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

3
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21
22 **Keywords:** red muscle; whole-animal performance; repeatability; goldfish

23
24 **Abstract**

25 A fundamental issue in the metabolic field is whether it is possible to understand underlying
26 mechanisms that characterize individual variation. Whole-animal performance relies on
27 mitochondrial function as it produces energy for cellular processes. However, individual
28 variation in mitochondrial traits lack of longitudinal measures to evaluate how mitochondrial
29 function can change within and among individuals and with environmental context. The aims
30 of this study were to test the repeatability of muscle mitochondrial metabolism thanks to two
31 biopsies performed in red muscle, and to evaluate effects of biopsies on whole-animal
32 performance in goldfish *Carassius auratus*. Our results show that basal mitochondrial
33 respiration and net phosphorylation efficiency are repeatable at 14-day intervals. We also show
34 that swimming performance (optimal cost of transport and critical swimming speed) was
35 repeatable in biopsied fish, whereas the repeatability of individual oxygen consumption
36 (standard and maximal metabolic rates) seemed not stable over time. However, we noted that
37 the means of individual and mitochondrial traits did not change over time in biopsied fish. This
38 study shows that muscle biopsies allow the measurements of mitochondrial metabolism without

39 sacrificing animals and that two muscle biopsies 14 days apart affect the intraspecific variation
40 in fish performance without affecting average performance of individuals.

41 **Introduction**

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

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

75 There is thus a real need for studies that assess mitochondria from samples that can be
76 collected without sacrificing animals (Salin et al., 2015; Stier et al., 2017). Recently, researchers
77 have sought to conduct non-lethal studies of mitochondrial function (Jacobs et al., 2012;
78 Quéméneur et al., 2022; Stier et al., 2017). Such new approaches, based on biopsy (Pesta and
79 Gnaiger, 2012; Quéméneur et al., 2022; Teulier et al., 2012) or blood samples (Al Amir Dache
80 et al., 2020; Braganza et al., 2020; Fang et al., 2014; Karamercan et al., 2013; Stier et al., 2017),
81 now make it possible to gather information on the temporal dynamics of whole-organism and
82 mitochondrial traits. In birds, non-lethal sampling of blood has shown a relationship between
83 mitochondrial traits in red blood cells and reproductive performance (Stier et al., 2017; Stier et
84 al., 2022) and whole-animal metabolic rate (Malkoc et al., 2021; Thorat et al., submitted). In
85 European Sea bass *Dicentrarchus labrax*, non-lethal sampling of the red muscle has linked
86 variation in mitochondrial properties and individual growth performance (Quéméneur et al.,
87 2022). However, in this last study, the mitochondrial traits that explained growth variation
88 differed between the growth rates determined before and after the mitochondrial assay: past
89 growth was correlated with the activity of the cytochrome c oxidase, a measure of mitochondrial
90 density, whereas future growth was linked to LEAK respiration. A possible explanation is that
91 mitochondrial metabolism varies over time (Bouchard and Guderley, 2003; Boutilier and St-
92 Pierre, 2002; Salmón et al., 2023). This might be representative of natural variation in
93 mitochondrial metabolic traits or because sampling procedure for mitochondrial assay have
94 consequences for upcoming whole-organism performance. It has been shown that muscle
95 biopsy has no consequence on the mortality and body condition in fish as Russell's snapper
96 *Lutjanus russeli* and Grass emperor *Lethrinus laticaudis* (Henderson et al., 2016). However,
97 growth rate of Sea bass declined after their muscle being biopsied (Quéméneur et al., 2022).
98 Furthermore, muscle biopsy procedure might be expected to have important consequences for
99 organismal traits that directly rely on bioenergetics, such as growth, locomotory activity, and
100 whole-organism metabolism, but these consequences remain largely unexplored.

