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To cite this version:

Alexandre Mignucci, Jérôme Bourjea, Fabien Forget, Hossein Allal, Gilbert Dutto, et al.. Cardiac and behavioural responses to hypoxia and warming in free-swimming gilthead seabream, Sparus aurata. Journal of Experimental Biology, 2021, 224 (14), pp.jeb242397. 10.1242/jeb.242397 . hal-03415613

HAL Id: hal-03415613
https://hal.umontpellier.fr/hal-03415613v1

Submitted on 22 Nov 2021

Cardiac and behavioural responses to hypoxia and warming in free-swimming gilthead seabream (*Sparus aurata*)

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

SUMMARY STATEMENT

Biologging of cardiac responses to hypoxia and warming in a free-swimming fish revealed that confinement in respirometer chambers raises heart rate, with consequences for estimates of metabolic rates.

ABSTRACT

Gilthead seabream were equipped with intraperitoneal biologging tags to investigate cardiac responses to hypoxia and warming, comparing when fish were either swimming freely in a tank with conspecifics or confined to individual respirometers. After tag implantation under anaesthesia, heart rate (f_H) required 60 hours to recover to a stable value in a holding tank. Subsequently, when undisturbed under control conditions (normoxia, 21°C), mean f_H was always significantly lower in the tank than respirometers. In progressive hypoxia (100 - 15% oxygen saturation), mean f_H in the tank was significantly lower than respirometers at oxygen levels until 40%, with significant bradycardia in both holding conditions below this. Simultaneous logging of tri-axial body acceleration revealed that spontaneous activity, inferred as the variance of external acceleration (VAR_m), was low and invariant in hypoxia. Warming (21 to 31°C) caused progressive tachycardia with no differences in f_H between holding conditions. Mean VAR_m was, however, significantly higher in the tank during warming, with a positive relationship between VAR_m and f_H across all temperatures. Therefore, spontaneous activity contributed to raising f_H of fish in the tank during warming. Mean f_H in respirometers had a highly significant linear relationship with mean rates of oxygen uptake, considering data from hypoxia and warming together. The high f_H of confined seabream indicates that respirometry techniques may bias estimates of metabolic traits in some fishes, and that biologging on free-swimming fishes will provide more reliable insight into cardiac and behavioural responses to environmental stressors by fishes in their natural environment.

List of symbols and abbreviations:

- f_H: heart rate frequency
- $\dot{M}O_2$: rate of oxygen uptake
- EA: external acceleration
- VAR_m: Variance of external acceleration

INTRODUCTION

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

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

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

We implanted biologgers into gilthead seabream (*Sparus aurata*) to compare cardiac responses to progressive hypoxia and warming when each animal was either shoaling in a tank with conspecifics, or confined in an individual respirometer chamber. We expected that, although fish would exhibit hypoxic bradycardia and warming tachycardia when swimming freely in the tank (Claireaux et al., 1995a,b; Lefrançois et al., 1998), the ability to express spontaneous activity would alter responses compared to when confined in a respirometer. We expected that f_H of undisturbed animals would be higher in the tank in normoxia at acclimation temperatures, due to spontaneous swimming activity. Progressive hypoxia might cause progressive declines in fH in the tank, which initially and prior to the chemoreflexive hypoxic bradycardia, were due to reduced spontaneous swimming activity (Remen et al., 2015). In the respirometer, by contrast, fH would be low and relatively stable until onset of chemoreflexive bradycardia (Perry et al., 2009). We expected f_H to increase more rapidly in the tank with warming, due to stimulation of activity (Claireaux et al., 1995a), with a maximum f_H reached at a lower temperature. We expected to demonstrate that swimming activity was a determinant of cardiac activity in the tank, by revealing a direct relationship between logged EA and f_H. Finally, the respirometers allowed us to investigate whether f_H was a predictor of metabolic rate in *S. aurata* during exposure to hypoxia and warming, as it is during aerobic exercise (Hachim et al., 2020).

MATERIAL AND METHODS

Ethical approval

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

Animals

Experiments were performed on n = 12 seabream with a mass of approximately 500 g and age of approximately 18 months. Sex was not verified, but fish were most probably male, as *S. aurata* is a protandrous hermaphrodite species. The seabream were obtained from the Ferme Marine du Douhet (La Brée les Bains, France) as post-larvae then reared at the Ifremer Aquaculture Research Station in Palavas-les-Flots, in indoor cylindrical tanks (vol 2 m^3) under seasonal photoperiods, provided with a flow of biofiltered and UV-treated seawater at 21°C. Fish were fed daily with commercial pellets (B-Grower Marin, Le Gouessant, www.legouessant.com) but fasted for 24 h prior to surgery.

Surgery

Fish were anesthetized by immersion in 0.1 g l^{-1} benzocaine (Benzocaine ethyl 4-Aminobenzoate, VWR, www.vwr.com) in aerated seawater, until active ventilation ceased, then weighed and placed on an operating table with their gills irrigated with aerated seawater containing 0.05 g l^{-1} benzocaine. Heart rate loggers (DST milli HRT-ACT, 13 mm × 39.5 mm, 12 g, Star-Oddi, Iceland, www.star-oddi.com) were implanted in the intraperitoneal space, via an a ~2 cm longitudinal incision along the ventral midline, ~0.5 cm posterior to the pectoral fins. Loggers were advanced as close as possible to the pericardium and fixed with sutures (silk suture and non-absorbable monofilament) such that their ECG electrodes lay against the body wall, with the incision then closed with sutures (non-absorbable monofilament). Fish were left to recover in a 1 m^3 cylindrical tank provided with a flow of aerated, biofiltered and UV-treated seawater at 21°C. The tank was isolated in a room dedicated exclusively to the study, with a natural photoperiod through skylights. The tank was shielded behind an opaque black plastic curtain. During recovery fish were checked by visual observation through small holes in the curtain (McKenzie et al., 2007) in the morning (08:30) and evening (17:00). Fish were not fed during recovery or subsequent experiments.

