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

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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'.

1. Introduction

Metabolic rate determines the energy expenditure of individual animals and, as such, is a fundamental trait underlying organismal performance [1]. Metabolic rate is often determined from the organismal rate of oxygen consumption; it is referred to 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 [2]. The MMR is the excess energy an individual can allocate to growth, reproduction [3] or exercise [4,5]. 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

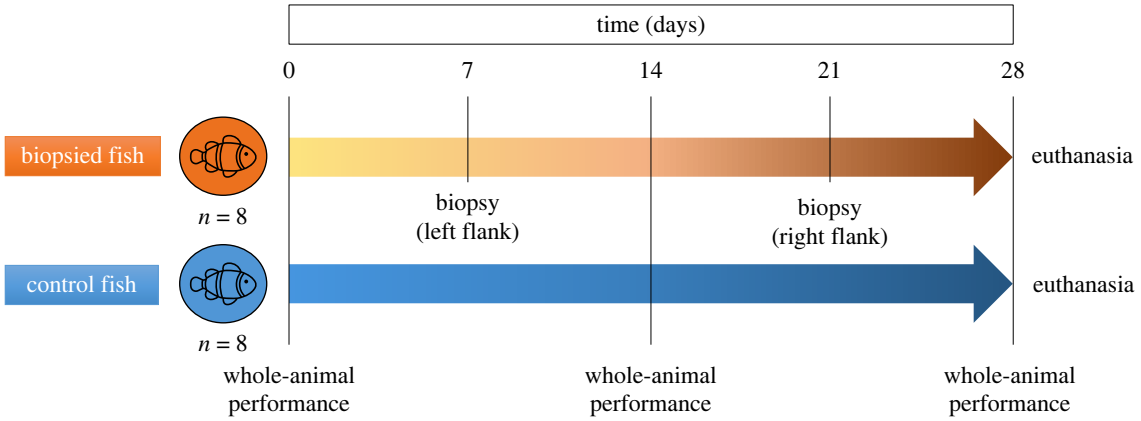

Figure 1. Experimental protocol description. Whole-animal performance was estimated from measurements of whole-animal oxygen consumption rate ($\dot{M}O_2$) and swimming speed (U_{crit}) and repeatability was evaluated three times across a 28-day period at 14-day intervals. Biopsied fish had two biopsies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

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 [6,7].

Investigating variation in mitochondrial metabolism provides an opportunity for integrating how mechanisms at the cellular level constrain whole-organism traits [6–9]. For example, individual variation in SMR and MMR were positively associated with proton leak respiration in hepatic mitochondria (i.e. oxygen consumption in the absence of ATP production, or LEAK respiration) of brown trout *Salmo trutta* [10]. 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. oxygen consumption linked to ATP production, or OXPHOS respiration) in liver mitochondria [11]. Similarly, high rates of OXPHOS respiration in heart mitochondria were found in individuals Gulf killifish *Fundulus grandis* that had the highest MMR [12], 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 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 [13,14]. Recently, researchers have sought to conduct non-lethal studies of mitochondrial function [14–16]. Such new approaches, based on biopsy [16–18] or blood samples [14,19–22], 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 [14,23] and whole-animal metabolic rate [24,25]. In European sea bass *Dicentrarchus labrax*, non-lethal sampling of the red muscle has linked variation in mitochondrial properties and individual growth performance [16]. 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 [26–28]. This might be representative of natural variation in mitochondrial metabolic traits or because sampling procedures 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 such as Russell's snapper *Lutjanus russeli* and grass emperor *Lethrinus laticaudis* [29]. However, growth rate of sea bass declined after their muscle was biopsied [16]. 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 analysed

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 [30,31] and has a major contribution in whole-animal oxygen consumption and aerobic swimming in fish [32]. To date, muscle biopsy has only been employed in kilograms endotherm organisms (Humans [17]; King penguins [33]) with the sole exception of the recent study using a species of fish, the sea bass [16]. 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 approximately 30 g goldfish.

2. Material and methods

(a) Animal 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 200 l tanks ($n = 16$ experimental fish per tank) and allowed to acclimate for 11 days at room temperature (mean ± s.d.: 19.2 ± 0.3°C) in continuously fully aerated water with a light cycle of 12 h : 12 h. Fish were fed to satiety 6 days a week with commercial pellets (Tetra, Goldfish Granules). Fish were then randomly distributed in eight replicate 50 l 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 §2e below and results in the electronic supplementary material).

