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Oxygen uptake, heart rate and activities of locomotor muscles during a critical swimming speed protocol in the gilthead sea bream *Sparus aurata*

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Abstract

Oxygen uptake, heart rate, and contraction frequencies of slow oxidative (SO) and fast glycolytic (FG) muscle, were measured simultaneously in gilthead seabream *Sparus aurata* submitted to stepwise increases in current speed in a swimming respirometer. Variation in oxygen uptake was closely related to variation in heart rate, over initial steps these rose in concert with an increase in contraction frequency of SO muscle. There was an asymptote in oxygen uptake and heart rate at high speeds, that reflected a transition from exclusive use of aerobic SO muscle to a combination of SO and anaerobic FG muscle, and which preceded fatigue.

The critical swimming speed (U_{crit}) protocol (Brett, 1964) is a well-established method to measure exercise performance in fishes (Beamish, 1978; Webb, 1998; McKenzie & Claireaux, 2010). It has been used widely to investigate effects on performance of environmental factors such as temperature or salinity (Randall & Brauner, 1991; Farrell *et al.*, 2008; McKenzie & Claireaux, 2010) or as a biomarker of toxicological effects of aquatic pollutants (e.g. Beaumont et al., 1995; McKenzie et al., 2007; Wood et al., 1996), and has had important applications in research for conservation of valuable migratory species (Farrell *et al.*, 2008; Eliason *et al.*, 2011). The protocol exposes fish to stepwise increases in speed in a swimming respirometer, until fatigue. The fish swims against the current by rheotactic reflex, holding position in the swim channel of the respirometer.

In fishes that use body-caudal fin locomotion (Webb, 1998), slow-twitch oxidative (SO) 'red' muscle is used to power swimming at low to intermediate swimming speeds. Strips of muscle along the flanks beat the tail at steady frequencies directly proportional to swimming speed and the fish maintains a relatively static position in the respirometer. Contraction of SO muscle relies on ATP generated aerobically, it is well vascularized and supplied with nutrients and oxygen in the blood, pumped by the heart. So, increased aerobic swimming speed is linked to marked increases in oxygen uptake rate (M_{O2}) and heart beat frequency (f_H) (Chatelier *et al.*, 2005; McKenzie & Claireaux, 2010). At a certain high current speed in a U_{crit} test, however, the fish starts to engage its large myotomal blocks of fast-twitch glycolytic (FG) 'white' muscle, with irregular powerful tailbeats. These propel the fish forward in the swim channel, after which they drift back on the current, a 'burst and coast' swimming mode (Webb, 1998). The FG muscle is poorly vascularised and uses endogenous fuel stores to generate ATP anaerobically so, when it is recruited at high swim speeds, there can be an asymptote or even slight decline in M_{O2} and f_H (Beamish, 1978; Lee *et al.*, 2003; Chatelier *et al.*, 2005). This may be linked to reduced rates of SO muscle contraction but this has not been explicitly investigated. Once fishes recruit FG muscle and engage burst and coast swimming, they typically fatigue quite rapidly.

Although patterns of SO and FG muscle recruitment have been studied during forced swimming in fishes (Roberts & Graham, 1979; Rome *et al.*, 1985; Rome & Alexander, 1990; Geist *et al.*, 2003) no study has, to the best of our knowledge, simultaneously measured M_{O2}, f_H and muscle activity during a U_{crit} trial. This is interesting better to understand how patterns of oxygen uptake and heart rate relate to sequential recruitment of aerobic oxidative and then anaerobic glycolytic muscle. Specifically, to demonstrate that an asymptote in f_H and M_{O2} at high speeds coincides with recruitment of FG muscle, and to ascertain what SO muscle activity patterns are at the highest speeds prior to fatigue.

Experiments were performed on gilthead seabream *Sparus aurata* L. 1758, a coastal marine teleost that is highly prized and widely farmed in the Mediterranean. It uses a sub-carangiform swimming mode and, in the wild, adults can migrate over hundreds of kilometres (Lasserre, 1976). 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#10130-201704071516523 v3.