Hypoxia and temperature challenges

Fish were instrumented and studied in two groups of six individuals. To allow us to compare each individual's responses to the challenges when either swimming freely in the tank or confined in a respirometer, we used a protocol where they were sequentially exposed in one holding condition and then in the other.

At 90h after surgery (Fig 1), three of the six fish were netted from the 1 m^3 tank and placed in individual rectangular clear plastic respirometer chambers (vol. 9 l) which were submerged in a small raceway in the same room, shielded behind an opaque black curtain and provided with the same water as the tank. The other three fish were left in the tank without any handling. After 24h recovery from this disturbance (hence at 5 days after surgery for all animals, Fig 1), experiments were conducted over five days. Over the first two days, the fish were exposed to warming and hypoxia challenges, with overnight recovery between the stressors. After this, fish were exchanged into their reciprocal holding condition and allowed to recover for 24h. Then, they were once again exposed to the two challenges over two successive days. The exact order in which each fish experienced warming and hypoxia, in the tank or in a respirometer, is provided in Tab S1. Fish were observed throughout all exposure protocols, at all exposure levels, through small holes in the curtains (McKenzie et al., 2007b). Care was taken to reduce all disturbance to a minimum during experiments, therefore experimenters entered at 08:20 to set up the trials then gave fish 30 min to recover from any disturbance before commencement.

For progressive hypoxia, oxygen partial pressure in the tank and raceway was decreased simultaneously by bubbling with 100% nitrogen, from 100% (normoxia) to 80%, then in steps of 10% from 80% to 20%, then finally 15%. Each step had a duration of 30 min, water oxygen levels were recorded using optical oxygen probes (Firesting sturdy dipping probes, Pyroscience, www.pyroscience.com) and meter (FireSting FSO2-4), with data displayed and recorded in the Pyro Oxygen Logger software, with nitrogen flow and setpoints controlled manually. For acute warming, temperature was raised simultaneously in the tank and respirometers, in steps of 1°C every 30 min, from 21 to 31°C, using automated temperature control systems (AquaMedic T controller twin, www.aqua-medic.de) that reached incremental setpoints precisely by controlling activity of a submerged pump, in the tank or the raceway, that generated a flow of water through heat exchange coils immersed in a reservoir (1 m^3) of tapwater held at 40°C. All fish were exposed to these levels of hypoxia and warming. The limit of 15% saturation in hypoxia was chosen because seabream of a similar size have been reported to be able to tolerate exposure to below 10% at 21°C (Remen et al., 2015). A limit of 31°C in warming is some degrees below CT_{max} for *S. aurata*, which is at least 34°C (Kır et al., 2020; Madeira et al., 2014; Madeira et al., 2016). Thus, these exposures were sublethal but should nonetheless have engendered significant cardiac and behavioural responses (Claireaux et al., 1995a,b. Remen et al., 2015).

Rates of oxygen uptake ($\dot{M}O_2$, mmol kg^{-1} h^{-1}) were measured on the fish in the respirometers, using automated intermittent stopped-flow respirometry (Steffensen, 1989), over a 15 min cycle with a 6 min closure and 9 min flush period, providing two measures of $\dot{M}O_2$ for each 30 min step of hypoxia or warming. Water oxygen concentrations were recorded continuously in each respirometer using Firesting sturdy dipping probes and meter, with data displayed and recorded in Pyro Oxygen Logger. Each fish's $\dot{M}O_2$ was then calculated considering rate of decline in oxygen concentration in the chamber, chamber volume and the mass of the fish (McKenzie et al., 1995; McKenzie et al., 2007a). Background measurements, on empty chambers, were made prior to placing the fish and at the end of each series. These were always negligible and so no corrections were applied.

Logger programming and data processing

The loggers were programmed with Mercury software (Star-Oddi) according to the manufacturer's instructions. Fish were all operated in the morning, with tags activated at 07:00 the following day. For f_H, the ECG data were sampled at 200 Hz for 4 s. During recovery from surgery and at night during experiments, f_H was measured once every two hours. It was measured once every 5 min from 07:00 to 18:00 during the exposure trials. An ECG trace was saved with each measure of f_H, for visual confirmation of data quality. For EA, data were sampled at 10 Hz for 1 min, only during experiments, once every 5 min from 07:00 to 16:00 for the first series, and from 09:00 to 17:00 for the second series. Water temperature, date and time were also recorded with each f_H and EA measurement.

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

Data and statistical analysis

Statistics were performed with R version 3.5.3 (R Core Team 2019) with p = 0.05 taken as the fiducial level for statistical significance. To evaluate f_H across recovery days following surgery, data for each individual were averaged into 6h bins (3 2-hourly measures), starting at tag activation until 84h post-surgery, when fish were handled for experimentation. A one-way analysis of variance ANOVA with repeated measures was used to compare the sequential 6h values, using the aov function from the stats package (R Core Team 2019). Holm-Bonferroni post-hoc tests were used to identify where significant differences lay. For the five-day experimental protocol, 'undisturbed' f_H at 21°C in normoxia was evaluated from a single measure at 07:00 for each fish each morning. A two-way ANOVA was used to compare the undisturbed values with one factor being holding condition and the second being the sequential days of the protocol. The overall mean for each individual was compared between the tank and the respirometers by paired t-test. For these tests, normality, homoscedasticity and independence of residuals were verified visually.