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

(b) Muscle biopsies

Fish were randomly assigned to two treatments: 'biopsy' and 'control'. The biopsied fish experienced handling, anaesthesia and two biopsies, one on each flank 14 days apart. The non-biopsied (control) fish experienced handling and anaesthesia but no biopsy (figure 1). The body mass, body length and Fulton index (an index of body condition calculated as 100 x (body mass / body length^3); [34]) at the start of the experiment did not differ between the groups (linear models: body mass: $p = 0.740$; body length: $p = 0.680$, Fulton index: $p = 0.564$; see electronic supplementary material Data §1, table S1).

Each fish was isolated in a 20 L aquarium and fasted for 24 h before the procedure. On day of the procedure, anaesthesia and analgesia were performed on fish with a solution of MS-222 (150 mg l^{-1}) and lidocaine (5 mg l^{-1}) until loss of equilibrium and dyspnea [35]. The addition of an analgesic compound such as lidocaine was used to prevent the animals from experiencing pain [36].

Deeply anesthetized fish (total loss of equilibrium, slow and irregular opercular movement; see [37]) were immediately placed on a surgical table with a constant flow of the anaesthetic solution to the mouth throughout the surgical procedure (see electronic supplementary material, Data §2, figure S1). A moistened tissue was placed over the body to prevent the animal from drying out. Biopsy was performed on the lateral line to collect a sample of red muscle [38]. First, three scales were removed with forceps and the skin was opened over a 4 mm-superficial line with a scalpel. The muscle was collected using a biopsy punch (Biopsy Punch, Kai medical, diameter: 1.5 mm) and the muscle sample (4.1 ± 0.5 mg) was immediately placed in a respiration buffer on ice (MiRO5: 0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g.l^{-1} free-fatty-acid bovine serum albumin, pH 7.1; [39]). The wound was then sutured with a stitch (JV398, Vicryl) and a protective gelling powder (Orahesive, ConvaTec) was applied to the wound before the fish was placed in an oxygenated recovery bath (duration of full recovery from anaesthesia: 9 ± 5 min). When the fish was fully awake, it was placed back into the 20 l aquarium in isolation for 4 h before being returned to its original aquarium. Control fish had no surgical procedure (i.e. no scales removed, no scalping and no biopsy) but remained on the surgical table with a continuous supply of anaesthetic solution to the mouth for a duration similar to those having the biopsy procedure (non-biopsied fish: 6 ± 1 min; biopsied-fish: 8 ± 2 min). Control fish were then placed in a recovery bath (8 ± 7 min) before being returned to their respective aquarium after 4 h in isolation.

(c) 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 days before and 7 days after the biopsies (figure 1). The day before each U_{crit} protocol, the fish were anaesthetized with MS-222 (100 mg l^{-1}) and then were measured for body length and mass and placed in a 30 l swim tunnel respirometer (Loligo Systems, Tjele, Denmark) overnight at a speed of 0.5 body lengths per second (BL/s). The water was maintained at the room temperature (17.9 ± 0.5°C). The fish were fasted for 24 h before being placed in the respirometer. The oxygen consumption ($\dot{M}O_2$) of the fish was recorded from around 4 PM to 8 AM the next day (minimum of 13 h) and the SMR was calculated as the average 10% of the lowest oxygen consumption values [40].

The next morning, the U_{crit} protocol was performed as in [41]. Individuals were exposed to a gradual increase in the current (0.5 BL/s every 30 min) until the fish was exhausted (resting on the bottom grid of the swimming area for more than 10 s; [41]). During the U_{crit} protocol, the $\dot{M}O_2$ was also measured, and the MMR was calculated as the maximum oxygen consumption.

U_{crit} (i.e. critical swimming speed) was calculated using Brett's equation [42]:

$$U_{crit} = Vf + \left(\frac{Tf}{Ti}\right) Vi,$$

where Vf is the maximum speed at which the fish was able to swim for 30 min (BL/s), Tf is the time spent swimming at the maximum speed at which the fish became exhausted (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:

$$\mathrm{COT} = \left(\frac{\dot{M}O_2}{\mathrm{speed}}\right) \times 1000,$$

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 confirmed that 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 (LM; see electronic supplementary material, table S1). At day 0, the SMR, U_{crit} and

OptCOT did not differ between control and biopsied fish (LM: $p > 0.05$), while the MMR was higher in biopsied fish (LM: $F = 5.018$, $p = 0.042$).

