surgery presumably indicates an acute stress response, which may have

352   included a release of circulating catecholamines (Reid et al., 1998, Gallo and Civinini 2003) and/or

353   removal of inhibitory cholinergic neural control (Randall 1982, reviewed in Farrell, 1984). The

354   progressive decline in f_H during ensuing recovery presumably indicates an associated decline in stress

355   and recovery of autonomic control (Campbell et al., 2004; McKenzie et al., 2007b; Sandblom and

356   Axelsson, 2011; Taylor et al., 2010). Recovery of mean f_H by 60h is somewhat faster than studies on

357   salmonids. Brijs et al., (2019b) reported that a minimum of three days was necessary for free-

358   swimming rainbow trout (*Oncorhynchus mykiss*) to recover a stable fH after implantation of these

359   loggers, while Føre et al., (2021) found that an average of four days was required in free-swimming

360   Atlantic salmon (*Salmo salar*). The undisturbed mean f_H of the seabream following 60h recovery, at

361   around 60 beats min^{-1} at 21°C, are amongst the lowest resting values reported for this species

362   (Aissaoui et al., 2000; Aissaoui et al., 2005; Altimiras et al., 1997; Hachim et al., 2020).

363   Throughout the five-day exposure protocol, the seabream were necessarily subjected to

364   daily disturbance from presence of experimenters, plus the exposures to hypoxia and warming.

365   Nonetheless, when undisturbed in the morning, seabream consistently had higher f_H when in the

366   respirometers, irrespective of whether they had been handled or exposed to stress on the previous

367   day. The most obvious explanation would be a stress response to confinement, as stress is known to

368   increase heart rate in fishes (Farrell, 1991; Lefrançois et al., 1998; Rabben and Furevik, 1993; Sopinka

369   et al., 2016; Svendsen et al., 2021). The proximate mechanism for the high f_H in seabream confined in

370   chambers requires further investigation. This implies, nonetheless, that allowing the seabream to

371   shoal with conspecifics was less stressful than being confined alone. This finding indicates that

372   confinement may introduce bias into studies of physiological responses by fishes to environmental

373   stressors, in a manner that may differ among species. Notably, it could bias measures of metabolic

374   rate by static respirometry, given that f_H can be a predictor of $\dot{M}O_2$, which is the case for *S. aurata*

375   (Hachim et al., 2020). The experiments also revealed how sensitive seabream were to disturbance as,

376   despite taking great care, our simple presence was enough to obscure differences in f_H between the

377   tank and the respirometers at trial commencement in normoxia at 21°C.

378   **Responses to hypoxia**

379   The data demonstrate that hypoxic bradycardia is observed in free-swimming fish but the cardiac

380   response did not comprise a progressive decline linked to a progressive drop in spontaneous activity.

381   Instead, it was very similar to 'typical' responses reported for many species under confined

382   experimental conditions (Perry et al., 2009). Hypoxic bradycardia is a reflex response in teleosts, the

383   sensory arm being chemoreceptor nerve cells in and around the gills that sense oxygen levels in

384   ventilatory water and blood streams and transmit this information to the brainstem. The reflex

385   response occurs via cholinergic fibres in the cardiac branch of the vagus nerve, which slow the heart

386   (reviewed by Farrell and Smith, 2017; Stecyk, 2017; Taylor, 1992). The functional significance of

387   hypoxic bradycardia is still debated but it may protect function of the cardiac pump, a purely aerobic

388 organ, by conserving contractility and reducing myocardial energy requirements when oxygen supply
389 in the blood is below a critical level (Farrell, 2007; Iversen et al., 2010; Joyce et al., 2016; McKenzie et
390 al., 2009).

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

399 The low VAR_m during progressive hypoxia, and absence of differences between the tank and
400 respirometers, presumably indicates that movements in the tank did not involve changes in speed,
401 which are necessary to engender variation in acceleration (Hinch et al., 2002; Kawabe et al., 2003;
402 Palstra et al., 2021; Tanaka et al., 2001). Thus, the gentle movements observed during hypoxia in the
403 tank were clearly below the sensitivity of the accelerometer in the tag.