Effects of progressive hypoxia or warming on f_H were evaluated and compared between holding conditions by two-way ANOVA with repeated measures using the aov_car function from the afex package (Singmann et al., 2021) where one factor was holding condition and the other was either oxygen level or temperature. Since measures were repeated, both factors were treated as within subject factors. Undisturbed values at 07:00 prior to each trial were included in the ANOVA. As a two-way ANOVA with repeated measures does not tolerate missing data, 1.7% of the f_H measures were imputed using either nearest neighbours' or linear regression method, using the method kNN function from DMwR package (Torgo 2010). Linear regression models were always significant and calculated f_H was always plausible. Normality of the data was verified with a Shapiro-Wilk test. Sphericity of the data was not met, therefore a Greenhouse-Geisser correction was applied. Homoscedasticity of the residuals was verified visually by plotting them as a function of their fitted values. Holm-Bonferroni post-hoc tests were used to identify where significant differences occurred. A paired t-test was also used to compare maximum and minimum f_H during trials and the oxygen partial pressure or temperatures at which these occurred, with normality, homoscedasticity and independence of the residuals verified visually.

As VAR_m data were not normal, effects of the stressors and comparison between holding conditions were evaluated with a generalized mixed linear model, with fixed factors being holding condition and either oxygen partial pressure or temperature, and individuals as a random effect. Tukey post-hoc tests were used to identify where any significant differences lay. Models were calculated with the function glmer from lme4 package (Bates et al., 2020) and post-hoc analysis was performed with emmeans package (Lenth et al., 2021).

A linear relationship between VAR_m and f_H was established for all individuals using a generalized mixed linear model, and between f_H and $\dot{M}O_2$ using a mixed linear model, with individuals as a random effect. Regression slopes between temperature and hypoxia trials were compared using the function lstrends from the lsmeans R package (Lenth, 2018). A linear relationship was also established between f_H and $\dot{M}O_2$ for each individual, using a linear model. Homoscedasticity and independence of the residuals were verified visually.

RESULTS

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

Undisturbed f_H

Over 72h of recovery with absolutely minimal disturbance (Fig 1), all individuals showed a progressive decline in f_H (Fig 1). The mean (± S.E.) f_H declined significantly until 60h, after which it showed no further significant change (Fig 1). At 90h of recovery, corresponding to 15:00 in the afternoon, fish were disturbed by netting and transfer of individuals to chambers, to start experiments. Over the ensuing five-day protocol, the mean undisturbed fH (i.e. the mean fH at 07:00 each day) was significantly lower in the tank than in the respirometers. This was irrespective of the days elapsed or individual exposure history during the protocol (Tab 1, Figs 2 and 3, Fig S1).

Responses to hypoxia

During progressive hypoxia, *S. aurata* displayed bradycardia in both the tank and respirometers (Fig 2). Within each condition, f_H did not vary significantly from undisturbed normoxia down to 40%, but then f_H decreased significantly at 30%, 20% and 15%. There was, however, a significant difference in f_H between the tank and the respirometers during hypoxia trials ($p < 0.01$) and a significant interaction between holding condition and oxygen level ($p < 0.01$). Thus, mean f_H was significantly lower in the tank than in the respirometers at all oxygen steps between 80% and 40%. Although undisturbed normoxic fH differed significantly (Tab 1, Fig 2), this was not true of the measures taken in normoxia at the first step of the exposure trial, likely because of increased individual variation in fH in the respirometers (Fig 2). This was presumably because the fish had been slightly disturbed by presence of experimenters. Once bradycardia occurred, namely at 30%, 20% and 15%, there were no significant differences in f_H between the tank and the respirometers (Fig 2). These different patterns of f_H during hypoxia, between the two holding conditions, were reflected in the fact that mean maximum f_H, whenever this might have occurred during hypoxia trials, was significantly lower ($p < 0.01$) in the tank than in the respirometers (Tab 1). On the other hand, the mean minimum f_H was similar and occurred at a similar very low oxygen saturation (Tab 1).

The VAR_m of all individuals was generally low in hypoxia, with no significant differences between the tank and the respirometers at any level of hypoxia. (Fig S2). Visual inspection of the tank revealed that the seabream were moving slowly around the perimeter in hypoxia and tended to stop swimming entirely and rest on the bottom of the tank at hypoxic levels that caused bradycardia. Inspection of the respirometers showed no signs of agitation at any of the hypoxic levels.

Oxygen uptake in hypoxia showed a typical teleost response, where it was regulated at levels similar to normoxia until 40%, from where there was a significant and progressive decline in mean $\dot{M}O_2$ (Fig S3).

Responses to warming

During progressive warming, *S. aurata* displayed tachycardia in both the respirometers and in the tank (Fig 3). Mean f_H was statistically similar between holding conditions at all temperatures, despite having been different when undisturbed at 21°C (Tab 1, Fig 3). Once again, f_H at the initial 21°C step of the exposure protocol was variable among individuals, presumably due to mild disturbance. There was, however, a significant interaction between holding condition and temperature ($p < 0.01$). Thus, f_H was similar between undisturbed and 21°C steps, but then increased significantly from 21 up until 27°C in the tank, but only increased from 21 up until 23°C in respirometers (Fig 3). Furthermore, the mean temperature at which maximum f_H occurred was significantly higher in the tank (30.3 ± 0.34°C) than in the respirometers (28.6 ± 0.68°C), being closer to the maximum temperature tested (31°C) in the tank (Tab 1). One fish in a respirometer (Fish n. 4, Tab S1) showed an aberrant cardiac response to temperature, with a drastic drop in f_H at 28°C, which then remained low until 31°C (Fig 3), which probably contributed to the interaction between holding condition and temperature. Visual inspection revealed that the fish was very dark coloured and striped at 28°C, and then showed signs of losing equilibrium at the end of trial. It was immediately removed and transferred to the tank. It then performed normally in subsequent trials, so we had no reason to exclude this animal from analyses.