Six *S. aurata* with a mean (± SD) mass of 612 ± 10g and forklength of 294 ± 13 mm were studied, from a population of animals obtained from Cannes Aquaculture fish farm and maintained at Ifremer Experimental Aquaculture Station at Palavas-les-Flots, in outdoor 3000 l tanks provided with a flow of local seawater at prevailing seasonal temperatures and an average salinity over the study period of 34‰. Fish were held for over 18 months prior to experiments, fed commercial pellets daily but fasted for 24 h prior to trials. Experiments were in April and May, when seawater temperature was 15 to 17 °C.

Fish were anesthetized by immersion in 0.1 g l^{-1} benzocaine in aerated seawater, until active ventilation ceased, then positioned on an operating table with gills irrigated with aerated seawater containing 0.05 g l^{-1} benzocaine. Insulated stranded stainless steel wire electrodes (0.05 mm wire diameter, 0.23 mm total diameter; Steel 7 Strand, A-M Systems, www.phymep.com), 800mm total length, insulated to within 0.5 cm of the tip, were placed to measure ECG and EMGs. For ECG, two electrodes were inserted on either side of the animal, under the cleithrum behind the 4th gill arch in close proximity to the heart. For EMGs, pairs of electrodes were inserted on one flank, along the axis of the animal beginning at the insertion of the dorsal fin and at a distance of 4 cm from each other; for SO muscle superficially just above midline (avoiding any contact with the lateral line); for FG muscle into epaxial muscle dorsally. The position of electrodes in SO and FG muscle was confirmed by dissection post mortem. All wires were held in place with sutures at point of insertion, then gathered and held in place with a common suture in front of the dorsal fin, so they trailed above fish during swimming trials (Blasco *et al.*, 2016). After surgery, fish were recovered in a Steffensen-type swim-tunnel respirometer (vol. 49 l) for at least 24h in aerated seawater at 16°C, swimming in a current equivalent to 0.5 body lengths per second (BL s^{-1}).

The respirometer is designed to provide a non-turbulent water flow with a uniform velocity profile, in which to exercise fish at controlled current speeds while measuring their rates of oxygen uptake (McKenzie *et al.*, 2001). The anterior portion of the swim section was shielded with black plastic sheeting to avoid visual disturbance of fish, which spontaneously occupied this area. After recovery, seabream were exposed to progressive increments of swimming speed of 0.5 BL s^{-1} each 40 min, until fatigue. Fish were considered fatigued when they rested their caudal fins on the downstream grid for at least 10s. Critical swimming speed (U_{crit}, in BL s^{-1}) was calculated as described by Brett (1964). The Mo_2 was measured in mmol O_2 kg^{-1} h^{-1} by respirometry twice at each speed by cyclical "intermittent stopped-flow" (Steffensen, 1989; Svendsen *et al.*, 2016), using the Aquaresp program (University of Copenhagen, www.aquaresp.com). Briefly, the cycle alternates two phases; firstly, the swim tunnel receives no water and the fish consumes oxygen within the closed system, measured by an optical sensor (Firesting O2, Pyro-Science, www.pyro-science.com) and associated software (Pyro Oxygen Logger), with data taken into Aquaresp. Secondly, Aquaresp starts a pump by USB relay (Cleware, www.cleware-shop.de) that flushes aerated seawater through the tunnel, so renewing oxygen and removing wastes. Each cycle was 20 min, 8 of measurement and 12 of flushing; two cycles were completed at each swimming speed. Standard metabolic rate (SMR; basal metabolic rate at acclimation temperature) was estimated by back-extrapolating the relationship between MO_2 and speed to a notional speed of zero (Brett, 1964; Chabot *et al.*, 2016). Active Metabolic Rate (AMR; maximal MO_2 at acclimation temperature) was identified during swimming and usually occurred at speeds approaching U_{crit} (McKenzie *et al.*, 2003). Absolute aerobic scope (AAS) was the net difference between AMR and SMR, factorial scope (FAS) was AMR/SMR (Beamish, 1978).

To record f_H, (beats min^{-1}) and frequencies of SO and FG muscle contractions (f_{SO} and f_{FG}, respectively, in Hz), ECG and EMG electrodes were connected to a BIOPAC MP36R data acquisition system linked to a computer with BIOPAC Acqknowledge software (www.biopac.com). EMG signals were filtered and recorded using the Acqknowledge 'EMG 30-1000 Hz' acquisition package, which sampled at 2000 Hz with low band pass at 1000 Hz and high band pass at 30 Hz. Signals were displayed on Acqknowledge throughout trials, then recorded twice for 5 min at each swimming speed. The f_H was calculated based on mean time required for 10 R-R intervals of ECG waveforms. The f_{SO} and f_{FG} were obtained from the interval between 5 EMG burst onsets, at each speed.