101

102 The aims of our study were (i) to measure the repeatability over time of mitochondrial
103 traits as well as whole-animal performance parameters and (ii) to determine the consequences
104 of muscle biopsy procedure on whole-animal performance of goldfish *Carassius auratus*. We
105 conducted a longitudinal experiment to evaluate repeatability of red muscle mitochondrial traits
106 14 days apart (OXPHOS and LEAK respirations, as well as Basal respiration) using biopsied
107 samples of red muscle, and whole organism performance measured three times 14 days apart
108 (whole-organism metabolic rate and swimming performance, Figure 1). We evaluated the effect

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

123

124 **Material and Methods**

125

126 *Animals husbandry*

127 In January 2022, goldfish juveniles (*Carassius auratus*, $n = 32$) were obtained from a
128 commercial breeder (Anthias Aquariologie, Les Chères, France). Fish were transported to the
129 University of Lyon 1 where they were held in two replicate 200L tanks ($n = 16$ experimental
130 fish per tank) and allowed to acclimate for 11 days at room temperature (mean \pm SD: $19.2 \pm$
131 0.3°C) in a continuously fully aerated water with a light cycle of 12h:12h. Fish were fed to
132 satiety 6 days a week with commercial pellets (Tetra, Goldfish Granules). Fish were then
133 randomly distributed in eight replicate 50L tanks ($n = 4$ per tank). To identify individual fish,
134 variation in the body colour was used. Half of the water of each aquarium was replaced every
135 week and water quality was monitored throughout the experiment with test strips (JBL Easy
136 Test 6 in 1, JBL GMBH;). Additional fish ($n = 8$ per tank) were present in the two initial tanks
137 (final density: $n = 24$ per tank). These additional fish were used in pilot experiments (see the
138 ‘Preliminary studies’ section below and results in the Supplemental data).

139 All experimental procedures were approved by the French ethical committee (APAFIS
140 #34451-2021122117327959 v2).

141

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 $100 * (\text{body}$
147 $\text{mass} / \text{body length}^3)$; 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 KH₂PO₄, 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.

176

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

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

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

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

195
$$U_{crit} = V_f + \left(\frac{T_f}{T_i}\right) V_i$$

196 Where V_f is the last speed reached by the fish for which the fish swam during the complete
197 speed step (BL/s), T_f is the time held at the last speed step reached (min), T_i is the full period
198 of a speed step (30 min) and V_i is the speed increment between steps (0.5 BL/s).

199 The optimal cost of transport (OptCOT) in $\mu\text{g O}_2 \text{ cm}^{-1} \text{ kg}^{-1}$ of fish was calculated as the minimal
200 cost of transport calculated during the U_{crit} protocol:

201
$$COT = \left(\frac{\dot{M}O_2}{Speed}\right) * 1000$$

202 With the $\dot{M}O_2$ expressed in $\text{mg O}_2 \text{ s}^{-1} \text{ kg}^{-1}$ of fish and the *Speed* in cm s^{-1} .

203 We verified whether control and biopsied fish did not differ in terms of whole-animal
204 performance at the beginning of the experiment, on Day 0, using linear models (see Table S1).

205 At day 0, the SMR, U_{crit} and OptCOT were not different between control and biopsied fish (LM:
206 $P > 0.05$), while the MMR was higher in biopsied fish (LM: $F = 5.018$, $P = 0.042$).

207

208 ***Mitochondrial metabolism***

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

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

220 A sequential substrate and inhibitor titration protocol, adapted from Teulier et al., 2019
221 and Thoral et al., 2022a, was immediately run for each fish. First, the Basal respiration was
222 obtained following the addition of pyruvate (final concentration in the respirometry chamber:
223 5 mM), malate (2.5 mM) and succinate (5 mM). Then, the ATP synthase was activated with the
224 injection of ADP (1 mM) to obtain the phosphorylating respiration (i.e., OXPHOS respiration).
225 Non-phosphorylating respiration was then measured (i.e., LEAK respiration) by adding
226 oligomycin (1.25 µg/mL) that inhibited the ATP synthase. Finally, the residual oxygen
227 consumption (i.e., ROX respiration) was determined by adding antimycin A (2.5 µM); ROX
228 was then removed from all other respiration rates. One negative respiration value of Basal
229 respiration has been subsequently removed from the analyses. The respiratory control ratio
230 (RCR) has been calculated as the rate of respiration in presence of ADP and cytochrome c
231 divided by the rate of respiration in presence of oligomycin. The mean RCR value was 3.22 (±
232 0.40), suggesting a good integrity of the inner mitochondrial membranes (Chung et al., 2017;
233 Fongy et al., 2023; Hedges et al., 2019). The integrity of the outer mitochondrial membrane
234 was also tested by the injection of cytochrome *c* (0.01 mM) under OXPHOS state. The mean
235 cytochrome *c* effect in the biopsied fish was 24% (± 3%).