During the warming trials, VAR_m was highly variable in the tank but generally showed a progressive increase (Fig 4). It increased significantly from 21 to 23°C ($p <0.01$), the first warming step, and then showed various further significant changes, being highest overall at 31°C, the highest temperature ($p <0.001$) compared to 21°C (Fig 4). As would be expected in confined fish, VAR_m was usually low in the respirometers and did not vary significantly with temperature (Fig 4). As a result, mean VAR_m in the tank was significantly higher than in the respirometer at many temperatures (Fig 4). Visual inspection of the tank showed that the fish were swimming actively around the perimeter at high temperatures, with occasional bursts of speed, especially at 31°C. Inspection of the respirometers only revealed signs of activity in one individual, starting at 26°C, which was reflected in a high VAR_m at 26 and 28°C (Fig 4). Unfortunately, VAR_m data were not collected on this animal at higher temperatures due to an error in our programming of the Star-Oddi tag.

During warming, animals in respirometers generally showed an increase in $\dot{M}O_2$ with warming that was significant at temperatures above 23°C (Fig S4). The individual that showed the aberrant cardiac response also showed an aberrant metabolic response, with a decline in $\dot{M}O_2$ from 28°C (Fig S4).

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

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

There was a significant positive linear relationship between f_H and $\dot{M}O_2$ in the respirometers 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 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 was no significant difference between these two slopes, so a single linear relationship between f_H and $\dot{M}O_2$ was fitted for all hypoxia and warming values plotted together, which was highly significant ($p < 0.001$; Fig 6). Heart rate was also a predictor of metabolic rate for each individual fish (Tab S2).

DISCUSSION

This study provides the first explicit demonstration of cardiac responses to progressive hypoxia and progressive warming in a free-swimming fish, and comparison to responses by the same individuals confined in a respirometer. The cardiac loggers have, however, already been used on several species (Arvén Norling, 2017; Brijs et al., 2018; Brijs et al., 2019a; Brijs et al., 2019b; Davidsen et al., 2020; Prystay et al., 2017; Skeeles et al., 2020), including to demonstrate that f_H is significantly affected by water temperature in sockeye salmon (*Oncorhynchus nerka*) migrating in the wild (Prystay et al., 2017). A major unexpected result was that the f_H of undisturbed seabream was higher when they were confined compared to free-swimming. As a consequence, cardiac responses to hypoxia and warming in the free-swimming animals did not follow our predictions, although they did exhibit some interesting differences when compared to the fish when confined.

Undisturbed heart rates and the effects of confinement

The relatively high f_H after surgery presumably indicates an acute stress response, which may have included a release of circulating catecholamines (Reid et al., 1998, Gallo and Civinini 2003) and/or removal of inhibitory cholinergic neural control (Randall 1982, reviewed in Farrell, 1984). The progressive decline in f_H during ensuing recovery presumably indicates an associated decline in stress and recovery of autonomic control (Campbell et al., 2004; McKenzie et al., 2007b; Sandblom and Axelsson, 2011; Taylor et al., 2010). Recovery of mean f_H by 60h is somewhat faster than studies on salmonids. Brijs et al., (2019b) reported that a minimum of three days was necessary for free-swimming rainbow trout (*Oncorhynchus mykiss*) to recover a stable fH after implantation of these loggers, while Føre et al., (2021) found that an average of four days was required in free-swimming Atlantic salmon (*Salmo salar*). The undisturbed mean f_H of the seabream following 60h recovery, at around 60 beats min^{-1} at 21°C, are amongst the lowest resting values reported for this species (Aissaoui et al., 2000; Aissaoui et al., 2005; Altimiras et al., 1997; Hachim et al., 2020).

Throughout the five-day exposure protocol, the seabream were necessarily subjected to daily disturbance from presence of experimenters, plus the exposures to hypoxia and warming. Nonetheless, when undisturbed in the morning, seabream consistently had higher f_H when in the respirometers, irrespective of whether they had been handled or exposed to stress on the previous day. The most obvious explanation would be a stress response to confinement, as stress is known to increase heart rate in fishes (Farrell, 1991; Lefrançois et al., 1998; Rabben and Furevik, 1993; Sopinka et al., 2016; Svendsen et al., 2021). The proximate mechanism for the high f_H in seabream confined in chambers requires further investigation. This implies, nonetheless, that allowing the seabream to shoal with conspecifics was less stressful than being confined alone. This finding indicates that confinement may introduce bias into studies of physiological responses by fishes to environmental stressors, in a manner that may differ among species. Notably, it could bias measures of metabolic rate by static respirometry, given that f_H can be a predictor of $\dot{M}O_2$, which is the case for *S. aurata* (Hachim et al., 2020). The experiments also revealed how sensitive seabream were to disturbance as, despite taking great care, our simple presence was enough to obscure differences in f_H between the tank and the respirometers at trial commencement in normoxia at 21°C.

