A whole blood approach improves speed and accuracy when measuring mitochondrial respiration in avian hematocytes

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AN and EE conceived the idea and designed the experimental protocol together with IC. AN and IC performed the laboratory work. AN analysed the data and produced the graphical material. All authors interpreted the results. AN wrote the first draft of paper, which was edited by EE and IC. All authors approved submission and agreed to be accountable for all contents.

ABSTRACT

1. Mitochondria are the main producers of the cellular fuel (adenosine triphosphate, ATP) that eukaryotes require to function. Understanding mitochondrial biology and pathology is therefore important when enquiring about the evolution of animal form and function.
2. Mitochondrial measurement often involves invasive, or even terminal, sampling, which can be difficult to reconcile in wild models or in longitudinal studies. Non-mammal vertebrates contain mitochondria in their red blood cells. This has recently been exploited for minimally invasive mitochondrial measurement in a range of bird species.
3. Studies to date have measured mitochondrial function using isolated blood cells. This is time consuming and might result in technical complications if extended handling of blood cells impacts their integrity. Interpretation of homogenate measurement can also be problematic on biological grounds since homogenate production eliminates biological tissue context.
4. We developed a protocol to measure mitochondrial respiration in bird whole blood samples. Endogenous respiration was comparable between isolated blood cells and whole blood. However, respiration towards oxidative phosphorylation (whereby ATP is produced) was higher in whole blood. Moreover, whole blood mitochondria were better coupled and had higher maximum working capacity, as well as higher and more precise repeatability, compared to cell isolates. Whole blood measurement was feasible over a 10-fold range of sample volumes, though changes to respiratory traits were recorded for both very small and very large samples. We found that the blood can be stored for extended periods of time. After 24 h at 5 °C without agitation all respiration traits but maximum working capacity remained unchanged, the latter decreasing by 14%. Finally, we provide proof of principle that whole blood samples, like isolated hematocytes, can be quickly and reliably permeabilised.
5. Our study indicates that measurement of whole blood can provide faster, more precise, and more biologically (tissue context) and physiologically (mitochondrial integrity) relevant assessment of mitochondrial respiration in bird blood cells. On these grounds, we recommend future studies to take a whole blood approach unless specific circumstances require the use of isolated blood cells.

Keywords: bird, blood sampling, cell respiration, ecology, energy, erythrocyte, evolution, metabolism, mitochondria, respirometry

INTRODUCTION

The ability to convert energy contained in food into a currency that can be used to sustain bodily housekeeping functions and key fitness-related traits such as growth, reproduction, migration, and survival, is a fundamental requirement for life. This occurs by digestion of food items into macromolecules that are subsequently used to create energy for working cells in the form of adenosine triphosphate (ATP). More than 90% of this fuel is produced during cellular respiration in the mitochondria (Nicholls & Ferguson 2013). It is therefore not surprising that mitochondrial respiration and/or volume increase when organismal demand for energy increase (e.g. Liknes & Swanson 2011; Zheng *et al.* 2014; Dawson *et al.* 2016). Mitochondrial ATP is created by transport of an electron through a series of protein complexes (the electron transport system, ETS) whilst protons are being actively pumped across the inner mitochondrial membrane to establish an electrochemical potential. The energy required for oxidative phosphorylation (OXPHOS) is harnessed when protons backflow into the mitochondrial matrix at the end of the ETS. However, some protons forego OXPHOS by leaking back into the mitochondrial matrix spontaneously or under the influence of uncoupling proteins (Salin *et al.* 2015). Proton leak thus uncouples mitochondrial respiration from ATP production (LEAK) but generates heat and reduces the rate at which potentially biodegrading reactive oxygen species (ROS) are formed (Brand *et al.* 2004; Speakman *et al.* 2004). There is intricate regulation of the balance between LEAK and OXPHOS, such that mitochondria can be made more effective for a given substrate oxidation rate when energy is in short supply (Trzcionka *et al.* 2008; Bourguignon *et al.* 2017; Roussel *et al.* 2020), or more thermogenic to aid endothermic heat production (Walter & Seebacher 2009).

