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Non-lethal sampling for assessment of mitochondrial function does not affect metabolic rate and swimming performance

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Abstract :

A fundamental issue in the metabolic field is whether it is possible to understand underlying mechanisms that characterize individual variation. Whole-animal performance relies on mitochondrial function as it produces energy for cellular processes. However, our lack of longitudinal measures to evaluate how mitochondrial function can change within and among individuals and with environmental context makes it difficult to assess individual variation in mitochondrial traits. The aims of this study were to test the repeatability of muscle mitochondrial metabolism by performing two biopsies of red muscle, and to evaluate the effects of biopsies on whole-animal performance in goldfish Carassius auratus . Our results show that basal mitochondrial respiration and net phosphorylation efficiency are repeatable at 14-day intervals. We also show that swimming performance (optimal cost of transport and critical swimming speed) was repeatable in biopsied fish, whereas the repeatability of individual oxygen consumption (standard and maximal metabolic rates) seemed unstable over time. However, we noted that the means of individual and mitochondrial traits did not change over time in biopsied fish. This study shows that muscle biopsies allow the measurement of mitochondrial metabolism without sacrificing animals and that two muscle biopsies 14 days apart affect the intraspecific variation in fish performance without affecting average performance of individuals. This article is part of the theme issue ‘The evolutionary significance of variation in metabolic rates’.

Keywords : red muscle, whole-animal performance, repeatability, goldfish

Introduction

Metabolic rate determines the energy expenditure of individual animals and, as such, is a fundamental trait underlying organismal performance (Metcalfe et al., 2016). Metabolic rate is often determined from the organismal rate of oxygen consumption; it is refereed as minimal, or standard, and maximal metabolic rates (SMR and MMR, respectively) in ectotherms. SMR is the minimum of energy that animals expend on their tissues maintenance and homeostatic mechanisms (Fry, 1971). The MMR is the excess energy an individual can allocate to growth, reproduction (Norin and Clark, 2016) or exercise (McKechnie and Swanson, 2010; Weibel and Hoppeler, 2005). Therefore, SMR and MMR represent lower and upper limits of aerobic capacity. However, oxygen consumption alone is unlikely to be a sufficient marker of aerobic metabolism in many situations. This is due to the variability in the link between the amount of adenosine triphosphate (ATP) generated per molecule of oxygen consumed by mitochondria. Because ATP is the main form of energy fueling cellular function, such as biomass production and muscle contraction, the efficacy with which mitochondria produce ATP can be a major determinant of organismal performance (Hood et al., 2018; Koch et al., 2021).

Investigating variation in mitochondrial metabolism provides an opportunity for integrating how mechanisms at the cellular level constraints whole-organism traits (Cominassi et al., 2022; Conley, 2016; Hood et al., 2018; Koch et al., 2021). For example, individual variation in SMR and MMR were positively associated with proton leak respiration in hepatic mitochondria (i.e., LEAK respiration) of brown trout *Salmo trutta* (Salin et al., 2016). These results suggest that a high SMR or MMR, rather than signalling a higher ability for respiration-driven ATP synthesis, may actually reflect a greater dissipation of energy. Conversely, the basal metabolic rate in capped chickadees *Poecile atricapillus* was positively correlated with the phosphorylating respiration (i.e., OXPHOS respiration) in liver mitochondria (Milbergue et al., 2022). Similarly, high rates of OXPHOS respiration in heart mitochondria was found in individuals Gulf killifish *Fundulus grandis* that had the highest MMR (Rees et al., 2022), supporting the idea that mitochondrial properties determine the whole-organism metabolism. Individuals that have higher metabolic rates – and so higher mitochondrial phosphorylating respiration – may therefore have a higher rate of ATP production and perform better. However, the interpretation of cross-sectional correlational studies is limited since any relationship between the mitochondrial and whole-animal performance cannot be presumed causal. The main reason for results derived from correlative approaches is that studying mitochondrial metabolic traits usually involves terminal sampling, where determination of whole-organism performance can only precede the mitochondrial measurement.

There is thus a real need for studies that assess mitochondria from samples that can be collected without sacrificing animals (Salin et al., 2015; Stier et al., 2017). Recently, researchers have sought to conduct non-lethal studies of mitochondrial function (Jacobs et al., 2012; Quéméneur et al., 2022; Stier et al., 2017). Such new approaches, based on biopsy (Pesta and Gnaiger, 2012; Quéméneur et al., 2022; Teulier et al., 2012) or blood samples (Al Amir Dache et al., 2020; Braganza et al., 2020; Fang et al., 2014; Karamercan et al., 2013; Stier et al., 2017), now make it possible to gather information on the temporal dynamics of whole-organism and mitochondrial traits. In birds, non-lethal sampling of blood has shown a relationship between mitochondrial traits in red blood cells and reproductive performance (Stier et al., 2017; Stier et al., 2022) and whole-animal metabolic rate (Malkoc et al., 2021; Thoral et al., submitted). In European Sea bass *Dicentrarchus labrax*, non-lethal sampling of the red muscle has linked variation in mitochondrial properties and individual growth performance (Quéméneur et al., 2022). However, in this last study, the mitochondrial traits that explained growth variation differed between the growth rates determined before and after the mitochondrial assay: past growth was correlated with the activity of the cytochrome c oxidase, a measure of mitochondrial density, whereas future growth was linked to LEAK respiration. A possible explanation is that mitochondrial metabolism varies over time (Bouchard and Guderley, 2003; Boutilier and St-Pierre, 2002; Salmón et al., 2023). This might be representative of natural variation in mitochondrial metabolic traits or because sampling procedure for mitochondrial assay have consequences for upcoming whole-organism performance. It has been shown that muscle biopsy has no consequence on the mortality and body condition in fish as Russell's snapper *Lutjanus russeli* and Grass emperor *Lethrinus laticaudis* (Henderson et al., 2016). However, growth rate of Sea bass declined after their muscle being biopsied (Quéméneur et al., 2022). Furthermore, muscle biopsy procedure might be expected to have important consequences for organismal traits that directly rely on bioenergetics, such as growth, locomotory activity, and whole-organism metabolism, but these consequences remain largely unexplored.

The aims of our study were (i) to measure the repeatability over time of mitochondrial traits as well as whole-animal performance parameters and (ii) to determine the consequences of muscle biopsy procedure on whole-animal performance of goldfish *Carassius auratus*. We conducted a longitudinal experiment to evaluate repeatability of red muscle mitochondrial traits 14 days apart (OXPHOS and LEAK respirations, as well as Basal respiration) using biopsied samples of red muscle, and whole organism performance measured three times 14 days apart (whole-organism metabolic rate and swimming performance, Figure 1). We evaluated the effect

of the muscle sampling procedure on whole-animal performance with whole-organism metabolic rate (SMR and MMR) and swimming performance (optimal cost of transport - OptCOT and critical swimming speed – U_{crit}). We also tested how intraspecific variation in organismal performance across the experimental period relates to variation in their mitochondrial metabolic properties. We analyzed mitochondrial properties in the red muscle, as it can be sampled using biopsy punches to collect a muscle tissue plug in a non-lethal manner (Jeffries et al., 2021; Porter et al., 2015) and has a major contribution in whole-animal oxygen consumption and aerobic swimming in fish (McKenzie, 2011). To date, muscle biopsy has only been employed in kilograms endotherm organisms (Humans (Pesta and Gnaiger, 2012); King penguins (Monternier et al., 2014)) with the sole exception of the recent study using a species of fish, the Sea Bass (Quéméneur et al., 2022). By doing so, we provide a detailed description of how to develop the biopsy procedure in organism for which the method has never been applied before, and we illustrate that the red muscle biopsy can be used in smaller animal as small as tens of grams, in our case ~30 g goldfish.