Responses to hypoxia

The data demonstrate that hypoxic bradycardia is observed in free-swimming fish but the cardiac response did not comprise a progressive decline linked to a progressive drop in spontaneous activity. Instead, it was very similar to 'typical' responses reported for many species under confined experimental conditions (Perry et al., 2009). Hypoxic bradycardia is a reflex response in teleosts, the sensory arm being chemoreceptor nerve cells in and around the gills that sense oxygen levels in ventilatory water and blood streams and transmit this information to the brainstem. The reflex response occurs via cholinergic fibres in the cardiac branch of the vagus nerve, which slow the heart (reviewed by Farrell and Smith, 2017; Stecyk, 2017; Taylor, 1992). The functional significance of hypoxic bradycardia is still debated but it may protect function of the cardiac pump, a purely aerobic organ, by conserving contractility and reducing myocardial energy requirements when oxygen supply in the blood is below a critical level (Farrell, 2007; Iversen et al., 2010; Joyce et al., 2016; McKenzie et al., 2009).

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

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

Responses to warming

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

It is interesting that, unlike in hypoxia, the accelerometer detected activity in the tank during warming, especially at the higher temperatures. The consistently higher VAR_m in the tank relative to the respirometer, for most warming steps, would explain why f_H did not differ significantly between the holding conditions. Thus, the VAR_m data confirm that, in free-swimming individuals, the tachycardia was not only due to the warming itself, but also to behavioural responses to increasing temperature (Claireaux et al., 1995a). When free to express spontaneous behaviour, the seabream were extremely sensitive: the significant increase in VAR_m upon initial exposure to 23°C may have been because the fish were reared their whole post-larval life at 21°C. The fact that the mean temperature for maximum f_H was reached at a lower temperature in the respirometer than in the tank was contrary to our expectation, but entirely consistent with the fact that the confined fish already exhibited tachycardia when undisturbed at 21°C. The activity observed in the tank, especially the bursts of speed at high temperatures, may have reflected attempts to escape the conditions, although fish did not become agitated at the same temperature when confined in the respirometer. Overall, the behavioural responses to warming may provide useful indicators of tolerance that are much more sensitive than, for example, loss of equilibrium at a critical thermal maximum (CT_{max}) (McDonnell and Chapman, 2015). In *S. aurata*, CT_{max} ranges from about 34.3°C to 36.6°C, depending on acclimation temperature (Kır, 2020; Madeira et al., 2014; Madeira et al., 2016).

Relationships of heart rate to acceleration and metabolic rate

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

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

$\dot{M}O2$ from their fH when in the tank. Further research is required to establish the extent to which this variation among individuals is methodological, for example because f_H and $\dot{M}O_2$ were measured over different time scales, or is physiological. Nonetheless, the results are promising in terms of calibrating the relationship of f_H to $\dot{M}O_2$ using respirometry and then using logged f_H data to estimate patterns of energy use by free-swimming seabream (Clark et al., 2010; Cooke et al., 2016; Lucas, 1994; Treberg et al., 2016). The need to retrieve the tag is still a limitation on performing such studies on fish released into their natural environment (Prystay et al., 2017, 2019).

CONCLUSIONS

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

FUNDING

This work was supported by the French National program EC2CO 2019 (Ecosphère Continentale et Côtière) N°DEC20045DR16, Région Occitanie PhD funding initiative (ALDOCT 00374 – 2018001408) and IFREMER.

ACKNOWLEDGMENTS

The authors are grateful to Marc Vandeputte and Ferme du Douhet for donating the fish. The authors are also grateful Ásgeir Bjarnason of Star-Oddi Ltd for technical advice and assistance, and to Germain Salou and Aurélien Leddo of Ifremer Palavas-les-Flots for help in setting up the experiments. The authors also thank Tobias Wang for stimulating discussions about the experiments.

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TABLE

Tab 1. Elements of mean (± S.E.) heart rate (f_H, beats min^{-1}) of *Sparus aurata* fitted with biologging tags and exposed to hypoxia or warming, comparing when swimming as groups of three in a tank or confined individually in a respirometer chamber (n = 10). Undisturbed f_H indicates as measured between 07:00 and 08:00 in normoxia at 21°C, prior to the respective trial. Maximum refers to the mean of the highest, and minimum to mean of the lowest, f_H observed in each fish in each trial. For hypoxia, PO_2 at max or min refers to the mean oxygen partial pressure at which maximum or minimum measures occurred, respectively. For warming, T at max or min refers to the mean temperature at which these measures occurred. Asterisks indicate difference between holding conditions for that variable, * < 0.05, *** < 0.001

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

FIGURES CAPTIONS

Fig 1. **Heart rate (f_H) of *Sparus aurata* as a function of time elapsed after surgery.** Each line is a single fish (n = 10) and each point corresponds to the mean of heart rate values collected once every two hours over 6 hours. The mean f_H dropped significantly until 60h (black dashed line) and then showed no further significant change (One-way ANOVA with repeated measures, $p < 0.001$).

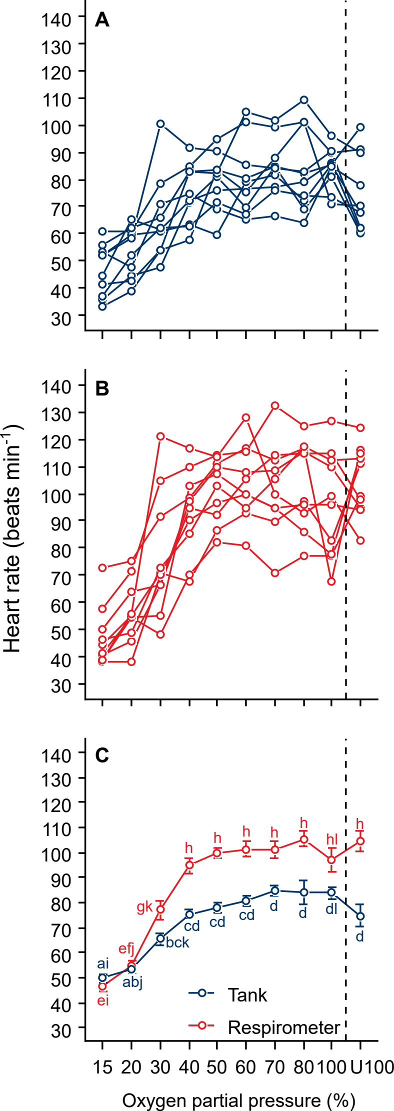
Fig 2. **The effect of progressive hypoxia on heart rate (f_H) of *S. aurata* (n = 10) showing individual responses when in a tank (A) or box (B), and the mean (± S.E.) response in both holding conditions (C).** "U100" refers to f_H of fish when undisturbed in normoxia (21°C) at 07:00, before hypoxia trials. The vertical dashed line indicates the beginning of the trial. In (C) a common superscript indicates no significant difference in the mean (Two-way ANOVA with repeated measures, $p < 0.05$).