It is clear that studies of mitochondrial function may provide proximate and ultimate depth in many fundamental areas of research within ecology and evolution (see Koch *et al.* 2021 for a

83 recent review). However, such studies are rare in comparison to the rich body of literature on
84 organismal-level adaptation, function, and performance, particularly in the wild. This probably
85 reflects logistic and ethical considerations, because mitochondrial measurement often requires
86 terminal sampling for organ harvest, anaesthesia to collect biopsies, and speedy handling of the
87 samples to ensure organelle integrity; all of which are easier to reconcile with work on
88 laboratory models. However, birds and some other non-mammal vertebrates retain the nucleus
89 and functional organelles throughout erythroblast maturation and so contain mitochondria in
90 their erythrocytes (red blood cells) (Moritz, Lim & Wintour 1997; Moras, Lefevre & Ostuni
91 2017). Studies on birds have found that this makes it possible to assess mitochondrial function
92 even from small (25-50 µl) blood samples (Stier *et al.* 2013; Stier *et al.* 2017; Stier *et al.* 2019)
93 that can be safely collected in organisms < 5 g (Kramer & Harris 2010). This is not possible
94 when measurements are performed using other mitochondria-rich hematocytes, which is
95 necessary in mammals (e.g. Sjövall *et al.* 2013a; 2013b). While much is still unknown about
96 the function and regulation of blood cell mitochondria relative those in other tissues, several
97 recent bird studies indicate that blood cell respiration is predictably regulated in line with
98 variation in organismal-level energy demand over long (Stier *et al.* 2019; Casagrande *et al.*
99 2020) and short (Garcia Diaz et al., *in prep*.) time frames, and in the contexts of seasonal
100 adaptation (Nord *et al.* 2021) and metabolic senescence (Dawson & Salmón 2020). Moreover,
101 blood cell mitochondrial function seems amenable to epigenetic programming during
102 embryonic development (Udino *et al.* 2021; Stier, Monaghan & Metcalfe 2022), much like what
103 is a well-known fact within human medicine (Gyllenhammer *et al.* 2020). Studies have even
104 found that blood cell respiration can potentially be used to predict variation in whole-organism
105 metabolic rate (Malkoc, Casagrande & Hau 2021). Thus, there is ample evidence that
106 mitochondrial measurement in avian blood cells can provide minimally invasive insights into

107 several key areas within the broader fields of ecology, ecological physiology, and evolutionary
108 biology.
109
110 To the best of our knowledge, all studies on avian blood cell mitochondria to date have been
111 performed using isolated blood cells, which are prepared using standard laboratory techniques
112 including pipetting, vortexing and centrifugation, all of which could impact the status of the
113 cells going into the experiment (e.g. Wiegmann *et al.* 2017). If standard handling impacts cell
114 health, it can be speculated normal mitochondrial function in isolated hematocytes is not
115 representative of the *in vitro* state, but this notion remains untested. Furthermore, while
116 erythrocytes will be responsible for most of the respiration in the avian circulation (c.f. Scanes
117 2022), it may be difficult to reliably separate cell types by centrifugation in the small samples
118 that are typically collected from wild birds. Thus, the exact composition of a cell isolate may,
119 or may not, correspond to that in whole blood. Moreover, it can be argued that isolate-based
120 measurements lack some biological value on conceptual grounds since the removal of serum
121 before measurement effectively also removes the tissue-context of the blood. On this
122 background, we asked whether mitochondrial measurement on avian whole blood samples
123 would produce different results compared to when measurements were performed on the
124 isolated hematocytes from the same blood volume. If handling of samples in conjunction with
125 isolate production impacts mitochondrial integrity, we predicted that measurements would be
126 indicative of leaker mitochondria with lower overall respiration compared to whole blood
127 samples. To explore the utility of whole blood measurement, we proceeded by testing the
128 resilience of the assay to variation in sample volume and long-term storage, and by undertaking
129 initial validation of a protocol for permeabilising avian whole blood.
130
131 MATERIALS AND METHODS
132 **Animals and housing**