Data were tested for normality using Shapiro-Wilk Test prior to parametric tests. The effects of swimming speed on M_{O2}, f_H, f_{SO} and f_{FG} were assessed by one way-analysis of variance (ANOVA) for repeated measures. When significant effects were observed, Bonferroni post-hoc tests were used to compare means. For descriptive purposes of patterns in mean data during the stages of the U_{crit} trial, linear or exponential regressions were applied, as detailed below. The relationship of f_H to M_{O2} was described by linear regression. All analyses were performed with SigmaStat 4.0 (Systat Software Inc., www. systatsoftware.com).

Mean (± SE) U_{crit} was 3.2 ± 0.2 BL s^{-1}, all animals completed at least 20 min swimming at a speed of 3 BL s^{-1}, so data were collected for all variables for speeds of 0.5 to 3 BL s^{-1} (Fig 1). Mean M_{O2} increased significantly ($P<0.001$ by ANOVA) with swimming speed, steeply until 2 BL s^{-1} followed by an asymptote at higher speeds (fig 1A). Application of an exponential relationship to mean data prior to the asymptote, namely 0.5 BL s^{-1} to 2 BL s^{-1}, revealed a high correlation coefficient (R^2= 0.994). Mean SMR was 3.37 ± 0.28 mmol O_2 kg^{-1} h^{-1}, mean AMR was

11.45 ± 0.60 mmol O_2 kg^{-1} h^{-1}and occurred at 2.83 ± 0.14 BL s^{-1}. The AAS was 8.07 ± 0.49 mmol O_2 kg^{-1} h^{-1} and FAS 3.52 ± 0.29. Mean f_H increased significantly ($P<0.001$ by ANOVA) with swimming speed, this was exponential between 0.5 and 2 BL s^{-1} ($R^2 = 0.996$) with evidence of an asymptote at speeds beyond, coinciding with the asymptote in MO_2 (fig 1B). Maximum f_H was 96 ± 4 beats min^{-1}, at an average swimming speed of 2.92 ± 0.19 BL s^{-1}. There was a highly significant linear relationship between f_H and MO_2 (fig 2) whereby $MO_2 = 0.208(f_H) - 5.648$ ($R^2 = 0.987$, $P < 0.0001$). The reciprocal relationship was used to estimate mean f_H at mean SMR, being 50 beats min^{-1} at 3.37 mmol O_2 kg^{-1} h^{-1}, such that average factorial cardiac scope was 1.9 (96/50).

Fig. S1 in Supplementary Material shows representative traces of EMG signals for SO and FG muscle activity, such as were used to calculate their contraction frequency based upon intervals between burst onsets. There was a significant ($P<0.001$ by ANOVA) increase in mean f_{SO} up to 2 BL s^{-1} after which contraction frequency dropped and, at 3 BL s^{-1}, it was significantly lower than at 2.5 BL s^{-1} (fig 1C). Among fish, the maximum f_{SO} of 4.00 ± 0.31 Hz occurred at a mean speed of 2.67 ± 0.2 BL s^{-1}, providing a mean stridelength (BL swum per SO muscle contraction) of 0.69 ± 0.07 BL. By contrast, FG muscle had no contractile activity until a speed of 2 BL s^{-1}, after which it increased rapidly and significantly ($P<0.001$ by ANOVA) to reach a maximum of 1.07 ± 0.30 Hz at 3 BLs^{-1}, the last swimming speed that all fish achieved prior to fatigue (fig 1D). The engagement of FG muscle was associated with a gait transition to a 'burst and coast' swimming mode, where sea bream used irregular powerful tailbeats that 'burst' it forward in the swim channel, after which it 'coasted' back on the current until repeating the action.