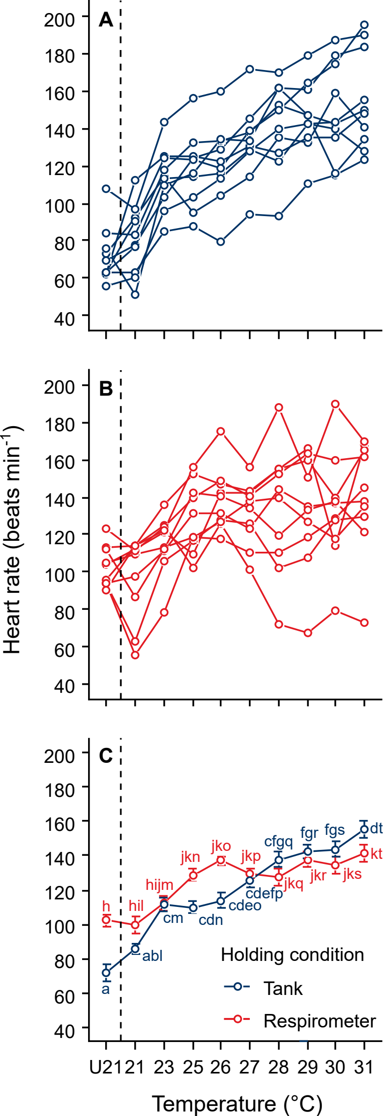
Fig 3. **The effect of progressive warming on heart rate (fH) of S. aurata (n = 10) showing individual responses when in a tank (A) or box (B), and the mean (± S.E.) response in both holding conditions (C).** "U21" refers to f_H of fish when undisturbed at 21°C, at 07:00 before warming trials. The vertical dashed line indicates the beginning of the trial. In (C) a common superscript indicates no significant difference in the mean (Two-way ANOVA with repeated measures, $p < 0.05$).

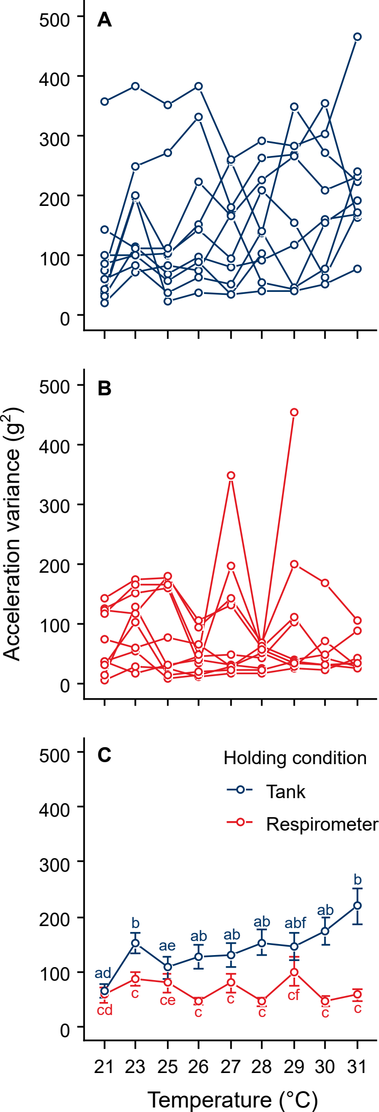
Fig 4. **The effect of progressive warming on the variance of external acceleration (VAR_m) of *S. aurata* (n = 10) showing individual responses when in a tank (A) or box (B), and the mean (± S.E.) response in both holding conditions (C).** In (C) a common superscript indicates no significant difference in the mean (generalized mixed linear model, $p < 0.001$,).