Material and Methods

Animals husbandry

In January 2022, goldfish juveniles (*Carassius auratus*, n = 32) were obtained from a commercial breeder (Anthias Aquariologie, Les Chères, France). Fish were transported to the University of Lyon 1 where they were held in two replicate 200L tanks (n = 16 experimental fish per tank) and allowed to acclimate for 11 days at room temperature (mean ± SD: 19.2 ± 0.3°C) in a continuously fully aerated water with a light cycle of 12h:12h. Fish were fed to satiety 6 days a week with commercial pellets (Tetra, Goldfish Granules). Fish were then randomly distributed in eight replicate 50L tanks (n = 4 per tank). To identify individual fish, variation in the body colour was used. Half of the water of each aquarium was replaced every week and water quality was monitored throughout the experiment with test strips (JBL Easy Test 6 in 1, JBL GMBH;). Additional fish (n = 8 per tank) were present in the two initial tanks (final density: n = 24 per tank). These additional fish were used in pilot experiments (see the 'Preliminary studies' section below and results in the Supplemental data).

All experimental procedures were approved by the French ethical committee (APAFIS #34451-2021122117327959 v2).

142 ***Muscle biopsies***

143 Fish were randomly assigned to two treatments: 'Biopsy' and 'Control'. The biopsied-
144 fish experienced handling, anaesthesia and two biopsies, one on each flank 14 days apart. The
145 non-biopsied (control) fish experienced handling and anaesthesia but no biopsy (Figure 1). The
146 body mass, body length and Fulton index (an index of body condition calculated as 100 * (body
147 mass / body length^3); Gilliers et al., 2004) at the start of the experiment were not different
148 between the groups (Linear models: Body mass: $P = 0.740$; Body length: $P = 0.680$, Fulton
149 index: $P = 0.564$, see Supplemental Data Section 1 Table S1).
150 Each fish was isolated in a 20L-aquarium and fasted for 24 hours before the procedure. On day
151 of the procedure, anaesthesia and analgesia were performed on fish with a solution of MS-222
152 (150 mg/L) and lidocaine (5 mg/L) until loss of equilibrium and dyspnea (AVMA, 2020). The
153 addition of an analgesic compound such as lidocaine was used to prevent animal's pain
154 (Sneddon, 2012).
155
156 Deeply anesthetized fish (total loss of equilibrium, slow and irregular opercular
157 movement, see Hoseini et al., 2015) was immediately placed on a surgical table with a constant
158 flow of the anaesthetic solution to the mouth throughout the surgical procedure (see
159 Supplemental Data Section 2 Figure S1). A moistened tissue was placed over the body to
160 prevent the animal dryness. Biopsy was performed on the lateral line to collect a sample of red
161 muscle (Rome et al., 1988). First, three scales were removed with forceps and the skin was
162 opened over a 4mm-superficial line with a scalpel. The muscle was collected using a biopsy
163 punch (Biopsy Punch, Kai medical, diameter: 1.5 mm) and the muscle sample (4.1 ± 0.5 mg)
164 was immediately placed in a respiration buffer on ice (MiRO5: 0.5 mM EGTA, 3 mM MgCl2,
165 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1
166 g.L^{-1} free-fatty-acid bovine serum albumin, pH 7.1, Gnaiger, 2020). The wound was then
167 sutured with a stitch (JV398, Vicryl) and a protective gelling powder (Orahesive, ConvaTec) was
168 applied to the wound before the fish was placed in an oxygenated recovery bath (duration for
169 full recovery from anaesthesia: 9 ± 5 min). When the fish was fully awake, it was placed back
170 into the 20L aquarium in isolation for 4 hours before being placed back into its original
171 aquarium. Control fish had no surgical procedure (*i.e.* no scales removed, no scalping and no
172 biopsy) but remained on the surgical table with a continuous supply of anaesthetic solution to
173 the mouth for a duration similar to those having the biopsy procedure (non-biopsied fish: 6 ± 1
174 min; biopsied-fish: 8 ± 2 min). Control fish were then placed in a recovery bath (8 ± 7 min)
175 before being returned to their respective aquarium after 4 hours in isolation.

Whole-animal measurements: swimming performance and whole-animal metabolism

Each biopsied and control fish experienced three whole-animal performance measurements to determine their swimming performance (U_{crit} and OptCOT) and whole-animal metabolism (SMR and MMR) 7-day before and 7-day after the biopsies (see Figure 1). The day before each U_{crit} protocol, the fish were anaesthetized with MS-222 (100mg/L) and then were measured for body length and mass and placed in a 30L swim tunnel respirometer (Loligo Systems, Tjele, Denmark) over night at a speed of 0.5 body length per second (BL/s). The temperature was at the room temperature (17.9 ± 0.5°C). The fish were fasted for 24 hours before being placed in the respirometer. The oxygen consumption ($\dot{M}O_2$) of the fish was recorded from around 4pm to 8am the next day (minimum of 13 hours) and the standard metabolic rate (SMR) was calculated as the average 10% of the lowest oxygen consumption values (Chabot et al., 2016).

The next morning, the U_{crit} protocol was performed as in Thoral et al., 2022a. Individuals were exposed to a gradual increase in the current (0.5 BL/s every 30 minutes) until the fish was exhausted (resting on the bottom grid of the swimming area for more than 10 seconds; Thoral et al., 2022a). During the U_{crit} protocol, the $\dot{M}O_2$ was also measured, and the maximum metabolic rate (MMR) was calculated as the maximum oxygen consumption.

U_{crit} (*i.e.* critical swimming speed) was calculated using Brett's equation (Brett, 1964):

$$Ucrit = Vf + \left(\frac{Tf}{Ti}\right) Vi$$

Where Vf is the last speed reached by the fish for which the fish swam during the complete speed step (BL/s), Tf is the time held at the last speed step reached (min), Ti is the full period of a speed step (30 min) and Vi is the speed increment between steps (0.5 BL/s).

The optimal cost of transport (OptCOT) in µg O_2 cm^{-1} kg^{-1} of fish was calculated as the minimal cost of transport calculated during the U_{crit} protocol:

$$COT = \left(\frac{\dot{M}O2}{Speed}\right) * 1000$$

With the $\dot{M}O_2$ expressed in mg O_2 s^{-1} kg^{-1} of fish and the *Speed* in cm s^{-1}.

We verified whether control and biopsied fish did not differ in terms of whole-animal performance at the beginning of the experiment, on Day 0, using linear models (see Table S1). At day 0, the SMR, U_{crit} and OptCOT were not different between control and biopsied fish (LM: $P > 0.05$), while the MMR was higher in biopsied fish (LM: $F = 5.018$, $P = 0.042$).

Mitochondrial metabolism

Mitochondrial metabolism of biopsied red muscle was measured using high-resolution respirometers (Oxygraphs 2k high-resolution respirometers, Oroboros Instruments, Innsbruck, Austria). The respirometers were calibrated every morning at zero and air saturation oxygen levels. All measurements were done at 20°C and the data were acquired using DatLab *v.* 7.