404 **Responses to warming**

405 Although these tags have been used to study cardiac responses to acute warming in an anaesthetised
406 fish (Skeeles et al. 2020), this is the first report of responses to progressive warming in a fully-
407 recovered free-swimming animal. As for hypoxia, cardiac responses were generally similar between
408 the tank and respirometer, with a pronounced tachycardia in both cases. Warming tachycardia in
409 fishes presumably represents a response to increased oxygen demand when metabolism is
410 accelerated by warming, as demonstrated by the linear relationship of f_H and $\dot{M}O_2$ during warming in
411 the respirometer. In terms of the heart itself, this response may reflect both direct effects of
412 temperature on pacemaker function and modulation of autonomic control (see Eliason and Anntilla,
413 2017, for a detailed review). In the seabream, the maximum f_H observed during warming, 205 beats
414 min^{-1} at a temperature of 31°C, was about double the maximum achieved during forced exercise in a
415 swim-tunnel at 16°C in this species (Hachim et al., 2020). These high f_H in the seabream were all
416 confirmed by visual inspection of the traces, with clear ECG waveforms.

417 It is interesting that, unlike in hypoxia, the accelerometer detected activity in the tank during
418 warming, especially at the higher temperatures. The consistently higher VAR_m in the tank relative to
419 the respirometer, for most warming steps, would explain why f_H did not differ significantly between
420 the holding conditions. Thus, the VAR_m data confirm that, in free-swimming individuals, the
421 tachycardia was not only due to the warming itself, but also to behavioural responses to increasing
422 temperature (Claireaux et al., 1995a). When free to express spontaneous behaviour, the seabream

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423 were extremely sensitive: the significant increase in VAR_m upon initial exposure to 23°C may have
424 been because the fish were reared their whole post-larval life at 21°C. The fact that the mean
425 temperature for maximum f_H was reached at a lower temperature in the respirometer than in the
426 tank was contrary to our expectation, but entirely consistent with the fact that the confined fish
427 already exhibited tachycardia when undisturbed at 21°C. The activity observed in the tank, especially
428 the bursts of speed at high temperatures, may have reflected attempts to escape the conditions,
429 although fish did not become agitated at the same temperature when confined in the respirometer.
430 Overall, the behavioural responses to warming may provide useful indicators of tolerance that are
431 much more sensitive than, for example, loss of equilibrium at a critical thermal maximum (CT_{max})
432 (McDonnell and Chapman, 2015). In *S. aurata*, CT_{max} ranges from about 34.3°C to 36.6°C, depending
433 on acclimation temperature (Kır, 2020; Madeira et al., 2014; Madeira et al., 2016).

434 **Relationships of heart rate to acceleration and metabolic rate**

435 The significant dependence of f_H on VAR_m during warming in the tank is further proof that
436 spontaneous activity was responsible for raising f_H of the free-swimming fish. The relationship was,
437 nonetheless, rather noisy with low predictive power. This may be because increases in VAR_m,
438 especially at high temperatures, reflect agitation and burst swimming movements powered by fast-
439 twitch glycolytic muscle (Webb, 1978). The metabolic costs of such movements are paid during
440 recovery, rather than during the activity itself (Webb, 1978; Kieffer*, 2000), so changes in f_H may
441 have been out of phase with changes in VAR_m. Palstra et al. (2021) concluded that acceleration was
442 most reliable as an index of unsteady burst swimming activity in the seabream, for example when
443 fish are feeding. Although measures of body acceleration have also been used to predict metabolic
444 rate in fishes (Bouyoucos et al., 2017; Gleiss et al., 2010; Metcalfe et al., 2016; Wilson et al., 2013;
445 Wright et al., 2014), it seems unlikely they will ever have the same predictive power as f_H, not least
446 because movement is only one component of metabolic activity in fishes.