(d) Mitochondrial metabolism

Mitochondrial metabolism of biopsied red muscle was measured using high-resolution respirometers (Oxygraphs 2 k 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 (Oroboros Instruments, Innsbruck, Austria).

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 [41,43]; we can thus assert that the plasma membranes in our experimental conditions were permeabilized.

A sequential substrate and inhibitor titration protocol, adapted from [44] and [41], 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 measure phosphorylating respiration (i.e. OXPHOS respiration). Non-phosphorylating respiration was then measured (i.e. LEAK respiration) by adding oligomycin (1.25 µg ml^{-1}) 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 the 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 good integrity of the inner mitochondrial membranes [45–47]). 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 [48] was calculated as follows:

$$\text{net phosphorylation efficiency} = 1 - \frac{\text{LEAK respiration}}{\text{OXPHOS respiration}}.$$

(e) 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 electronic supplementary material, Data §3). Mitochondrial metabolism, whole-animal metabolism and swimming performance did not differ between fish exposed or not to lidocaine (electronic supplementary material, figure S3 and figure S4).

In a separate group of goldfish ($n = 8$), we compared the measurements made of mitochondrial metabolism using different techniques of tissue sampling, and the technical repeatability of mitochondrial metabolic traits was assessed (see electronic supplementary matrial, Data §4). These fish were anaesthetized and biopsied as above and on the next day 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 done in [41]. Sampling technique was not found to have any effect on the mitochondrial metabolic traits. However, the technical repeatability between the two red muscle samples from the same individual was low to moderate, depending on the respiration rates (see electronic supplementary material data §4).

(f) Statistical analyses

We examined the repeatability of mitochondrial parameters across a two-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 [49], which allows the examination of whether repeatability between trials remained while accounting for individual body mass (model: 'mitochondrial trait ~ trial (biopsy 1 or 2) x body mass + (1 | individual)'). Repeatability was also assessed using intraclass correlation coefficients (ICC [50]).

We also examined the repeatability of whole-animal performance in biopsied fish between trials 1 and 2 to measure the effect of the first biopsy. The *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 was tested between trials 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 electronic supplementary material, Data §5 for the biopsied fish and electronic supplementary material, Data §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) x time (day 7 or 21) + body mass + (1 | individual)' and the model for the other whole-animal traits was: 'trait ~ treatment x 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 electronic supplementary material, Data §7 (see electronic supplementary material, table S2).

The data from whole-animal measurements on day 28 for one biopsied fish were excluded from the experiment because this fish's wound 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 (jumped 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