The results demonstrate how patterns of M_{O2} and f_H during a U_{crit} protocol relate to sequential recruitment of aerobic oxidative and anaerobic glycolytic muscle in a teleost with sub-carangiform locomotion. The U_{crit} values are similar to reports for farmed *S. aurata* (Basaran et al., 2007; Svendsen et al., 2015), comparisons are confounded by differences in body size and water temperature, which both affect U_{crit} performance (Beamish, 1978; McKenzie & Claireaux, 2010). The changes in M_{O2} with swimming speed, comprising an initial exponential increase followed by an asymptote, have been reported in various species (Chatelier et al., 2005; Lee et al., 2003; Tudorache et al., 2015; this study), although others report an exponential increase up until fatigue (McKenzie *et al.*, 2003; Steinhausen *et al.*, 2005; Methling *et al.*, 2011; Tudorache *et al.*, 2015). The FAS during U_{crit}, approximately 3.5, is less than a previous report of around 5 (Svendsen et al., 2015), which may be due to factors such as fish size and rearing conditions. The f_H at low speeds, and as estimated at SMR from the linear relationship between f_H and M_{O2}, were similar to a previous reports of 'routine' f_H for *S. aurata* at this temperature (Aissaoui *et al.*, 2000).

The exponential increase in M_{O2} and f_H during initial stages of the U_{crit} was clearly a response to increasing oxygen and nutrient demands of mitochondria in working SO muscle (Teulier *et al.*, 2019), required to contract the muscle and beat the tail faster as speed steps were imposed. It is well-established that M_{O2} rises exponentially with speed of aerobic swimming in fishes (Beamish, 1978; Webb, 1998). The estimated stridelength of SO muscle, approximately 0.7 BL per tailbeat, is typical for teleosts with sub-carangiform locomotion (Beamish, 1978). The asymptote in M_{O2} and f_H at 2 BL s^{-1} coincided with f_{SO} also reaching an asymptote coupled with engagement of FG muscle. The fact that SO muscle continued to contract at relatively high frequencies, alongside recruitment of the large, powerful FG muscle blocks, may explain why M_{O2} and f_H did not decline but essentially remained stable up until fatigue at U_{crit}. Although FG muscle is poorly vascularised, it does receive some blood flow and is a very large organ, representing up to 70% of the mass of the seabream (Teulier *et al.*, 2019). The very close coupling of M_{O2} to f_H, throughout the U_{crit} protocol, was confirmed by the fact that variation in f_H explained almost 99% of variation in M_{O2}.

The f_{FG} was much lower than f_{SO}, with much more variation around the mean because contractions were aperiodic. It has been suggested that fatigue in a U_{crit} swim test is in fact a behavioural response, that occurs when a fish cannot engage the full power of FG muscle in the limited confines of the swim tunnel, so 'chooses' to fall back against the rear screen (Peake & Farrell, 2006; Tudorache et al., 2007).

In conclusion, the data provide the first simultaneous measure of M_{O2}, f_H and contraction frequencies of SO and FG muscle in a fish during a U_{crit} protocol. The results demonstrate unequivocally that variation in M_{O2} is closely related to variation in f_H and that an asymptote in M_{O2} and f_H, at high speeds, reflects a transition from exclusive use of aerobic SO muscle to a combination of SO and anaerobic FG muscle.