After removing potential white muscle fibres under a binocular magnifying glass, the red muscle sample was gently dried on aluminium foil and then weighed before being placed in the respirometer chamber containing 2.1 mL of MiR05 without undergoing chemical or mechanical permeabilization. We have shown in previous studies that similar tissue preparation in fish allows for stimulation of respiratory fluxes by exogenous substrates (Thoral et al., 2021; Thoral et al., 2022a); we can thus assert that the plasma membranes in our experimental conditions were permeabilized.

A sequential substrate and inhibitor titration protocol, adapted from Teulier et al., 2019 and Thoral et al., 2022a, was immediately run for each fish. First, the Basal respiration was obtained following the addition of pyruvate (final concentration in the respirometry chamber: 5 mM), malate (2.5 mM) and succinate (5 mM). Then, the ATP synthase was activated with the injection of ADP (1 mM) to obtain the phosphorylating respiration (i.e., OXPHOS respiration). Non-phosphorylating respiration was then measured (i.e., LEAK respiration) by adding oligomycin (1.25 µg/mL) that inhibited the ATP synthase. Finally, the residual oxygen consumption (i.e., ROX respiration) was determined by adding antimycin A (2.5 µM); ROX was then removed from all other respiration rates. One negative respiration value of Basal respiration has been subsequently removed from the analyses. The respiratory control ratio (RCR) has been calculated as the rate of respiration in presence of ADP and cytochrome c divided by the rate of respiration in presence of oligomycin. The mean RCR value was 3.22 (± 0.40), suggesting a good integrity of the inner mitochondrial membranes (Chung et al., 2017; Fongy et al., 2023; Hedges et al., 2019). The integrity of the outer mitochondrial membrane was also tested by the injection of cytochrome *c* (0.01 mM) under OXPHOS state. The mean cytochrome *c* effect in the biopsied fish was 24% (± 3%).

The Net Phosphorylation Efficiency (Shama et al., 2016) was calculated as follows:

$$Net\ Phosphorylation\ Efficiency = 1 - \frac{LEAK\ respiration}{OXPHOS\ respiration}$$

Preliminary studies

To evaluate the effect of lidocaine on mitochondrial metabolism, whole-animal metabolism and swimming performance, some fish were anaesthetized with lidocaine (n = 8) in addition to MS-222 while others were anaesthetized with no lidocaine (n = 8; See Supplemental Data section 3). Mitochondrial metabolism, whole-animal metabolism and swimming performance did not differ between fish exposed or not to lidocaine (Figure S3 and Figure S4).

In a separate group of goldfish (n = 8), a comparison in the mitochondrial metabolism between technics of tissue sampling was performed, and the technical repeatability of mitochondrial metabolic traits was assessed (see Supplemental Data section 4). These fish were anaesthetized and biopsied as above and, the day after, they were anaesthetized and euthanized (cervical dislocation) and dissected to collect red muscle. Collection of red muscle was performed by cutting a square of skin and removing a piece of red muscle with a scalpel as used in Thoral et al., 2022a. No effect of the sampling technique on the mitochondrial metabolic traits was found. However, the technical repeatability between the two red muscle samples from the same individual was low to moderate depending on the respiration rates (See Supplemental data section 4).

Statistical analyses

We examined the repeatability of mitochondrial parameters across a 2-week period (between the first and the second biopsy). To control for effects of body mass on the repeatability of mitochondrial parameters, we used the *RptR* package. *RptR* package (Stoffel and Nakagawa, 2017) allows to examine whether repeatability between trials remained while accounting for individual body mass (Model: "Mitochondrial trait ~ Trial (Biopsy 1 or 2) * Body mass + (1|Individual)"). Repeatability was also assessed using Intraclass Correlation Coefficients (ICC, Koo and Li, 2016).

We also examined the repeatability of whole-animal performance in biopsied fish between trial 1 and 2 to measure the effect of the first biopsy. *RptR* package was also used to examine whether repeatability between trials remained while accounting for variation in body mass for SMR and MMR expressed in mg $O_2.h^{-1}$. Thus, the model was "Whole-animal metabolic rate (SMR or MMR) ~ Trial (1 or 2) * Body mass + (1|Individual)". The repeatability of SMR and MMR using ICC was performed on the body-mass normalized metabolic rates (expressed in mg $O_2.h^{-1}.kg^{-1}$ of fish). Then, the repeatability of whole-animal performance in biopsied fish has been assessed between trial 2 and 3 to measure the effect of the second biopsy

as above. The repeatability of whole-animal performance over time has also been assessed in control (non-biopsied) fish.

Repeatability estimates with the *RptR* package are presented in the main document whereas ICC analyses are reported in the Supplemental Data Section 5 for the biopsied fish and Supplemental Data Section 6 for the control (non-biopsied) fish. We also performed paired t-tests to determine whether mean values of mitochondrial and whole-animal parameters changed over the course of the experiment.

We also performed linear mixed models (LMM, Chi-square test [X^2]) to test the effect of the biopsy procedure (fixed factor: biopsied or control fish) and time (fixed factor: day 7 and day 21) as well as their interaction, with individuals as a random effect, on the whole-animal traits (SMR, MMR, U_{crit}, OptCOT, body mass and Fulton index). The body mass was also integrated as a covariate in the models including SMR and MMR as the dependant variable. Thus, the model for the whole-animal metabolic rates was: "SMR or MMR ~ Treatment (control or biopsied) * Time (day 7 or 21) + Body mass + (1|Individual)" and the model for the other whole-animal traits was: "Trait ~ Treatment * Time + (1|Individual)". The model was then simplified according to a stepwise procedure by removing non-significant interaction and non-significant fixed effects (when not included in a significant interaction).

We finally tested correlation between mitochondrial and whole-animal parameters within the same fish by performing Pearson correlation tests. As all the results were not significant ($P > 0.05$), these results are presented in the Supplemental Data Section 7 (see Table S2).

The data from whole-animal measurements on day 28 for one biopsied fish were excluded from the experiment because the wound of this fish reopened during the swim. The whole-animal measurement data from one control fish on day 28 were missing because this fish died before day 28 (jump out of the aquarium). At the mitochondrial level, a negative value of Basal respiration due to a high ROX respiration was removed from the analyses in the biopsied fish anesthetized with MS-222 plus lidocaine. At the individual level, five SMR values are missing because of technical problems (n = 2 for control fish; n = 2 for biopsied fish anesthetized with MS-222 plus lidocaine; n = 1 for biopsied fish anesthetized with MS-222). Each statistical analysis including mitochondrial parameters was run with and without individuals for which the cytochrome *c* effect was higher than mean ± 2SD. Statistical outcomes were not different when including or excluding high cytochrome *c* effect; all mitochondrial data were kept in the dataset. We ran supplemental analyses because of an extreme value of cytochrome c effect of 73% for a fish. The RCR value of the mitochondria from this fish was

2.44, showing a good integrity of the inner membranes. The patterns of the analyses of mitochondrial properties were the same whether or not this individual was included in the models, so models including mitochondrial data from this fish are reported in the manuscript. A *P-value* was considered significant when ≤ 0.05. All statistical analyses were performed on R *v.* 4.0.3.

Results

I. Repeatability of mitochondrial parameters between first and second biopsy.

Between the first and second biopsy, the repeatability was high for the Basal respiration (R = 0.959, $P < 0.001$; Figure 2A) and Net Phosphorylation Efficiency (R = 0.625, $P = 0.024$; Figure 2D), whereas the repeatability was moderate for the LEAK respiration (R = 0.406, $P = 0.118$; Figure 2C) to low for the OXPHOS respiration (R = 0.061, $P = 0.432$; Figure 2B).