133 The study was performed using captive zebra finches (*Taeniopygia guttata* Vieillot) (n = 23, of
134 which 12 were female and 11 were males). All birds were in their second calendar year, or
135 older, and body masses ranged 12.1 to 19.4 g. The birds originated from a captive population
136 kept in outdoor flight aviaries at the Lund University field station Stensoffa (WGS84 DD:
137 N55.69534, E13.44739), and were brought to light- and temperature controlled indoor facilities
138 at Lund University 5-7 d before the experiment started. Birds were kept in cages measuring 120
139 × 80 × 100 cm (length × depth × height) at a density of ≤ 6 birds to a cage. Room temperature
140 was maintained at 19-20°C and photoperiod was 12:12 LD. Food (mixed millet seeds),
141 cuttlebone, and water was provided *ad libitum*.
142
143 **Blood sampling**
144 We sterilised the ventral skin area covering the distal part of the sternum using 70% ethanol
145 and immediately anaesthetised the birds by intraperitoneal injection of pentobarbital sodium (to
146 a concentration of 30-40 µg per g body mass). When the bird was reactionless to an external
147 stimulus (toe pinching), which occurred within 6-10 min after the injection, we collected a
148 maximal blood sample (250-650 µl) from the jugular vein and then euthanised the bird by
149 cervical dislocation. The sample was stored at 10-15°C in 2 mL K2-EDTA
150 (ethylenediaminetetraacetic acid) tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) without
151 agitation until analysed 15 to 45 min later.
152
153 **Mitochondrial respiration measurements and experiments**
154 Mitochondrial respiration was measured at bird body temperature (41°C) (Prinzinger, Pressmar
155 & Schleucher 1991) using Oxygraph O2k high-resolution respirometers (Oroboros Instruments,
156 Innsbruck, Austria). Respiration was inferred from the decline in O_2 concentration in a 2 ml
157 suspension of sample and respirometry medium (MiR05: 0.5 mM of EGTA, 3 mM of $MgCl_2$,
158 60 mM of K-lactobionate, 20 mM of taurine, 10 mM of KH_2PO_4, 20 mM of HEPES, 110 mM

159 of sucrose, and 1 g l^{-1} free fatty acid bovine serum albumin, pH 7.1). In three of the four
160 experiments, measurements were performed on intact cells. In the fourth experiment, we
161 developed a protocol to permeabilise bird whole blood.
162
163 In the first experiment, we compared mitochondrial measurements on blood cell isolates and
164 whole blood. Data were collected from 7 birds (3 males, 4 females) and all samples were run
165 in simultaneous duplicates within 45 min of collection. We manually mixed the blood by gently
166 tilting the sample tubes for 4-5 min. Then, we collected 100 µl whole blood samples either for
167 immediate respiration measurement, or to isolate blood cells following Stier et al. (2017) with
168 slight modifications according to Dawson & Salmón (2020) and Nord et al. (2021). Thus, the
169 blood sample was first centrifuged at 3000 g for 10 min at room temperature to separate plasma
170 from the blood cells. The pellet was then re-suspended in 500 µl cold MiR05, centrifuged at
171 1000 g for 5 min and the supernatant was discarded. The remaining pellet, containing all cells
172 from the 100 µl blood sample, was collected and immediately re-suspended in 750 µl MiR05
173 pre-equilibrated at measurement temperature, added to 1.35 ml MiR05 contained in the
174 respiration chamber (final volume 2 ml), and used in the assay. The sample volume used in this
175 experiment matches that used in the original protocol describing mitochondrial measurement
176 in bird blood cells (Stier *et al.* 2017) and is within range of that used in other recent bird studies
177 (e.g. Dawson & Salmón 2020; Nord *et al.* 2021).
178
179 In the second experiment, we asked how blood sample volume affected respiration
180 measurements, attempting to refine the protocol by defining the lowest possible volume
181 yielding representative data. Blood samples were drawn and handled as above (n = 7; 3 females,
182 4 males). Mitochondrial respiration traits were then assayed simultaneously in 10, 25, 50 and
183 100 µl whole blood samples.

184

185 In the third experiment, we investigated the effect of storage time on mitochondrial respiration
186 traits, using data from 6 birds (3 females, 3 males). Within 30 min of blood sampling
187 (henceforth, time = '0 h'), we followed the protocol above to measure respiration in 50 µl blood
188 samples. The tube with remaining blood was then stored without agitation at 5.2 ± 0.7 °C (mean
189 ± s.d.; range 3.9 to 6.5 °C) and a 50 µl was measured again (after 5 min of mixing) 24 h later
190 (henceforth, time = '24 h'). Samples from each of two birds were also opportunistically
191 measured at 72 h and 96 h after collection.