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

237
$$\text{Net Phosphorylation Efficiency} = 1 - \frac{\text{LEAK respiration}}{\text{OXPHOS respiration}}$$

238

239

240

241 ***Preliminary studies***

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

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

258

259 ***Statistical analyses***

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

267 We also examined the repeatability of whole-animal performance in biopsied fish
268 between trial 1 and 2 to measure the effect of the first biopsy. *RptR* package was also used to
269 examine whether repeatability between trials remained while accounting for variation in body
270 mass for SMR and MMR expressed in mg O₂.h⁻¹. Thus, the model was “Whole-animal
271 metabolic rate (SMR or MMR) ~ Trial (1 or 2) * Body mass + (1|Individual)”. The repeatability
272 of SMR and MMR using ICC was performed on the body-mass normalized metabolic rates
273 (expressed in mg O₂.h⁻¹.kg⁻¹ of fish). Then, the repeatability of whole-animal performance in
274 biopsied fish has been assessed between trial 2 and 3 to measure the effect of the second biopsy

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

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

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

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

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

309 2.44, showing a good integrity of the inner membranes. The patterns of the analyses of
310 mitochondrial properties were the same whether or not this individual was included in the
311 models, so models including mitochondrial data from this fish are reported in the manuscript.
312 A *P*-value was considered significant when ≤ 0.05 . All statistical analyses were performed on
313 R v. 4.0.3.

314

315 **Results**

316

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

318 Between the first and second biopsy, the repeatability was high for the Basal respiration ($R =$
319 $0.959, P < 0.001$; Figure 2A) and Net Phosphorylation Efficiency ($R = 0.625, P = 0.024$; Figure
320 2D), whereas the repeatability was moderate for the LEAK respiration ($R = 0.406, P = 0.118$;
321 Figure 2C) to low for the OXPHOS respiration ($R = 0.061, P = 0.432$; Figure 2B).

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

325

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

327 In the biopsied fish, the repeatability of SMR and MMR before and after the first biopsy was
328 poor (Figure 3A and 3B, $R = 0.126, P = 0.378$ and $R = 0, P = 1$, respectively). However, the
329 repeatability of U_{crit} and OptCOT was high (Figure 3C and 3D, $R = 0.643, P = 0.036$ and $R =$
330 $0.807, P = 0.006$, respectively). We found no significant difference for mean values of SMR
331 (Paired T-test: $P = 0.469, t = 0.784$), MMR ($P = 0.633, t = -0.500$), U_{crit} ($P = 0.098, t = 1.909$)
332 and OptCOT ($P = 0.427, t = -0.843$) before and after the first biopsy.

333 In contrast, the repeatability of SMR before and after the second biopsy was moderate (Figure
334 3A, $R = 0.201, P = 0.284$), but the repeatability of MMR, U_{crit} and OptCOT was null (Figure
335 3B to 3D, $R = 0, P = 1$). As above, we found no significant difference for mean values of SMR
336 ($P = 0.621, t = 0.517$), MMR ($P = 0.636, t = 0.498$), U_{crit} ($P = 0.324, t = 1.074$) and OptCOT
337 ($P = 0.546, t = -0.640$) before and after the second biopsy.

338 In the control (non-biopsied) fish, the repeatability between the first and the second trial was
339 low for the SMR ($R = 0.09, P = 0.407$, Figure S6A) and high for the MMR ($R = 0.656, P =$
340 0.023 , Figure S6B) and OptCOT ($R = 0.666, P = 0.015$ Figure S6D), and the U_{crit} tended to be
341 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

376 respiration from cellular substrates is stable over a 2-week time and within an environmental
377 context. LEAK and OXPHOS respirations can rapidly change in mitochondrion resulting from
378 new protein assembly in the respiratory chain (Somero and Hand, 1990), or changes in
379 phospholipid composition in the inner membranes (Kraffe et al., 2007). In our study, it is
380 possible that some individuals have been highly sensitive to the first biopsy, and they might
381 have released high level of stress hormone such as corticoids that caused changes in the
382 mitochondrial structure (Duclos et al., 2004). There may also be a change in mitochondrial
383 function associated with the fish handling with the measurement of whole-animal performance
384 7-day earlier, so that mitochondrial traits in some individuals are more flexible and can change
385 in response to an intensive swimming test or isolation in a respirometry chamber. Although
386 poorly studied, reaction norms of mitochondrial traits can differ among individuals in their
387 direction as well as their magnitude. Thus, repeatability of mitochondrial traits may be low in
388 variable environments because individuals might differ in their response to environmental
389 change. In the present study, we overcome the analytical challenges of longitudinal sampling
390 for mitochondrial assay, which offer future perspectives to explore the dynamics of
391 mitochondrial function across conditions.