However, the mean mitochondrial parameters did not differ between biopsy 1 and 2 (Paired T-test: all $P > 0.05$, Figures 2A to 2C), except for the Net Phosphorylation Efficiency that increased from the 1st to the 2nd biopsy (Paired T-test: t = -4.864, $P = 0.002$, Figure 2D).

II. Repeatability of whole-animal performance in biopsied and control fish.

In the biopsied fish, the repeatability of SMR and MMR before and after the first biopsy was poor (Figure 3A and 3B, R = 0.126, $P = 0.378$ and R = 0, $P = 1$, respectively). However, the repeatability of U_{crit} and OptCOT was high (Figure 3C and 3D, R = 0.643, $P = 0.036$ and R = 0.807, $P = 0.006$, respectively). We found no significant difference for mean values of SMR (Paired T-test: $P = 0.469$, t = 0.784), MMR ($P = 0.633$, t = -0.500), U_{crit} ($P = 0.098$, t = 1.909) and OptCOT ($P = 0.427$, t = -0.843) before and after the first biopsy.

In contrast, the repeatability of SMR before and after the second biopsy was moderate (Figure 3A, R = 0.201, $P = 0.284$), but the repeatability of MMR, U_{crit} and OptCOT was null (Figure 3B to 3D, R = 0, $P = 1$). As above, we found no significant difference for mean values of SMR ($P = 0.621$, t = 0.517), MMR ($P = 0.636$, t = 0.498), U_{crit} ($P = 0.324$, t = 1.074) and OptCOT ($P = 0.546$, t = -0.640) before and after the second biopsy.

In the control (non-biopsied) fish, the repeatability between the first and the second trial was low for the SMR (R = 0.09, $P = 0.407$, Figure S6A) and high for the MMR (R = 0.656, $P = 0.023$, Figure S6B) and OptCOT (R = 0.666, $P = 0.015$ Figure S6D), and the U_{crit} tended to be repeatable, but not significantly (R= 0.65, $P = 0.18$, Figure S6C). Between the second and the

342 third trial, the repeatability was null for the MMR (R = 0, P = 1; Figure S6B) and moderate (but
343 not significant) for the SMR (R = 0.285, P = 0.239. Figure S6A). However, the repeatability
344 was high for the OptCOT (R = 0.919, P < 0.001; Figure S6D) and (but not significant) for the
345 U_{crit} (R= 0.502, P = 0.077; Figure S6C). Moreover, we found no significant difference for mean
346 values of SMR, MMR, U_{crit} and OptCOT between the first and second trial, and also between
347 the second and third trial (P > 0.05, Figure S6).
348
349 **III. Effect of biopsy procedure on whole-animal performance.**
350 The body mass and Fulton index were not different between control and biopsied fish (LMM:
351 Body mass: X^2 = 0.056, P = 0.814; Fulton index: X^2 = 2.994, P = 0.084; Table 1). Moreover,
352 we observed that the SMR, regardless of the trial, did not differ between the biopsied and
353 control fish (X^2 = 0.289, P = 0.591). In addition, regardless of the biopsy procedure, fish did
354 not differ in their SMR between day 14 and day 28 (X^2 = 0.211, P = 0.646). The MMR and the
355 OptCOT were not affected by the biopsies (X^2 = 1.454, P = 0.228 and X^2 = 2.703, P = 0.100)
356 and did not differ between day 14 and day 28 (X^2 =0.067, P = 0.796 and X^2 = 2.187, P = 0.139).
357 Finally, the U_{crit} was also not affected by the biopsies (X^2 = 1.043, P =0.307) and by the
358 measurement day (X^2 = 2.802, P = 0.094).
359

360 ## Discussion

361 In this study, we evaluated the repeatability of mitochondrial metabolism as well as
362 whole-animal performance in goldfish, and we determined consequences of biopsy procedure
363 for whole-animal performance. Interestingly, the mitochondrial Basal respiration and Net
364 Phosphorylation Efficiency in red muscle were repeatable over time. However, even if
365 repeatability of whole-animal metabolism and swimming performance seemed lowered by
366 biopsy, our results show that two muscle biopsies made 14 days apart did not statistically affect
367 the mean whole-animal performance parameters.
368
369 Several recent articles have raised important issues regarding the changes in
370 mitochondrial traits over time (Quéméneur et al., 2022; Stier et al., 2019). Our fish showed
371 state-specific repeatability of the mitochondria, even with a stability of the environmental
372 conditions in which the goldfish were living between measurements. Basal respiration rate was
373 repeatable for a time interval of 14 days, whereas we found a lower repeatability in other traits
374 such as LEAK and OXPHOS respirations. Basal respiration is limited by oxidation of energy
375 substrates originally present in the cells, indicating that individual variation in the mitochondrial

respiration from cellular substrates is stable over a 2-week time and within an environmental context. LEAK and OXPHOS respirations can rapidly change in mitochondrion resulting from new protein assembly in the respiratory chain (Somero and Hand, 1990), or changes in phospholipid composition in the inner membranes (Kraffe et al., 2007). In our study, it is possible that some individuals have been highly sensitive to the first biopsy, and they might have released high level of stress hormone such as corticoids that caused changes in the mitochondrial structure (Duclos et al., 2004). There may also be a change in mitochondrial function associated with the fish handling with the measurement of whole-animal performance 7-day earlier, so that mitochondrial traits in some individuals are more flexible and can change in response to an intensive swimming test or isolation in a respirometry chamber. Although poorly studied, reaction norms of mitochondrial traits can differ among individuals in their direction as well as their magnitude. Thus, repeatability of mitochondrial traits may be low in variable environments because individuals might differ in their response to environmental change. In the present study, we overcome the analytical challenges of longitudinal sampling for mitochondrial assay, which offer future perspectives to explore the dynamics of mitochondrial function across conditions.

Our study showed that mitochondrial metabolism is measurable in red muscle biopsies performed in fish of a 10cm size and weighing less than 30g, with no consequence for their whole-animal performance. These results echoed those from Quéméneur et al., 2022 where one muscle biopsy did not affect the individual differences in the rank order of growth rate in Sea bass. The feasibility of measuring mitochondrial metabolism from tissue samples as small as milligrams of red muscle add on possibility in other types of tissues, such as liver (Kuznetsov et al., 2002) and gills (Dawson et al., 2020). As samples can be collected without sacrificing animals, biopsy procedure might provide a non-terminal approach to determine the mitochondrial metabolism of wild animals. We shall first determine whether tissue biopsies affect animal performance on long term, and future studies will have to be done over a period longer than 14 days. Yet, our results are encouraging for future projects focusing on small animal species, such as great tits *Parus major*, which are of interest for projects looking at changes in mitochondrial metabolism in natural setting (Casagrande et al., 2020; Nord et al., 2021). The biopsy of red muscle provide the means to move toward non-lethal sampling of animals, including small ectotherms, and toward ecological studies of mitochondrial metabolism (Morgan et al., 2019; Morgan et al., 2022; Touzot et al., 2019).