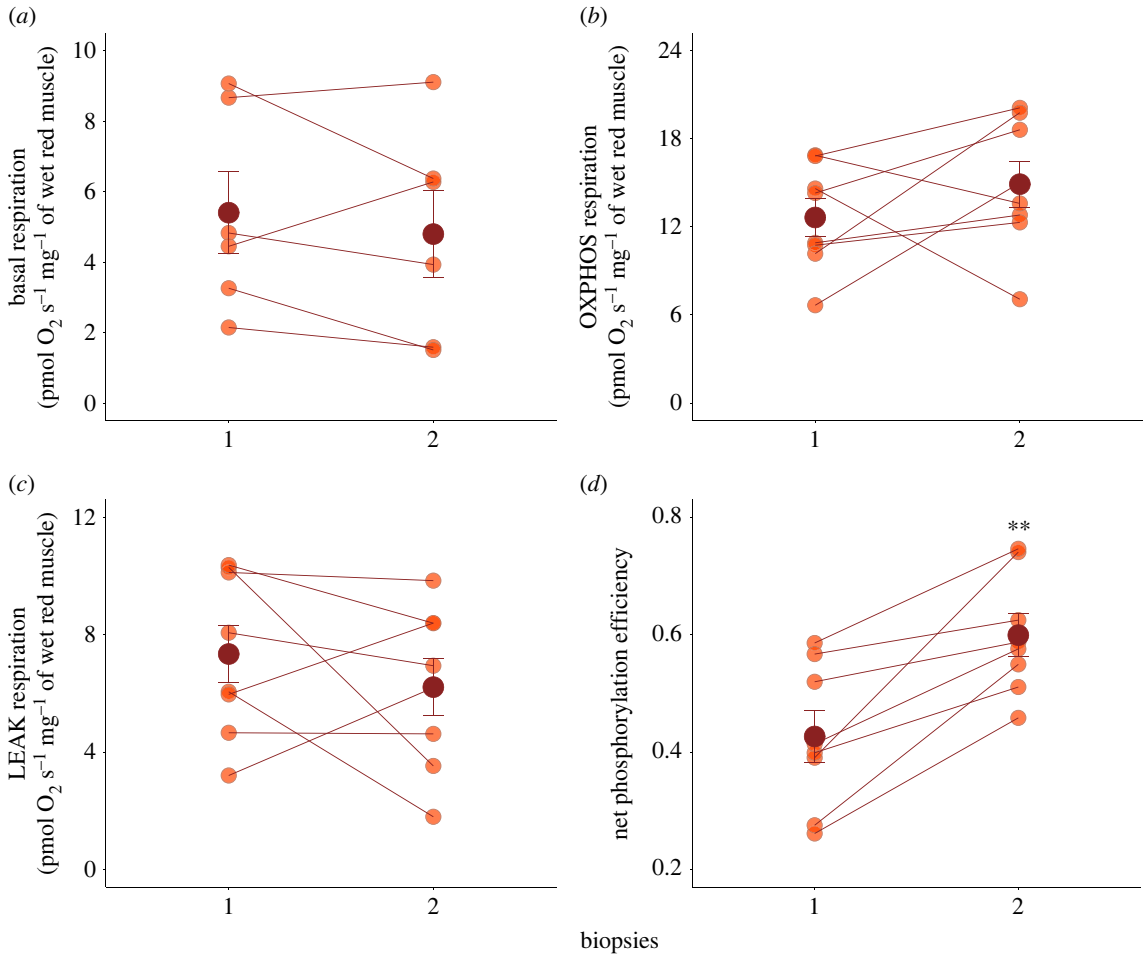

Figure 2. Repeated measurements of mitochondrial parameters at 14-day intervals. The mean ± sem are shown (dark dots) as well as the individuals measured in the two biopsies (light dots). ** Indicates a significant difference between biopsies 1 and 2 (**$p < 0.01$).

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 ± 2 *SD*. 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 one 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.

3. Results

(a) 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 2*a*) and net phosphorylation efficiency ($R = 0.625$, $p = 0.024$; figure 2*d*), whereas the repeatability was moderate for the LEAK respiration ($R = 0.406$, $p = 0.118$; figure 2*c*) to low for the OXPHOS respiration ($R = 0.061$, $p = 0.432$; figure 2*b*).

However, the mean mitochondrial parameters did not differ between biopsy 1 and 2 (paired *T*-test: all $p > 0.05$, figure 2*a–c*), except for the net phosphorylation efficiency that increased from the first to the second biopsy (paired *T*-test: $t = -4.864$, $p = 0.002$, figure 2*d*).

(b) 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 3*a,b*, $R = 0.126$, $p = 0.378$ and $R = 0$, $p = 1$, respectively). However, the repeatability of U_{crit} and *OptCOT* was high (figure 3*c,d*, $R = 0.643$, $p = 0.036$ 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.

By contrast, the repeatability of SMR before and after the second biopsy was moderate (figure 3*a*, $R = 0.201$, $p = 0.284$), but the repeatability of MMR, U_{crit} and *OptCOT* was null (figure 3*b–d*, $R = 0$, $p = 1$). 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 trials was low for the SMR ($R = 0.09$, $p = 0.407$; electronic supplementary material, figure S6A) and high for the MMR ($R = 0.656$, $p = 0.023$, electronic supplementary material, figure S6B) and *OptCOT* ($R = 0.666$, $p = 0.015$; electronic supplementary material, figure S6D) and the U_{crit} tended to be repeatable, but not significantly ($R = 0.65$, $p = 0.18$; electronic supplementary material, figure S6C).