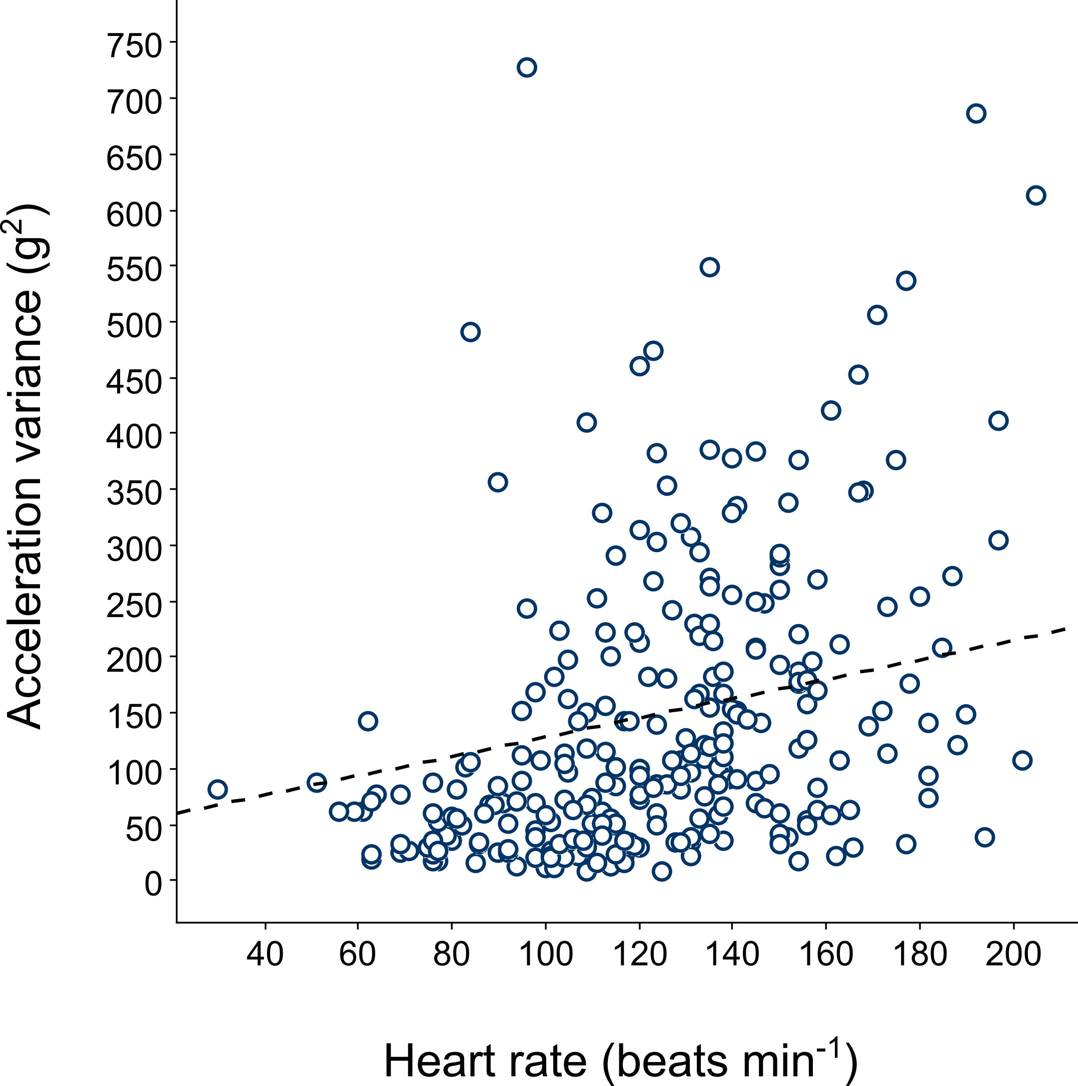
Fig 5. **The relationship of variance of acceleration (VAR_m) to heart rate (f_H) in *S. aurata* during warming trials in the tank.** Points are VAR_m calculated over 60 s after the corresponding f_H value logged for each fish (n = 10 in all cases). The relationship is described by $VAR_m = f_H (0.87) + 41.2$ (generalized mixed linear model, $p < 0.001$, marginal $R^2 = 0.061$; conditional $R^2 = 0.36$)

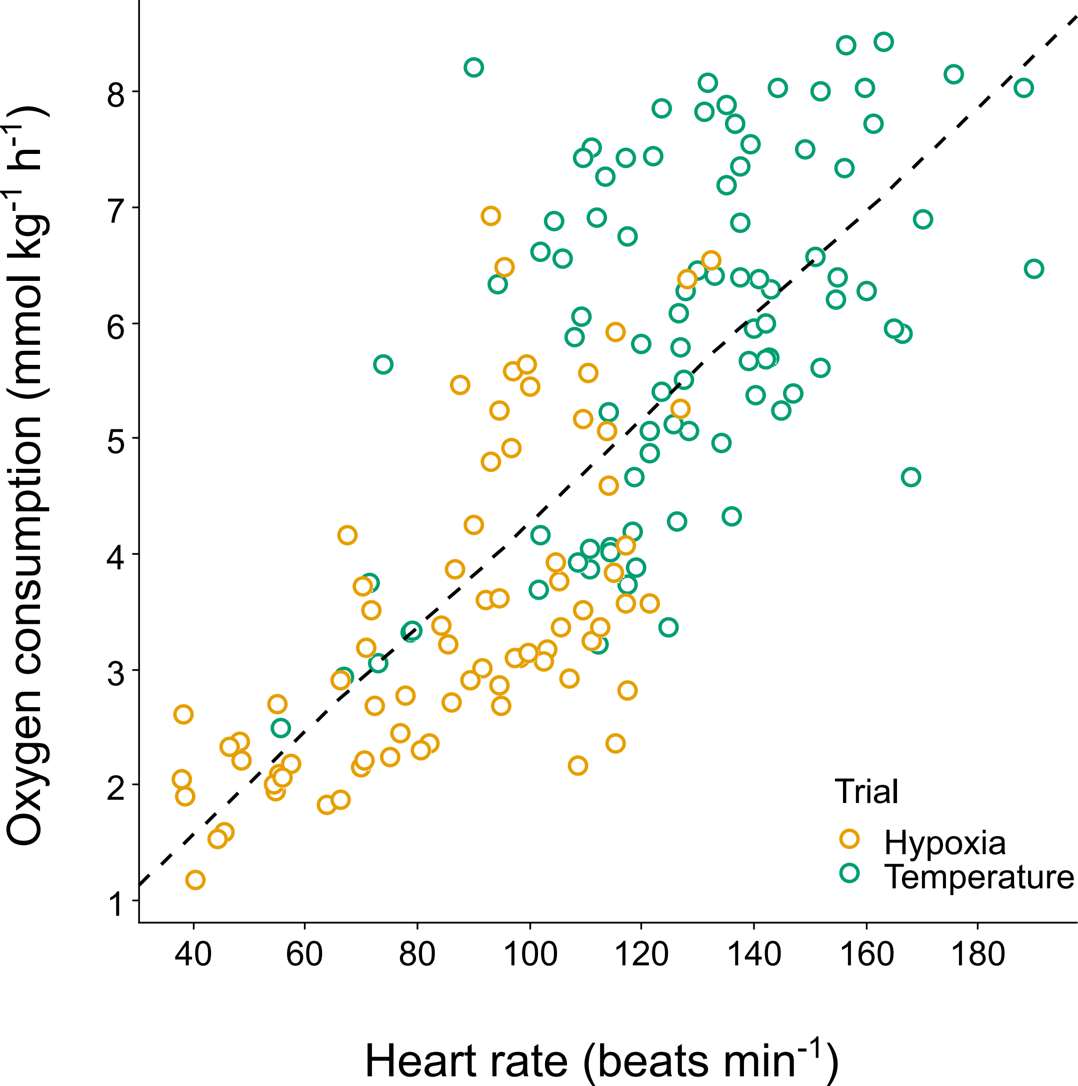
Fig 6. **The relationship of oxygen consumption ($\dot{M}O_2$) to heart rate (f_H) in *S. aurata* confined in respirometers, during exposure to hypoxia and warming.** Each point represents the mean oxygen consumption as a function of mean f_H for a single fish at a given oxygen partial pressure or temperature (n = 10 in all cases). The relationship is described by $\dot{M}O_2 = f_H (0.045) - 0.23$ (mixed linear model, $p < 0.001$, marginal $R^2 = 0.52$; conditional $R^2 = 0.76$) for all trials combined, represented by the black dashed line.