447 The data demonstrate that f_H is a predictor of metabolic rate in *S. aurata* during hypoxia and
448 warming, as it is during aerobic swimming (Hachim et al., 2020). The fact that a single linear
449 relationship between mean f_H and mean $\dot{M}O_2$ could be described, irrespective of whether data
450 derived from exposure to hypoxia or warming, demonstrates a tight coupling of cardiac pumping
451 activity to metabolic oxygen demand under diverse environmental conditions in this species. The
452 relationships for individual animals were highly significant, which Thorarensen et al., 1996 cite as a
453 necessary condition to use fH as a predictor for metabolic rate. However, their predictive power
454 differed markedly among fish, with variation in fH explaining less than 70% of variation in $\dot{M}O2$ in six
455 of the ten seabream. Also, the relationship may break down under multiple stressors and elevated
456 workloads, or at high temperatures where heart rate can become thermally limited (Thorarensen et
457 al., 1996, Brijs et al., 2019) For this reason, we did not perform the exercise of predicting individual

458    $\dot{M}O_2$ from their fH when in the tank. Further research is required to establish the extent to which

459   this variation among individuals is methodological, for example because f_H and $\dot{M}O_2$ were measured

460   over different time scales, or is physiological. Nonetheless, the results are promising in terms of

461   calibrating the relationship of f_H to $\dot{M}O_2$ using respirometry and then using logged f_H data to estimate

462   patterns of energy use by free-swimming seabream (Clark et al., 2010; Cooke et al., 2016; Lucas,

463   1994; Treberg et al., 2016). The need to retrieve the tag is still a limitation on performing such

464   studies on fish released into their natural environment (Prystay et al., 2017, 2019).

465

466   **CONCLUSIONS**

467   The results demonstrate that hypoxic bradycardia and warming tachycardia are observed in fish

468   whether they are free to shoal in a tank or confined in a respirometer. The fact, however, that

469   confining *S. aurata* in a respirometer raised their f_H, presumably due to stress, and that f_H is a

470   predictor of metabolic rate, has clear implications for estimating metabolic traits by static

471   respirometry in some fish species. Tachycardia in free-swimming fish during warming was due, to

472   some degree, to increased spontaneous activity. Thus, the combined measures of f_H and VAR_m in

473   free-swimming fish provided novel insight into drivers of cardiac responses to temperature, and

474   revealed highly sensitive behavioural responses to warming. Overall, the results demonstrate that

475   biologging of physiological and behavioural responses to hypoxia and warming, in free-swimming

476   fish, can provide more valid and reliable data than on confined fish, and has potential to reveal

477   sensitive sub-lethal thresholds for impacts of these stressors.

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TABLE

790 Tab 1. Elements of mean (± S.E.) heart rate (f_H, beats min^{-1}) of *Sparus aurata* fitted with biologging

791 tags and exposed to hypoxia or warming, comparing when swimming as groups of three in a tank or

792 confined individually in a respirometer chamber (n = 10). Undisturbed f_H indicates as measured

793 between 07:00 and 08:00 in normoxia at 21°C, prior to the respective trial. Maximum refers to the

794 mean of the highest, and minimum to mean of the lowest, f_H observed in each fish in each trial. For

795 hypoxia, PO$_2$ at max or min refers to the mean oxygen partial pressure at which maximum or

796 minimum measures occurred, respectively. For warming, T at max or min refers to the mean

797 temperature at which these measures occurred. Asterisks indicate difference between holding