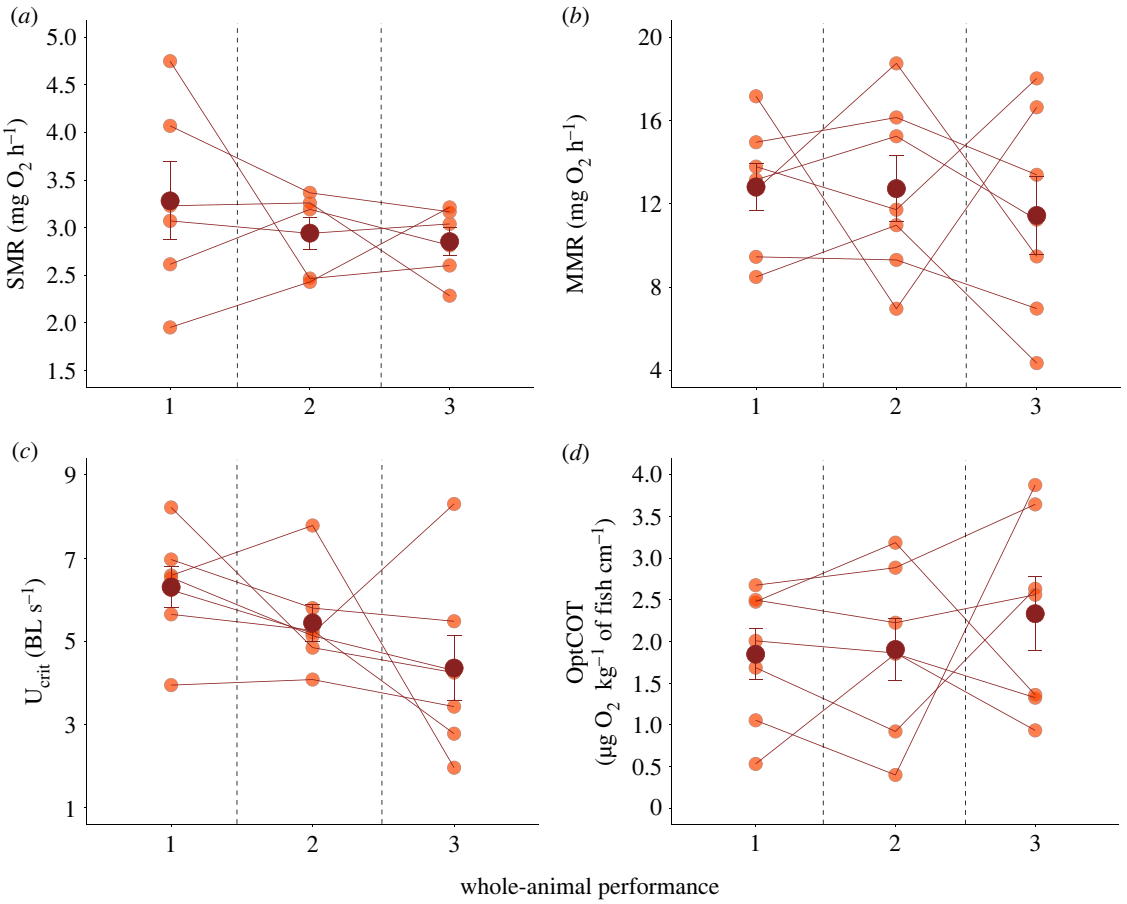

Figure 3. Repeated measurements of whole-animal metabolism and swimming performance in biopsied fish at trials 1, 2 and 3. Dashed vertical lines indicated the biopsies. (*a*) SMR: standard metabolic rate; (*b*) MMR: maximum metabolic rate; (*c*) U_{crit}: critical swimming speed; (*d*) *OptCOT*: optimal cost of transport. Means ± sem are shown (dark dots) as well as the individuals measured in the two whole-animal performances (paler dots).

Between the second and the third trials, the repeatability was null for the MMR ($R = 0$, $p = 1$; electronic supplementary material Figure S6B) and moderate (but not significant) for the SMR ($R = 0.285$, $p = 0.239$; electronic supplementary material, figure S6A). However, the repeatability was high for both the *OptCOT* ($R = 0.919$, $p < 0.001$; electronic supplementary material, figure S6D) and the U_{crit}, although the latter was not significant ($R = 0.502$, $p = 0.077$; electronic supplementary material, figure S6C). Moreover, we found no significant difference for mean values of SMR, MMR, U_{crit} and *OptCOT* between the first and second trials, and also between the second and third trials ($p > 0.05$, electronic supplementary material, figure S6).

(c) Effect of biopsy procedure on whole-animal performance

The body mass and Fulton index did not differ between control and biopsied fish (LMM: body mass: $X^2 = 0.056$, $p = 0.814$; Fulton index: $X^2 = 2.994$, $p = 0.084$; table 1). Moreover, we observed that the SMR, regardless of the trial, did not differ between the biopsied and control fish ($X^2 = 0.289$, $p = 0.591$). In addition, regardless of the biopsy procedure, fish did not differ in their SMR between day 14 and day 28 ($X^2 = 0.211$, $p = 0.646$). The MMR and the *OptCOT* were not affected by the biopsies ($X^2 = 1.454$, $p = 0.228$ and $X^2 = 2.703$, $p = 0.100$) and they did not differ between day 14 and day 28 ($X^2 = 0.067$, $p = 0.796$ and $X^2 = 2.187$, $p = 0.139$). Finally, the U_{crit} was also not affected by the biopsies ($X^2 = 1.043$, $p = 0.307$) nor by the measurement day ($X^2 = 2.802$, $p = 0.094$).