192

193 For the intact cell runs (i.e., Experiments 1-3), we allowed 10-15 min for O_2 consumption to
194 stabilise after adding sample to the chambers, and then recorded baseline phosphorylating
195 respiration on endogenous substrates for 2-4 min ("ROUTINE"). We then added 1 µg ml^{-1}
196 oligomycin to inhibit F_1F_0-ATP synthase, thus preventing oxidative phosphorylation. The
197 remaining respiration in this stage is used to offset the leak of protons that occur across the
198 inner mitochondrial membrane ("LEAK"). Thus, the part of respiration devoted to oxidative
199 phosphorylation ("OXPHOS") can be derived as the difference between the ROUTINE and
200 LEAK states. This was followed by titrating the mitochondrial uncoupler carbonyl cyanide-p-
201 trifluoro-methoxyphenyl-hydrazone (FCCP) in 0.5 µl, 1 mM, aliquots until maximum
202 respiration was reached (final concentration: 1-2 µM). At maximal non-inhibiting
203 concentration, FCCP abolishes the proton gradient across the inner mitochondrial membrane,
204 forcing the electron transport system to work at its maximum capacity ("ETS") to restore it.
205 After FCCP titration, we inhibited mitochondrial complex I using 2 µM rotenone, and then
206 added 1 µg ml^{-1} of the complex III inhibitor antimycin A to stop electron transport. Any
207 respiration remaining after addition of antimycin A is of non-mitochondrial origin. There was
208 never a meaningful further reduction in respiration when antimycin A was added onto rotenone

209 (data not shown). Thus, we adjusted respiration states for non-mitochondrial respiration using
210 whichever was lowest of respiration on rotenone and rotenone+antimycin A, under the
211 assumption that any absolute differences between the two were due to random noise.
212
213 In the fourth and final experiment, we developed a protocol for permeabilization of bird whole
214 blood. Blood was drawn and processed as above (*n* = 3; 2 females, 1 male). We ran 3 samples
215 of 25 µl per bird simultaneously, having previously established that this sample volume yielded
216 an optimal balance between phenotypic responses and O_2 saturation during the open cell run
217 (data not shown). Once we had collected 2-3 min of stable ROUTINE respiration, we added 0,
218 5, or 10 µl 5 mg ml^{-1} of the detergent digitonin (i.e., 0, 0.2 or 0.4 µg per µl blood) to permeabilise
219 the blood cell membrane. Using different concentrations of the permeabilising agent allowed
220 us to estimate which level of permeabilization that yielded the biologically most relevant results
221 (details below). Four to 5 minutes later, we added 5mM malate and 5 mM pyruvate to fuel
222 Complex I. When respiration on malate and pyruvate had remained stable for 2-3 min, we added
223 1 mM ADP to trigger phosphorylating respiration via Complex I. This allowed us to calculate
224 Complex I activity without (henceforth 'State 2') and with (henceforth '$OXPHOS_{CI}$')
225 concomitant ATP production. The additional Complex I-linked substrate glutamate (5 mM)
226 never increased respiration above $OXPHOS_{CI}$ and so these data are not presented below. We
227 then added 10 mM succinate, which is used by Complex II, to elicit maximum phosphorylating
228 respiration on excess substrate (henceforth '$OXPHOS_{CI+II}$'). We subsequently induced LEAK
229 state by inhibiting ATP synthase using 1 µg ml^{-1} oligomycin (henceforth '$LEAK_{Omy}$'), followed
230 by uncoupling of the electron transport system by titration of 0.5 µl aliquots of 1 mM FCCP
231 until maximum (henceforth 'ETS_{CI+II}') (final concentration: 1-2 µM). Afterwards, we added 2
232 µM rotenone to measure the contribution of Complex II to ETS (henceforth 'ETS_{CII}'). Finally,
233 we added 1 µg ml^{-1} antimycin A to gauge non-mitochondrial respiration.

234

235 **Data analyses**

236 We calculated three flux control ratios (FCR) to address integrity of mitochondrial respiration:
237 1) the fraction of maximum working capacity used during endogenous respiration (i.e.,
238 ROUTINE/ETS); 2) coupling efficiency of ATP production during endogenous respiration (i.e.,
239 LEAK/ROUTINE) where lower values indicate lower leakage of protons across the inner
240 mitochondrial membrane); 2) coupling efficiency of ATP production during a stimulated
241 cellular state (i.e., LEAK/ETS) where lower values again indicate little proton leakage even
242 when the electron transport system is maximally stimulated.