798 conditions for that variable, * < 0.05, *** < 0.001

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Stressor	Holding condition	
Hypoxia	Tank	Respirometer
Undisturbed	75 ± 4.4 ***	105 ± 4
Maximum	103 ± 3.2 **	118 ± 3.4
PO$_2$ at max	71 ± 7.7	62 ± 4.5
Minimum	37 ± 1.9	39 ± 1.9
PO$_2$ at min	17 ± 1.1	19 ± 1.7
Warming	Tank	Respirometer
Undisturbed	72 ± 4.8 ***	103 ± 3.4
Maximum	172 ± 6.3	166 ± 5.5
T at max	30.3 ± 0.34 *	28.6 ± 0.68
Minimum	71.2 ± 6.9	82 ± 5.3
T at min	21.4 ± 0.58	22.3 ± 0.83

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FIGURES CAPTIONS

804 Fig 1. **Heart rate (f_H) of *Sparus aurata* as a function of time elapsed after surgery.** Each line is a

805 single fish (n = 10) and each point corresponds to the mean of heart rate values collected once every

806 two hours over 6 hours. The mean f_H dropped significantly until 60h (black dashed line) and then

807 showed no further significant change (One-way ANOVA with repeated measures, p < 0.001).

808 Fig 2. **The effect of progressive hypoxia on heart rate (f_H) of *S. aurata* (n = 10) showing individual**

809 **responses when in a tank (A) or box (B), and the mean (± S.E.) response in both holding conditions**

810 **(C).** "U100" refers to f_H of fish when undisturbed in normoxia (21°C) at 07:00, before hypoxia trials.

811 The vertical dashed line indicates the beginning of the trial. In (C) a common superscript indicates no

812 significant difference in the mean (Two-way ANOVA with repeated measures, p < 0.05).

813 Fig 3. **The effect of progressive warming on heart rate (fH) of S. aurata (n = 10) showing individual**

814 **responses when in a tank (A) or box (B), and the mean (± S.E.) response in both holding conditions**

815 **(C).** "U21" refers to f_H of fish when undisturbed at 21°C, at 07:00 before warming trials. The vertical

816 dashed line indicates the beginning of the trial. In (C) a common superscript indicates no significant

817 difference in the mean (Two-way ANOVA with repeated measures, p < 0.05).

818 Fig 4. **The effect of progressive warming on the variance of external acceleration (VAR$_m$) of *S.***

819 ***aurata* (n = 10) showing individual responses when in a tank (A) or box (B), and the mean (± S.E.)**

820 **response in both holding conditions (C).** In (C) a common superscript indicates no significant

821 difference in the mean (generalized mixed linear model, p < 0.001,).

822 Fig 5. **The relationship of variance of acceleration (VAR$_m$) to heart rate (f_H) in *S. aurata* during**

823 **warming trials in the tank.** Points are VAR$_m$ calculated over 60 s after the corresponding f_H value

824 logged for each fish (n = 10 in all cases). The relationship is described by VAR$_m$ = f_H (0.87) + 41.2

825 (generalized mixed linear model, p < 0.001, marginal R^2 = 0.061; conditional R^2 = 0.36)

826 Fig 6. **The relationship of oxygen consumption ($\dot{M}O_2$) to heart rate (f_H) in *S. aurata* confined in**

827 **respirometers, during exposure to hypoxia and warming.** Each point represents the mean oxygen

828 consumption as a function of mean f_H for a single fish at a given oxygen partial pressure or

829 temperature (n = 10 in all cases). The relationship is described by $\dot{M}O_2$ = f_H (0.045) − 0.23 (mixed

830 linear model, p < 0.001, marginal R^2 = 0.52; conditional R^2 = 0.76) for all trials combined, represented

831 by the black dashed line.

Figure showing heart rate (beats min⁻¹) versus oxygen partial pressure (%) in three panels (A, B, C).

Panel A: individual traces in blue.

Panel B: individual traces in red.

Panel C: mean data with labels — Tank (blue, open circles) and Respirometer (red, open circles), with statistical group letters (ai, efj, ei, abj, gk, bck, cd, h, d, dl, hl, etc.).

Y-axis: Heart rate (beats min⁻¹), ranging 30–140.

X-axis: Oxygen partial pressure (%), values 15, 20, 30, 40, 50, 60, 70, 80, 100, U, 100.