392

393 Our study showed that mitochondrial metabolism is measurable in red muscle biopsies
394 performed in fish of a 10cm size and weighing less than 30g, with no consequence for their
395 whole-animal performance. These results echoed those from Quéméneur et al., 2022 where one
396 muscle biopsy did not affect the individual differences in the rank order of growth rate in Sea
397 bass. The feasibility of measuring mitochondrial metabolism from tissue samples as small as
398 milligrams of red muscle add on possibility in other types of tissues, such as liver (Kuznetsov
399 et al., 2002) and gills (Dawson et al., 2020). As samples can be collected without sacrificing
400 animals, biopsy procedure might provide a non-terminal approach to determine the
401 mitochondrial metabolism of wild animals. We shall first determine whether tissue biopsies
402 affect animal performance on long term, and future studies will have to be done over a period
403 longer than 14 days. Yet, our results are encouraging for future projects focusing on small
404 animal species, such as great tits *Parus major*, which are of interest for projects looking at
405 changes in mitochondrial metabolism in natural setting (Casagrande et al., 2020; Nord et al.,
406 2021). The biopsy of red muscle provide the means to move toward non-lethal sampling of
407 animals, including small ectotherms, and toward ecological studies of mitochondrial
408 metabolism (Morgan et al., 2019; Morgan et al., 2022; Touzot et al., 2019).

409

410 In our study, no significant relationship between the mitochondrial metabolism and
411 whole-animal performance has been found. An explanation for this discrepancy might lie in the
412 fact that rates of mitochondrial respiration are tissue-specific (Farhat et al., 2021; Ost et al.,
413 2018) and the correlation of mitochondrial respiration rates across different tissues in the same
414 individual can be poor (Salin et al., 2016; Salin et al., 2019). The red muscle might thus not be
415 representative of the overall rate of oxygen consumption and ATP production in the entire
416 animal, because this would be defined as the sum of the individual tissue-specific rates. For
417 instance, a recent study showed that mitochondrial metabolism in pectoral muscle was related
418 to thermogenic capacity of capped chickadees whereas liver metabolism, but not the muscle
419 one, was related to the bird BMR (Milbergue et al., 2022). An alternative explanation is based
420 on our sample sizes that might have been insufficient to detect statistically significant
421 covariation in traits such as mitochondrial functioning, swimming performance and whole-
422 animal metabolic rates. It has previously been shown that a sample size of 28-40 fish when
423 using a correlative approach allow to detect intraspecific variation in mitochondrial and whole
424 animal performance (Salin et al., 2016; Salin et al., 2019; Thorat et al., 2021). While
425 measurement of variation among individual fish were a secondary scope of the present study,
426 our work overcome an especially important hurdle for estimating individual variation of
427 mitochondrial traits, and non-lethal measures in relatively large sample size will provide
428 important insights in this area in coming years.