In our study, no significant relationship between the mitochondrial metabolism and whole-animal performance has been found. An explanation for this discrepancy might lie in the fact that rates of mitochondrial respiration are tissue-specific (Farhat et al., 2021; Ost et al., 2018) and the correlation of mitochondrial respiration rates across different tissues in the same individual can be poor (Salin et al., 2016; Salin et al., 2019). The red muscle might thus not be representative of the overall rate of oxygen consumption and ATP production in the entire animal, because this would be defined as the sum of the individual tissue-specific rates. For instance, a recent study showed that mitochondrial metabolism in pectoral muscle was related to thermogenic capacity of capped chickadees whereas liver metabolism, but not the muscle one, was related to the bird BMR (Milbergue et al., 2022). An alternative explanation is based on our sample sizes that might have been insufficient to detect statistically significant covariation in traits such as mitochondrial functioning, swimming performance and whole-animal metabolic rates. It has previously been shown that a sample size of 28-40 fish when using a correlative approach allow to detect intraspecific variation in mitochondrial and whole animal performance (Salin et al., 2016; Salin et al., 2019; Thoral et al., 2021). While measurement of variation among individual fish were a secondary scope of the present study, our work overcome an especially important hurdle for estimating individual variation of mitochondrial traits, and non-lethal measures in relatively large sample size will provide important insights in this area in coming years.

Conclusion

Our results showed that longitudinal and repeated sampling can be performed in a fish model as small as tens grams, testing for the first time how individual variation in mitochondrial metabolism is related to upcoming whole-organism performance. As shown in one of our previous studies (Thoral et al., 2022b), inter-individual variability in mitochondrial metabolism can be strongly affected by environmental conditions. Therefore, it seems essential, especially in a climate change context where individual responses will be more decisive than mean responses (Bestion et al., 2015; Fangue et al., 2009), to set up protocols that allow measuring metabolism of the same individuals at different times in order to assess its temporal evolution depending on environmental conditions. Our approach provides the means to move towards direct assessment of mitochondrial flexibility in individual animals from the size of tens grams. Studying mitochondrial function with no need to cull animals is also a great advantage as it prevents animal sacrifice and improves statistical power (Peters et al., 2012). Finally, analyses

of mitochondrial function from biopsy sampling could be used to investigate the supposed relationship between mitochondrial and whole-animal metabolism across a range of ecological context, in the laboratory and ultimately in the wild, which are relevant criteria for ecologists and evolutionary biologists.

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Authors' contribution

LT and KS conceptualized the project and designed the methodology. LD and IM-S collected the data with the help of LT, ET, AM, AC, LA and JS. ET analysed the data. ET and LT led the writing of the manuscript and all authors contributed to the drafts and final approval for publication.

References

Al Amir Dache, Z., Otandault, A., Tanos, R., Pastor, B., Meddeb, R., Sanchez, C., Arena, G., Lasorsa, L., Bennett, A., Grange, T., et al. (2020). Blood contains circulating cell-free respiratory competent mitochondria. *FASEB J.* **34**, 3616–3630.

AVMA (2020). Guidelines for the Euthanasia of Animals.

Bestion, E., Clobert, J. and Cote, J. (2015). Dispersal response to climate change: Scaling down to intraspecific variation. *Ecol. Lett.* **18**, 1226–1233.

Bouchard, P. and Guderley, H. (2003). Time course of the response of mitochondria from oxidative muscle during thermal acclimation of rainbow trout, Oncorhynchus mykiss. *J. Exp. Biol.* **206**, 3455–3465.

Boutilier, R. G. and St-Pierre, J. (2002). Adaptive plasticity of skeletal muscle energetics in hibernating frogs: Mitochondrial proton leak during metabolic depression. *J. Exp. Biol.*

205, 2287–2296.

Braganza, A., Annarapu, G. K. and Shiva, S. (2020). Blood-based bioenergetics: An emerging translational and clinical tool. *Mol. Aspects Med.* **71**, 100835.

Brett, J. R. (1964). The Respiratory Metabolism and Swimming Performance of Young Sockeye Salmon. *J. Fish. Res. Board Canada* **21**, 1183–1226.

Casagrande, S., Stier, A., Monaghan, P., Loveland, J. L., Boner, W., Lupi, S., Trevisi, R. and Hau, M. (2020). Increased glucocorticoid concentrations in early life cause mitochondrial inefficiency and short telomeres. *J. Exp. Biol.* **223**, 1–13.

Chabot, D., Steffensen, J. F. and Farrell, A. P. (2016). The determination of standard metabolic rate in fishes. *J. Fish Biol.* **88**, 81–121.

Chung, D. J., Morrison, P. R., Bryant, H. J., Jung, E., Brauner, C. J. and Schulte, P. M. (2017). Intraspecific variation and plasticity in mitochondrial oxygen binding affinity as a response to environmental temperature. *Sci. Rep.* **7**, 1–10.

Cominassi, L., Ressel, K. N., Brooking, A. A., Marbacher, P., Ransdell-Green, E. C. and O'Brien, K. M. (2022). Metabolic rate increases with acclimation temperature and is associated with mitochondrial function in some tissues of threespine stickleback. *J. Exp. Biol.* **225**,.

Conley, K. E. (2016). Mitochondria to motion: Optimizing oxidative phosphorylation to improve exercise performance. *J. Exp. Biol.* **219**, 243–249.

Duclos, M., Gouarne, C., Martin, C., Rocher, C., Mormède, P. and Letellier, T. (2004). Effects of corticosterone on muscle mitochondria identifying different sensitivity to glucocorticoids in Lewis and Fischer rats. *Am. J. Physiol. - Endocrinol. Metab.* **286**, 159–167.

Fang, J., Chen, K., Cui, H. M., Peng, X., Li, T. and Zuo, Z. C. (2014). Morphological and cytochemical studies of peripheral blood cells of Schizothorax prenanti. *J. Vet. Med. Ser. C Anat. Histol. Embryol.* **43**, 386–394.

Fangue, N. A., Richards, J. G. and Schulte, P. M. (2009). Do mitochondrial properties explain intraspecific variation in thermal tolerance? *J. Exp. Biol.* **212**, 514–522.

Farhat, E., Cheng, H., Romestaing, C., Pamenter, M. and Weber, J.-M. (2021). Goldfish Response to Chronic Hypoxia: Mitochondrial Respiration, Fuel Preference and Energy Metabolism. *Metabolites* **11**, 187.

Fongy, A., Romestaing, C., Blanc, C., Lacoste-garanger, N., Rouanet, J., Raccurt, M. and Duchamp, C. (2023). Ontogeny of muscle bioenergetics in Adélie penguin chicks (Pygoscelis adeliae). **1**, 1065–1075.

Fry, F. E. J. (1971). The effect of environmental factors on the physiology of fish. *Fish Physiol.* **6**, 1–98.

Gilliers, C., Amara, R., Bergeron, J. P. and Le Pape, O. (2004). Comparison of growth and condition indices of juvenile flatfish in different coastal nursery grounds. *Environ. Biol. Fishes* **71**, 189–198.

Gnaiger, E. (2020). Mitochondrial pathways and respiratory control - An introduction to OXPHOS analysis. *Bioenerg. Commun.* **5th Editio**, 1–23.

Hedges, C. P., Bishop, D. J. and Hickey, A. J. R. (2019). Voluntary wheel running prevents the acidosis-induced decrease in skeletal muscle mitochondrial reactive oxygen species emission. *FASEB J.* **33**, 4996–5004.

Henderson, C. J., Stevens, T. F. and Lee, S. Y. (2016). Assessing the suitability of a non-lethal biopsy punch for sampling fish muscle tissue. *Fish Physiol. Biochem.* **42**, 1521–1526.

Hood, W. R., Austad, S. N., Bize, P., Jimenez, A. G., Montooth, K. L., Schulte, P. M., Scott, G. R., Sokolova, I., Treberg, J. R. and Salin, K. (2018). The mitochondrial contribution to animal performance, adaptation, and life-history variation. *Integr. Comp. Biol.* **58**, 480–485.