243

244 Statistical analyses were performed using R 4.1.2 for Windows (R Core Team 2021). To
245 compare the utility of blood cells and whole blood (i.e., Experiment 1), we fitted trait- and FCR-
246 specific linear mixed effect models (lmer in the lme4 package) (Bates *et al.* 2015) with sample
247 type as a factor and bird ID as a random intercept to account for the dependence of observations
248 between duplicates and samples. Repeatability of traits (R) for each sample type was expressed
249 as the intraclass correlation coefficient with 95% confidence intervals based on 1000 bootstrap
250 iterations using the rptR package (Stoffel, Nakagawa & Schielzeth 2017). We used lmer models
251 to address the effect of sample volume on respiration rates and FCRs (i.e., Experiment 2) using
252 the same general model structure. Predicted values and their standard errors were calculated
253 using the emmeans package (Lenth 2019) and used the pairs function in this package to perform
254 post hoc tests for Experiment 2 data. The effects of storage time (i.e., Experiment 3) were
255 assessed using paired t-tests between the 0 h and 24 h samples (t.test function in R base). We
256 did not include 72 h and 96 h data in any statistical models since only two individuals each were
257 assayed at those time points. We restricted our evaluation of digitonin concentration in
258 Experiment 4 to a qualitative comparison of trait values since sample size for this part of the

259 study was low. Specifically, we assumed that the most biologically relevant level of
260 permeabilization would be characterised by stronger relative responses to ADP (i.e.,
261 $OXPHOS_{CI}$ / State 2 larger) and greater similarity between maximal uncoupled and
262 phosphorylating respiration (i.e., $OXPHOS_{CI+II}$ / ETS_{CI+II} smaller). ETS data were missing for
263 one individual in the 10 µl experiment because we erroneously injected rotenone before
264 maximal uncoupled respiration had been obtained.

265

266

267 RESULTS

268 Representative traces of experimental runs with intact and permeabilised whole blood are
269 provided in the Electron Supplementary Materials (ESM 1).

270

271 **Effects of sample type**

272 ROUTINE respiration did not differ between blood cell and whole blood samples (Table 1, Fig.
273 1A). However, OXPHOS and ETS were significantly higher when measured on whole blood
274 samples compared to on blood cell isolates (1.2 and 1.3-fold, respectively) (Figs 1B-C, Table
275 1). In contrast, LEAK was lower (by a factor 0.7) in the whole blood samples (Fig. 1D, Table
276 1). Consequently, $FCR_{ROUTINE/ETS}$ (i.e., endogenous work rate relative maximum) was
277 significantly lower in the whole blood samples (by a factor 0.8) (Fig. 2A, Table 1). Coupling
278 efficiency under both endogenous and stimulated was also higher in the whole blood samples
279 (i.e., $FCR_{LEAK/ROUTINE}$ and $FCR_{LEAK/ETS}$ where lower) (Fig. 2B-C, Table 1).

280

281 Respiration traits measured on both whole blood and isolated blood cells were significantly and
282 highly repeatable (blood cells: R_{min} = 0.790, R_{max} = 0.968; whole blood: R_{min} = 0.849, R_{max} =
283 0.985). However, whole blood samples were more repeatable, and repeatabilities had markedly
284 narrower confidence intervals, for all respiration traits and FCRs considered (Table 1).