429

430 **Conclusion**

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

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

447

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452

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457

458 **Authors' contribution**

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

463

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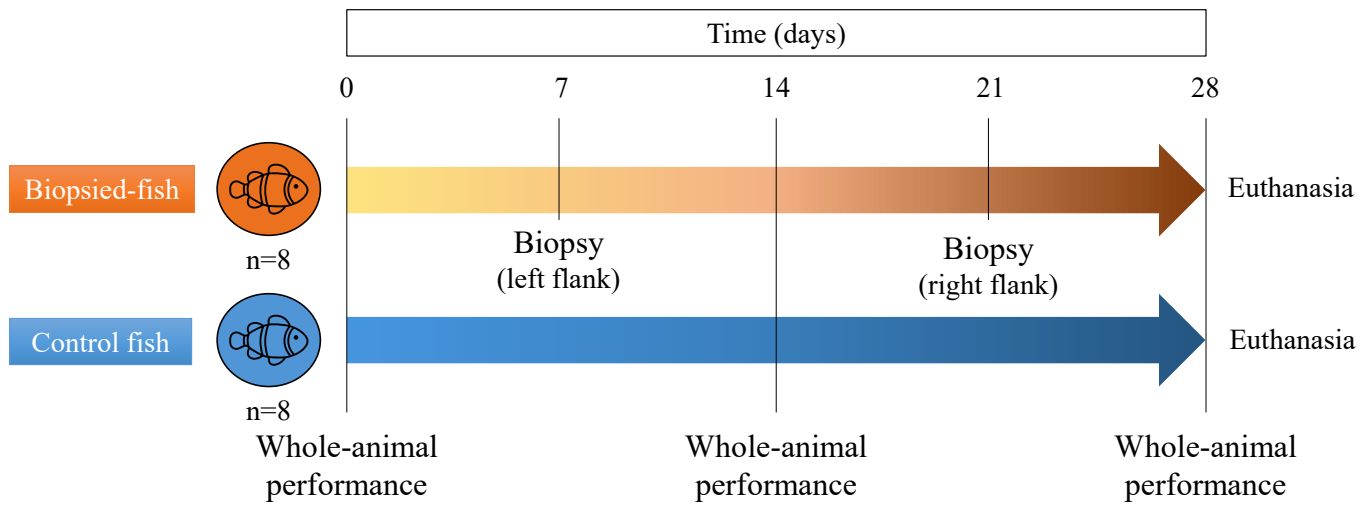
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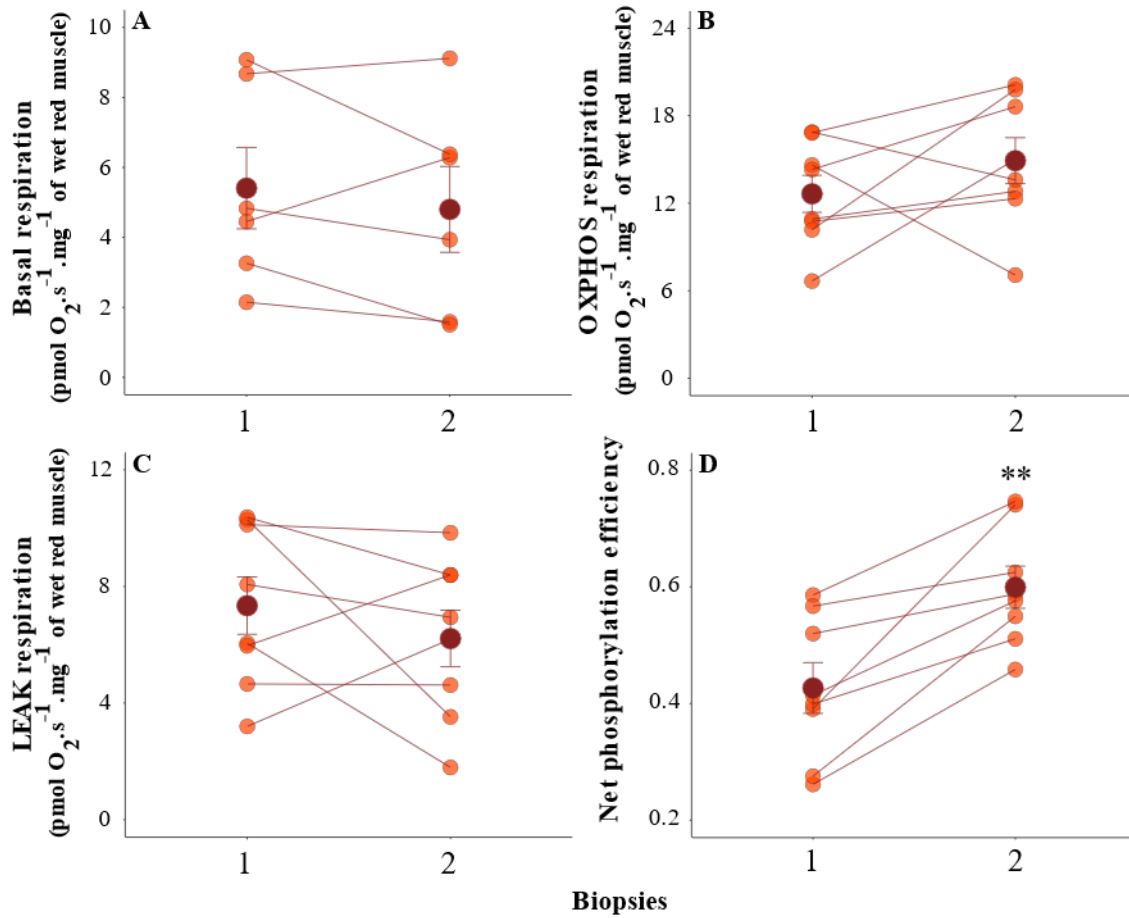
661 **Figures**