Hoseini, S. M., Rajabiesterabadi, H. and Tarkhani, R. (2015). Anaesthetic efficacy of eugenol on iridescent shark, *Pangasius hypophthalmus* (Sauvage, 1878) in different size classes. *Aquac. Res.* **46**, 405–412.

Jacobs, R. A., Siebenmann, C., Hug, M., Toigo, M., Meinild, A. and Lundby, C. (2012). Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria. *FASEB J.* **26**,.

Jeffries, K. M., Teffer, A., Michaleski, S., Bernier, N. J., Heath, D. D. and Miller, K. M. (2021). The use of non-lethal sampling for transcriptomics to assess the physiological status of wild fishes. *Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol.* **256**, 110629.

Karamercan, M. A., Weiss, S. L., Villarroel, J. P. P., Guan, Y., Werlin, E., Figueredo, R., Becker, L. B. and Sims, C. (2013). Can peripheral blood mononuclear cells be used as a proxy for mitochondrial dysfunction in vital organs during hemorrhagic shock and resuscitation? *Shock* **40**, 476–484.

Koch, R. E., Buchanan, K. L., Casagrande, S., Crino, O., Dowling, D. K., Hill, G. E., Hood, W. R., McKenzie, M., Mariette, M. M., Noble, D. W. A., et al. (2021). Integrating Mitochondrial Aerobic Metabolism into Ecology and Evolution. *Trends Ecol.*

Evol. **36**, 1–12.

Koo, T. K. and Li, M. Y. (2016). A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. *J. Chiropr. Med.* **15**, 155–163.

Kraffe, E., Marty, Y. and Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: Roles of membrane proteins, phospholipids and their fatty acid compositions. *J. Exp. Biol.* **210**, 149–165.

Kuznetsov, A. V., Strobl, D., Ruttmann, E., Königsrainer, A., Margreiter, R. and Gnaiger, E. (2002). Evaluation of mitochondrial respiratory function in small biopsies of liver. *Anal. Biochem.* **305**, 186–194.

Malkoc, K., Casagrande, S. and Hau, M. (2021). Inferring Whole-Organism Metabolic Rate From Red Blood Cells in Birds. *Front. Physiol.* **12**, 1–11.

McKechnie, A. E. and Swanson, D. L. (2010). Sources and significance of variation in basal, summit and maximal metabolic rates in birds. *Curr. Zool.* **56**, 741–758.

McKenzie, D. J. (2011). *Swimming and other activities | Energetics of Fish Swimming*. Elsevier Inc.

Metcalfe, N. B., Van Leeuwen, T. E. and Killen, S. S. (2016). Does individual variation in metabolic phenotype predict fish behaviour and performance? *J. Fish Biol.* **88**, 298–321.

Milbergue, M. S., Ve, F., Desrosiers, V. and Blier, P. U. (2022). How does mitochondrial function relate to thermogenic capacity and basal metabolic rate in small birds ? *J. Exp. Biol.* **225**,.

Monternier, P. A., Marmillot, V., Rouanet, J. L. and Roussel, D. (2014). Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks. *J. Exp. Biol.* **217**, 2691–2697.

Morgan, R., Sundin, J., Finnøen, M. H., Dresler, G., Vendrell, M. M., Dey, A., Sarkar, K. and Jutfelt, F. (2019). Are model organisms representative for climate change research? Testing thermal tolerance in wild and laboratory zebrafish populations. *Conserv. Physiol.* **7**, 1–11.

Morgan, R., Andreassen, A. H., Åsheim, E. R., Finnøen, M. H., Dresler, G., Brembu, T., Loh, A., Miest, J. J. and Jutfelt, F. (2022). Reduced physiological plasticity in a fish adapted to stable temperatures. *Proc. Natl. Acad. Sci. U. S. A.* **119**,.

Nord, A., Metcalfe, N. B., Page, J. L., Huxtable, A., McCafferty, D. J. and Dawson, N. J. (2021). Avian red blood cell mitochondria produce more heat in winter than in autumn. *FASEB J.* **35**, 1–12.

Norin, T. and Clark, T. D. (2016). Measurement and relevance of maximum metabolic rate in fishes. *J. Fish Biol.* **88**, 122–151.

Ost, M., Doerrier, C., Gama-Perez, P. and Moreno-Gomez, S. (2018). Analysis of mitochondrial respiratory function in tissue biopsies and blood cells. *Curr. Opin. Clin. Nutr. Metab. Care* **21**, 336–342.

Pesta, D. and Gnaiger, E. (2012). Chapter 3 - High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle. In *Mitochondrial Bioenergetics - Methods and Protocol*, pp. 25–58.

Peters, S. A. E., Palmer, M. K., Ruijter, H. M. Den, Diederick, E., Iii, J. R. C., Leary, D. H. O., Evans, G. W., Joel, S., Bots, M. L., Peters, S. A. E., et al. (2012). Sample size requirements in trials using repeated measurements and the impact of trial design. *Curr. Med. Res. Opin.* **28**, 681–688.

Porter, C., Reidy, P. T., Bhattarai, N., Sidossis, L. S. and Rasmussen, B. B. (2015). Resistance Exercise Training Alters Mitochondrial Function in Human Skeletal Muscle. *Med. Sci. Sports Exerc.* **47**, 1922–1931.

Quéméneur, J. B., Danion, M., Cabon, J., Collet, S., Luis, J., Infante, Z. and Salin, K. (2022). The relationships between growth rate and mitochondrial metabolism varies over time. *Sci. Rep.* 1–9.

Rome, L. C., Funke, R. P., Alexander, R. M., Lutz, G., Aldridge, H., Scott, F. and Freadman, M. (1988). Why animals have different muscle fibre types. *Nature* **335**,.

Roussel, D., Marmillot, V., Monternier, P. A., Bourguignon, A., Toullec, G., Romestaing, C. and Duchamp, C. (2020). Skeletal muscle metabolism in sea-acclimatized king penguins. II. Improved efficiency of mitochondrial bioenergetics. *J. Exp. Biol.* **223**,.

Salin, K., Auer, S. K., Rey, B., Selman, C. and Metcalfe, N. B. (2015). Variation in the link between oxygen consumption and ATP production, and its relevance for animal performance. *Proc. R. Soc. B Biol. Sci.* **282**,.

Salin, K., Auer, S. K., Rudolf, A. M., Anderson, G. J., Selman, C. and Metcalfe, N. B. (2016). Variation in metabolic rate among individuals is related to tissue-specific differences in mitochondrial leak respiration. *Physiol. Biochem. Zool.* **89**, 511–523.

Salin, K., Villasevil, E. M., Anderson, G. J., Lamarre, S. G., Melanson, C. A., McCarthy, I., Selman, C. and Metcalfe, N. B. (2019). Differences in mitochondrial efficiency explain individual variation in growth performance. *Proc. R. Soc. B Biol. Sci.* **286**,.

Salmón, P., Dawson, N. J., Millet, C., Selman, C. and Monaghan, P. (2023). Mitochondrial function declines with age within individuals but is not linked to the

pattern of growth or mortality risk in zebra finch. *Aging Cell* 1–4.

Shama, L. N. S., Mark, F. C., Strobel, A., Lokmer, A., John, U. and Mathias Wegner, K. (2016). Transgenerational effects persist down the maternal line in marine sticklebacks: gene expression matches physiology in a warming ocean. *Evol. Appl.* **9**, 1096–1111.