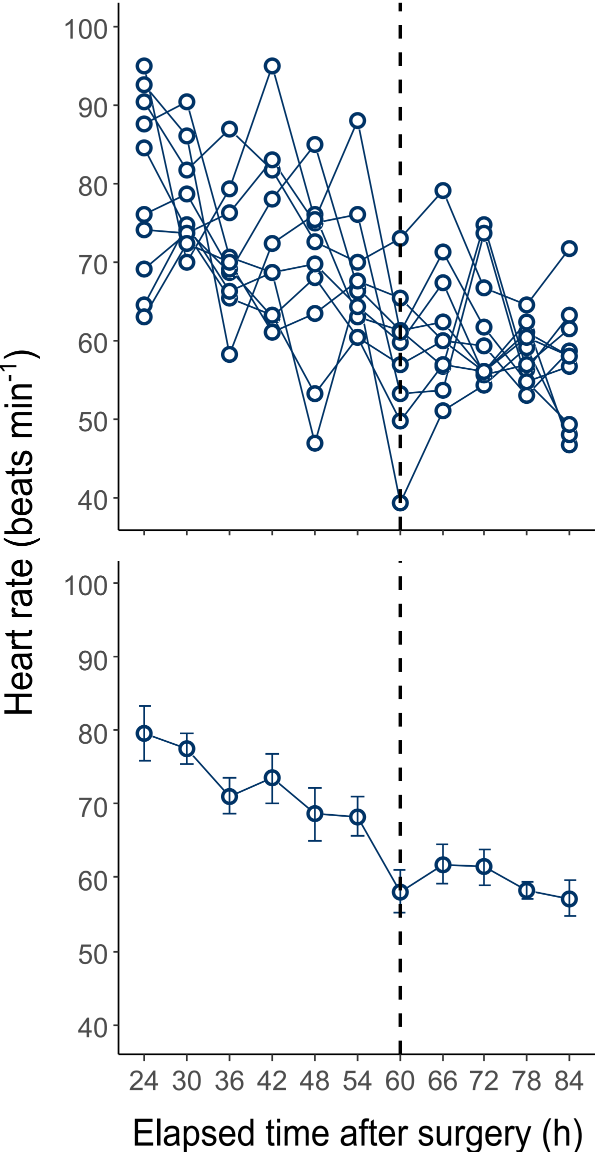

A
B
C
140
130
120
110
100
90
80
70
60
50
40
30
Heart rate (beats min^{-1})
ai
ei
abj
efj
bck
gk
cd
h
cd
h
cd
h
d
h
d
h
dl
hl
d
h
Tank
Respirometer
15
20
30
40
50
60
70
80
100
U100
Oxygen partial pressure (%)


A
B
C
200
180
160
140
120
100
80
60
40
Heart rate (beats min-1)
h
hil
hijm
jkn
jko
jkp
jkq
jkr
jks
kt
a
abl
cm
cdn
cdeo
cdefp
cfgq
fgr
fgs
dt
Holding condition
Tank
Respirometer
U21
21
23
25
26
27
28
29
30
31
Temperature (°C)


A
B
C
500
400
300
200
100
0
Acceleration variance (g^2)
Holding condition
Tank
Respirometer
ad
b
ae
ab
ab
ab
abf
ab
b
cd
c
ce
c
c
c
cf
c
c
21
23
25
26
27
28
29
30
31
Temperature (°C)


Acceleration variance (g^2)
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
40
60
80
100
120
140
160
180
200
Heart rate (beats min^{-1})


Oxygen consumption (mmol kg^{-1} h^{-1})
1
2
3
4
5
6
7
8
40
60
80
100
120
140
160
180
Heart rate (beats min^{-1})
Trial
Hypoxia
Temperature


Heart rate (beats min^{-1})
100
90
80
70
60
50
40
100
90
80
70
60
50
40
24
30
36
42
48
54
60
66
72
78
84
Elapsed time after surgery (h)