662



663

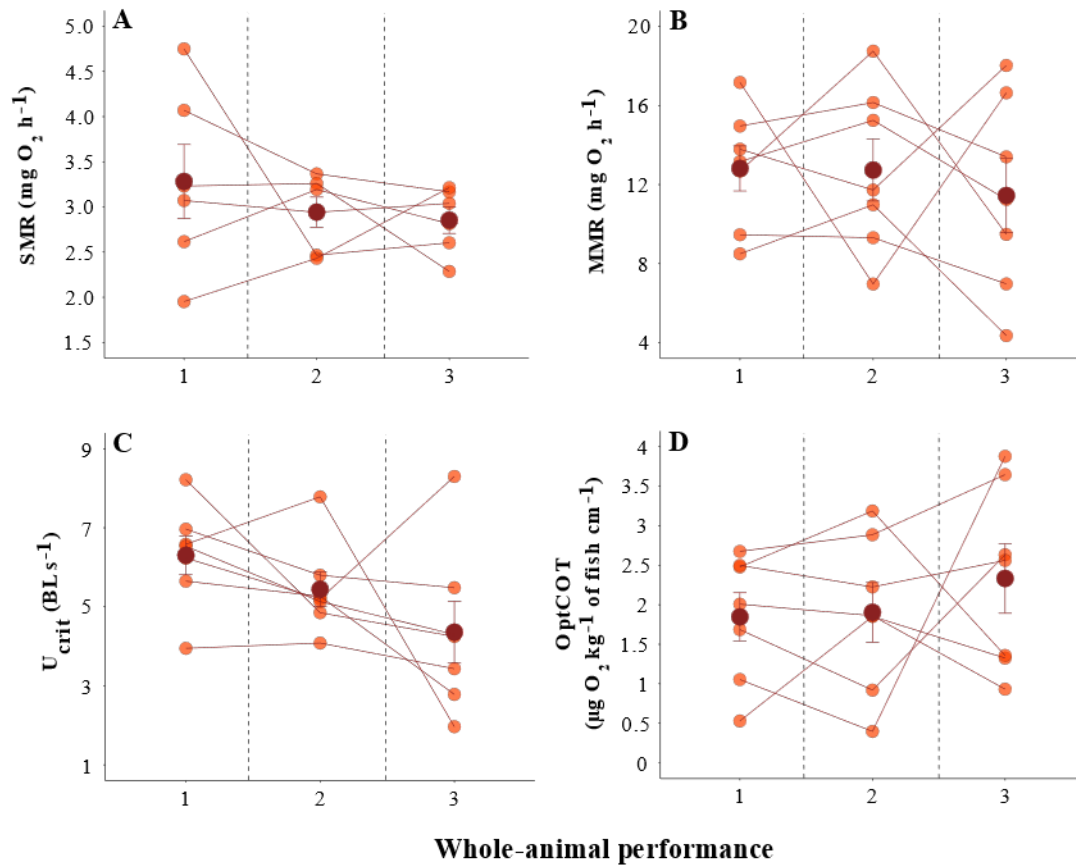
664 **Figure 1:** Experimental protocol description. Whole-animal performance was estimated from
665 measurements of whole-animal oxygen consumption rate ($\dot{M}O_2$) and swimming speed (U_{crit})
666 and repeatability was evaluated three times across a 28-day period at 14-days interval. Biopsied
667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



672

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).



676

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

682 **Tables**

683

Whole-animal performance	Biopsied fish		Control fish	
	Trial 2 (After 1 st biopsy)	Trial 3 (After 2 nd 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

684

685 **Table 1.** Mean changes in whole-animal traits in biopsied and control (non-biopsied) fish for686 trial 2 and trial 3. SMR: Standard metabolic rate. MMR: Maximum metabolic rate. U_{crit}: critical

687 swimming speed. OptCOT: optimal cost of transport.