Sneddon, L. U. (2012). Clinical Anesthesia and Analgesia in Fish. *J. Exot. Pet Med.* **21**, 32–43.

Somero, G. N. and Hand, S. C. (1990). Protein Assembly Regulation: Physiological and Perspectives Evolutionary. *Physiol. Zool.* **63**, 443–471.

Stier, A., Romestaing, C., Schull, Q., Lefol, E., Robin, J. P., Roussel, D. and Bize, P. (2017). How to measure mitochondrial function in birds using red blood cells: a case study in the king penguin and perspectives in ecology and evolution. *Methods Ecol. Evol.* **8**, 1172–1182.

Stier, A., Bize, P., Hsu, B. Y. and Ruuskanen, S. (2019). Plastic but repeatable: Rapid adjustments of mitochondrial function and density during reproduction in a wild bird species. *Biol. Lett.* **15**, 9–13.

Stier, A., Monaghan, P. and Metcalfe, N. B. (2022). Experimental demonstration of prenatal programming of mitochondrial aerobic metabolism lasting until adulthood. *Proc. R. Soc. B Biol. Sci.* **289**,.

Stoffel, M. A. and Nakagawa, S. (2017). rptR : repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods Ecol. Evol.* **8**, 1639–1644.

Teulier, L., Dégletagne, C., Rey, B., Tornos, J., Keime, C., de Dinechin, M., Raccurt, M., Rouanet, J. L., Roussel, D. and Duchamp, C. (2012). Selective upregulation of lipid metabolism in skeletal muscle of foraging juvenile king penguins: An integrative study. *Proc. R. Soc. B Biol. Sci.* **279**, 2464–2472.

Teulier, L., Thoral, E., Queiros, Q., McKenzie, D. J., Roussel, D., Dutto, G., Gasset, E., Bourjea, J. and Saraux, C. (2019). Muscle bioenergetics of two emblematic Mediterranean fish species: *Sardina pilchardus* and *Sparus aurata*. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **235**, 174–179.

Thoral, E., García-Díaz, C. C., Persson, E., Chamkha, I., Elmér, E., Ruuskanen, S. and Nord, A. Permeabilization status affects the relationship between resting metabolic rate and mitochondrial respiration in great tit blood cells. *Submitt. J. Exp. Biol.*

Thoral, E., Queiros, Q., Roussel, D., Dutto, G., Gasset, E., McKenzie, D. J., Romestaing, C., Fromentin, J. M., Saraux, C. and Teulier, L. (2021). Changes in foraging mode

caused by a decline in prey size have major bioenergetic consequences for a small pelagic fish. *J. Anim. Ecol.* **90**, 2289–2301.

Thoral, E., Farhat, E., Roussel, D., Cheng, H., Guillard, L., Pamenter, M. E., Weber, J.-M. and Teulier, L. (2022a). Different patterns of chronic hypoxia lead to hierarchical adaptative mechanisms in goldfish metabolism. *J. Exp. Biol.* **225**, 1–10.

Thoral, E., Roussel, D., Quispe, L., Voituron, Y. and Teulier, L. (2022b). Absence of mitochondrial responses in muscles of zebrafish exposed to several heat waves. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **274**, 111299.

Touzot, M., Teulier, L., Lengagne, T., Secondi, J., Théry, M., Libourel, P. A., Guillard, L. and Mondy, N. (2019). Artificial light at night disturbs the activity and energy allocation of the common toad during the breeding period. *Conserv. Physiol.* **7**, 1–9.

Weibel, E. R. and Hoppeler, H. (2005). Exercise-induced maximal metabolic rate scales with muscle aerobic capacity. *J. Exp. Biol.* **208**, 1635–1644.

Figures

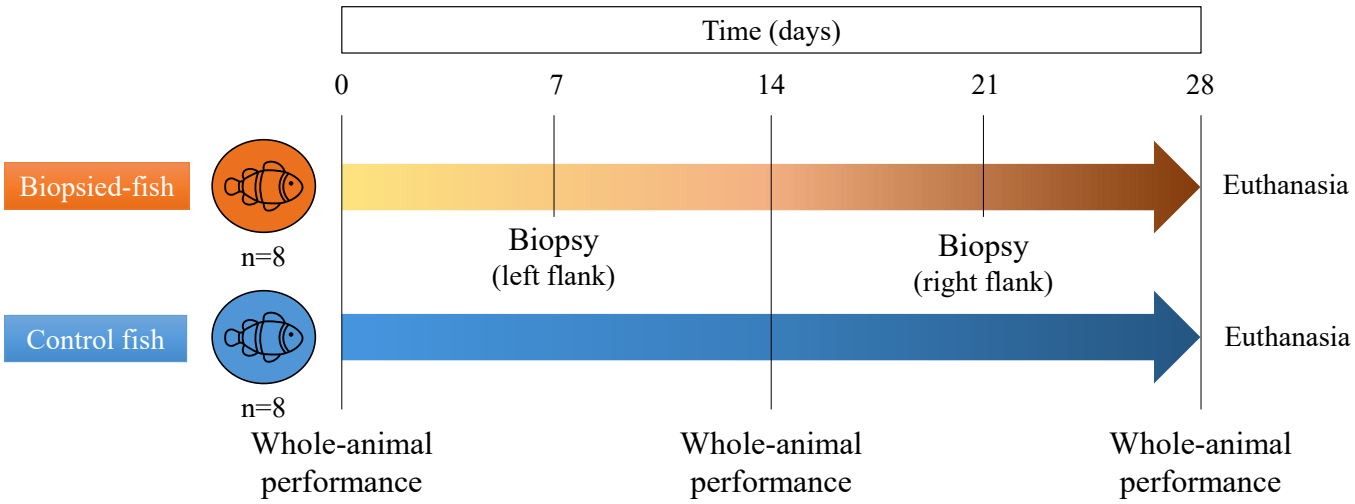
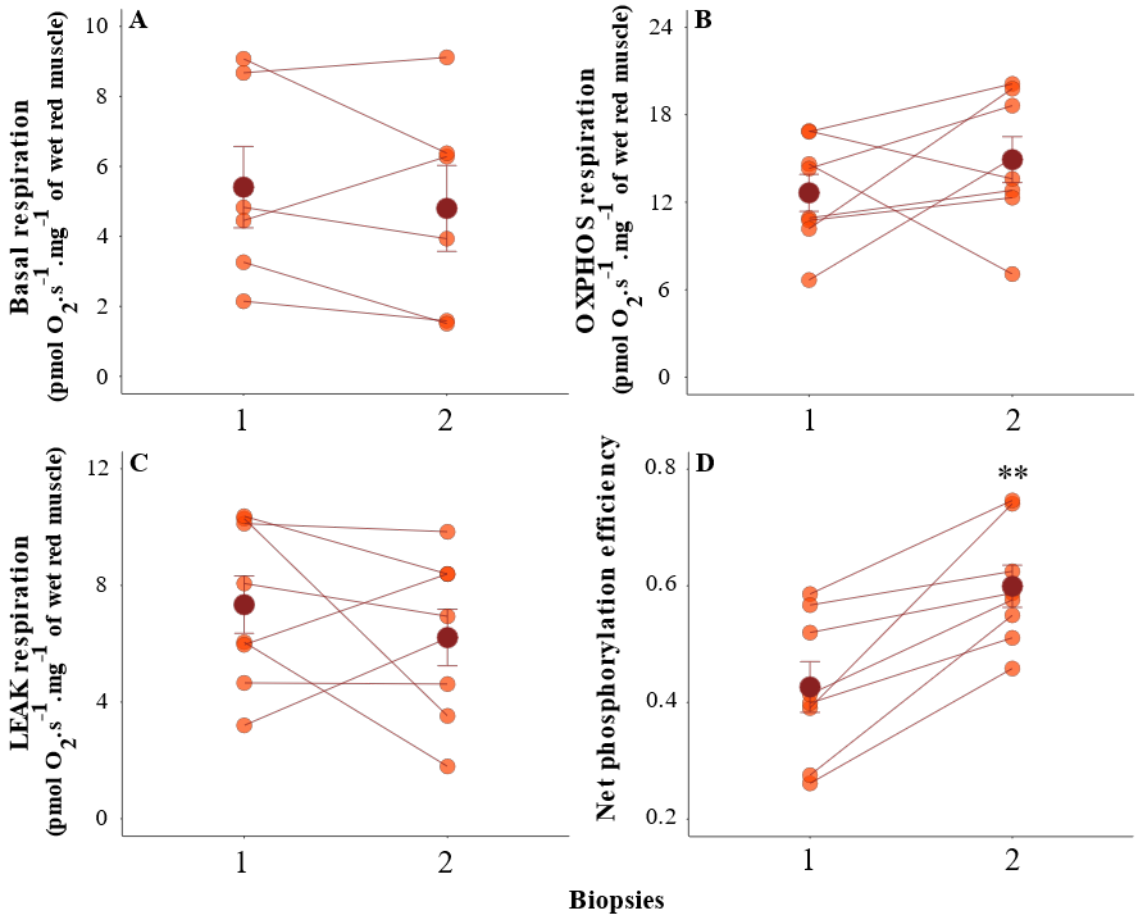