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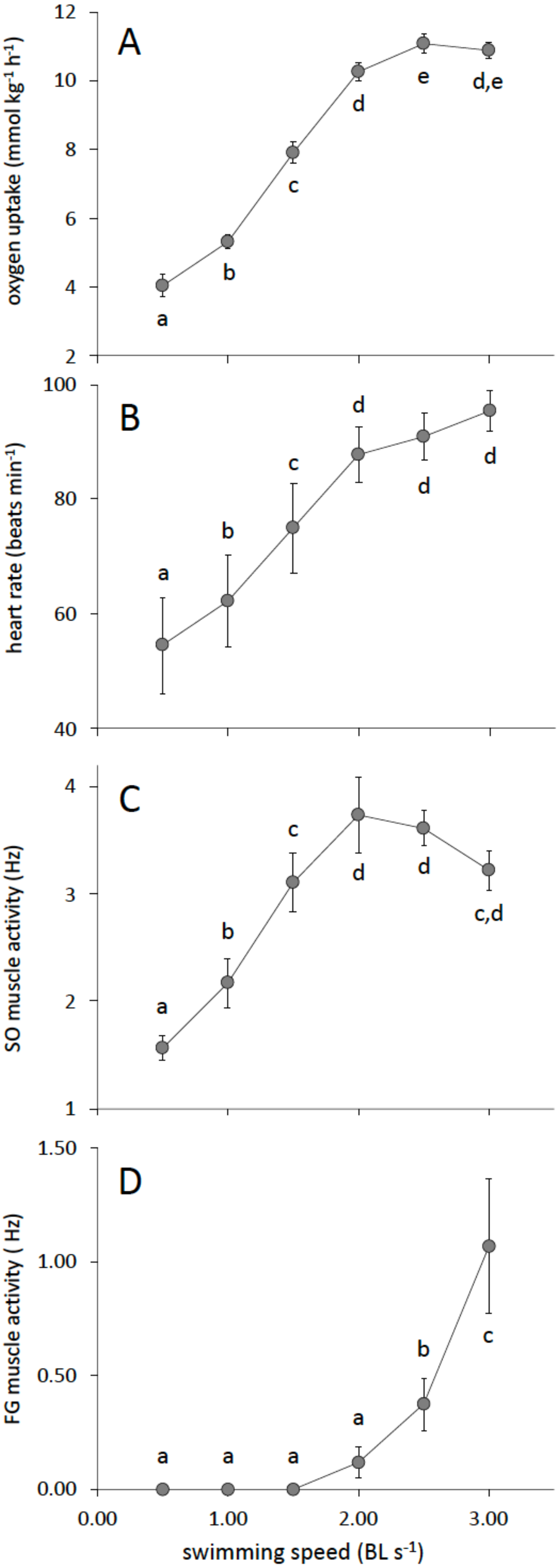
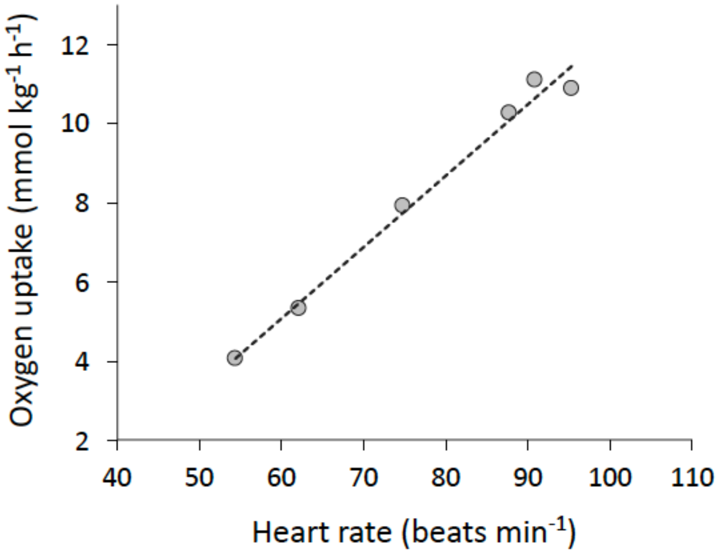
Figure legends

Figure 1. Effects of swimming speed, in bodylengths s^{-1}, on mean (± SEM) oxygen uptake rate (A); heart rate (B), and the contraction frequencies of slow-twitch oxidative (SO) and fast-twitch glycolytic (FG) muscles (C and D, respectively), in n =6 *Sparus aurata* submitted to a critical swimming speed protocol. On each panel, similar letters indicate no significant difference in the mean, by 1-way ANOVA for repeated samples and Bonferroni post-hoc test ($P > 0.05$).

Figure 2. Least squares linear relationship between mean heart beat frequency (f_H) and mean oxygen uptake rate (M_{O2}) in n =6 *Sparus aurata* submitted to a critical swimming speed protocol. The line is described by the equation $M_{O2} = 0.180(f_H) - 5.738$ ($R^2 = 0.987$).


A
oxygen uptake (mmol kg-1 h-1)
12
10
8
6
4
2
a
b
c
d
e
d,e
B
heart rate (beats min-1)
100
80
60
40
a
b
c
d
d
d
C
SO muscle activity (Hz)
4
3
2
1
a
b
c
d
d
c,d
D
FG muscle activity (Hz)
1.50
1.00
0.50
0.00
a
a
a
a
b
c
0.00
1.00
2.00
3.00
swimming speed (BL s-1)


Oxygen uptake (mmol kg^{-1} h^{-1})
2
4
6
8
10
12
40
50
60
70
80
90
100
110
Heart rate (beats min^{-1})