4. Discussion

In this study, we evaluated the repeatability of mitochondrial metabolism as well as whole-animal performance in goldfish, and we determined consequences of biopsy procedure for whole-animal performance. Interestingly, the mitochondrial basal respiration and net phosphorylation efficiency in red muscle were repeatable over time. However, even if repeatability of whole-animal metabolism and swimming performance seemed lowered by biopsy, our results show that two muscle biopsies made 14 days apart did not statistically affect the mean whole-animal performance parameters.

Several recent articles have raised important issues regarding the changes in mitochondrial traits over time [16,51]. The mitochondrial function in our fish showed state-specific repeatability, consistent with the stability of the environmental conditions in which the goldfish were living between measurements. Basal respiration rate was repeatable for a time interval of 14 days, whereas we found a lower repeatability in other traits such as LEAK and OXPHOS respirations. Basal respiration is limited by the oxidation of energy 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 a mitochondrion, resulting from new protein assembly in the respiratory chain [52] or changes in phospholipid composition in the inner membranes [53]. In our study, it is possible that some individuals were highly sensitive to the first biopsy, and they might have released high levels of stress hormone such as corticoids that

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.

	biopsied fish		control fish	
whole-animal performance	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 kg^{-1} of fish cm^{-1})	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

caused changes in the mitochondrial structure [54]. There may also have been a change in mitochondrial function associated with the fish handling during the measurement of whole-animal performance 7 days earlier, resulting in some individuals' mitochondrial traits being more flexible and changeable 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 overcame the analytical challenges of longitudinal sampling for mitochondrial assay, which offers 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 10 cm size and weighing less than 30 g, with no consequence for their whole-animal performance. These results echoed those from [16] 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 suggests that this may also become possible in other types of tissues, such as liver [55] and gills [56]. As samples can be collected without sacrificing animals, biopsy procedures might provide a non-terminal approach to determining the mitochondrial metabolism of wild animals. We shall first determine whether tissue biopsies affect animal performance in the long term, and future studies will have to be done over a period longer than 14 days. However, 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 settings [57,58]. The biopsy of red muscle provides the means to move toward non-lethal sampling of animals, including small ectotherms, and toward ecological studies of mitochondrial metabolism [59–61].

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 [62,63] and the correlation of mitochondrial respiration rates across different tissues in the same individual can be poor [10,64]. 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 the thermogenic capacity of capped chickadees, whereas the metabolism of liver—but not of muscle—was related to bird basal metabolic rate [11]. An alternative explanation is based on our sample sizes, which 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, when using a correlative approach, a sample size of 28–40 fish allows the detection of intraspecific variation in mitochondrial and whole-animal performance [10,43,64]. While measurement of variation among individual fish was a secondary aim in the present study, our work has overcome an especially important hurdle for estimating individual variation of mitochondrial traits, and non-lethal measures in relatively large sample sizes will provide important insights in this area over the coming years.

5. 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 [65], inter-individual variability in mitochondrial metabolism can be strongly affected by environmental conditions. It therefore seems essential—especially in the context of climate change, than mean responses [66,67]—to set up protocols that allow the measurement of the same individuals' metabolism 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 [68]. 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 contexts, in the laboratory and ultimately in the wild, which are relevant criteria for ecologists and evolutionary biologists.

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

Data accessibility. Supplementary material is available online from the Dryad Digital Repository: doi:10.5061/dryad.2v6wwpzvn [69].

Supplementary material is available online [70].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. E.T.: data curation, formal analysis, project administration, supervision, validation, visualization, writing—original draft, writing—review and editing; L.D.: investigation, writing—review and editing; I.M.-S.: investigation, writing—review and editing; A.C.: resources, writing—review and editing; L.A.: resources, writing—review and editing; J.S.: resources, writing—review and editing; A.M.: methodology, writing—review and editing; K.S.: conceptualization, methodology, resources, writing—original draft, writing—review and editing; L.T.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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