Figure 1: Experimental protocol description. Whole-animal performance was estimated from measurements of whole-animal oxygen consumption rate ($\dot{M}O_2$) and swimming speed (U_{crit}) and repeatability was evaluated three times across a 28-day period at 14-days interval. Biopsied fish had two biopsies of red muscle for measurement of mitochondrial metabolism. Repeatability of the biopsy was evaluated two times across 14-days interval, which occurred between whole-animal performance measurements. The control (non-biopsied) fish experienced handling and anaesthesia procedure, as well as whole-animal performance measurements similar to biopsied-fish, but no biopsy.



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673 **Figure 2**. Repeated measurements of mitochondrial parameters at 14-day interval. The mean ±
674 sem is shown (dark dots) as well as the individuals measured in the two biopsies (light dots).
675 **Indicates a significant difference between biopsies 1 and 2 (**$P < 0.01$).

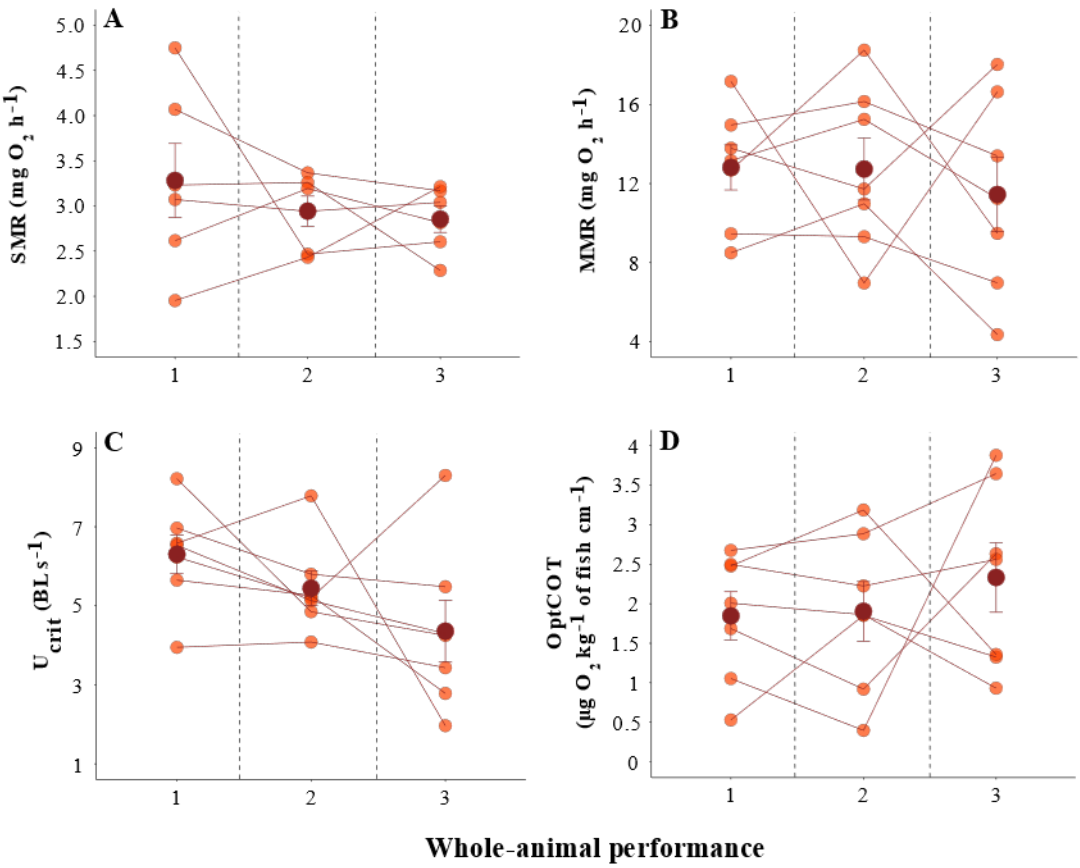

Figure 3: Repeated measurements of whole-animal metabolism and swimming performance in biopsied fish at trial 1, 2 and 3. Dashed lines indicated the biopsies. SMR (A): Standard metabolic rate; MMR (B): Maximum metabolic rate, U_{crit} (C): critical swimming speed; OptCOT (D): optimal cost of transport. The mean ± sem is shown (dark dots) as well as the individuals measured in the two whole-animal performances (light dots).

Tables

Whole-animal performance	Biopsied fish		Control fish	
	Trial 2 (After 1st biopsy)	Trial 3 (After 2nd biopsy)	Trial 2	Trial 3
N	8	7 - 8	8	6 - 7
SMR (mg O_2 h^{-1})	3.05 ± 0.15	2.95 ± 0.16	2.85 ± 0.23	2.87 ± 0.30
MMR (mg O_2 h^{-1})	13.80 ± 1.73	11.45 ± 1.88	10.12 ± 1.03	11.52 ± 1.62
U_{crit} (BL s^{-1})	5.41 ± 0.38	4.36 ± 0.78	4.56 ± 0.97	3.54 ± 0.75
OptCOT (µg O_2 cm^{-1} kg^{-1} of fish)	2.02 ± 0.35	2.33 ± 0.44	2.83 ± 0.55	3.56 ± 0.73
Body mass (g)	25.80 ± 1.39	26.53 ± 1.45	25.61 ± 2.44	25.03 ± 2.46
Fulton Index	2.24 ± 0.08	2.22 ± 0.08	2.16 ± 0.04	2.03 ± 0.04

Table 1. Mean changes in whole-animal traits in biopsied and control (non-biopsied) fish for trial 2 and trial 3. SMR: Standard metabolic rate. MMR: Maximum metabolic rate. U_{crit}: critical swimming speed. OptCOT: optimal cost of transport.