285

286 **Effects of blood volume**

287 There were non-linear effects on blood sample volume on respiration traits and FCRs, with the
288 lowest and highest sample volumes showing the most pronounced deviations. Accordingly,
289 ROUTINE was significantly higher (by a factor 1.5 to 1.6) for all volumes 10-50 µl compared
290 to the 100 µl sample (Fig. 3A, Table 2). OXPHOS was the highest, and similar for 25 and 50
291 µl, and the lowest for 10 and 100 µl (which did not differ) (Fig. 3A, Table 2). OXPHOS
292 measured using 50 µl was 1.3-fold higher than the measurement using 10 µl, though this
293 difference was not statistically significant. ETS was the highest on 50 µl samples (up to 1.4-
294 fold), and significantly different from all volumes but 25 µl (despite a 1.2-fold difference; Table
295 2). ETS was the lowest on 100 µl samples, though this volume differed statistically only from
296 50 µl (Table 2). LEAK was significantly higher on 10 µl compared to all other volumes, and
297 the lowest on 100 µl (though this volume was not statistically different from 50 µl) (Table 2).
298 FCRs, which provide rate-independent insight into mitochondrial function, were also volume-
299 specific. $FCR_{ROUTINE/ETS}$ was similar in 50 and 100 µl and significantly lower (by up to a factor
300 0.7) compared to the two other volumes (Fig. 3B). $FCR_{LEAK/ROUTINE}$ (i.e., coupling efficiency
301 under endogenous cellular conditions) was the highest in 10 µl (reflecting higher LEAK)
302 compared to the other volumes (Table 2, Fig. 3B). $FCR_{LEAK/ETS}$ (i.e., coupling efficiency under
303 a stimulated cellular state) was also the highest in 10 µl followed by 25 µl, but lower in both 50
304 and 100 µl (which were not statistically different) (Table 2, Fig. 3B).

305

306 **Effects of storage**

307 Endogenous (ROUTINE), phosphorylating (OXPHOS) and leak (LEAK) respiration did not
308 change significantly with 24 h storage at 5°C (all $P > 0.2$) (Fig. 4A, Table 3). However maximal
309 respiration capacity (ETS) was 16% lower at 24 h compared to at 0 h. As a result,
310 $FCR_{ROUTINE/ETS}$ and $FCR_{LEAK/ETS}$ was higher at 24 h (Fig. 4B, Table 3). There was no change
311 in $FCR_{LEAK/ROUTINE}$, in keeping with stable ROUTINE and LEAK over 24 h storage. Visual

312 inspection of Fig. 4 suggests there were relatively minor changes of respiration traits between
313 24 h and 72 h (though LEAK increased somewhat), but that 96-h storage induced more dramatic
314 effects on all traits considered.
315
316
317 **Permeabilization of whole blood**
318 We found that digitonin can be used to reliably permeabilise avian whole blood. On average,
319 the largest injected volume (0.4 µg per µl blood) yielded higher respiration for both maximal
320 phosphorylating (i.e., $OXPHOS_{CI+II}$) and non-phosphorylating (i.e., Complex I) respiration,
321 whereas phosphorylating respiration through complex 1 (i.e., $OXPHPOS_{CI}$), uncoupled (i.e.,
322 ETS_{CI}, ETS_{CI+II}) and LEAK respiration was rather similar. However, our measurements
323 revealed that the strongest response to ADP titration was obtained on permeabilization using
324 0.2 µg digitonin per µl blood (i.e., 5 µl 5 mg ml^{-1}), i.e., there was a smaller net increase in
325 respiration when ADP was added onto complex 1 substrates when the cells were permeabilised
326 using 10 µl digitonin (Fig. 5).
327
328
329 DISCUSSION
330 In this study, we asked whether measurement on whole blood could provide a feasible and
331 potentially more biologically contextualised approach to studying mitochondrial respiration in
332 avian blood cells. While endogenous respiration did not differ between whole blood samples
333 and isolated cells produced from the same blood volume, we found that both phosphorylating
334 (i.e., OXPHOS; Fig. 1B) and maximal (i.e., ETS; Fig. 1C) respiration was significantly higher
335 in whole blood. By contrast, blood cells displayed significantly higher LEAK than whole blood
336 (Fig. 1D). This translated to significant differences in flux control ratios (FCR), which indicated
337 that the isolated blood cells respired at a higher proportion of maximum (Fig. 2A) and that these

338 samples were more uncoupled under both endogenous and excited cellular conditions (Figs 2B-
339 C). Moreover, while our repeatability estimates for blood cell homogenate measurement were
340 consistently high and mostly within the range of those reported for the same traits reported
341 elsewhere (Stier et al. 2017), repeatabilities of the whole blood samples were consistently
342 higher (> 0.93) and considerably less variable for all traits considered (Table 1). Thus, in our
343 study whole blood measurement was more precise, and biological inference differed in several
344 key traits depending on whether measurements were performed on the intact tissue or on a
345 derived sample. We believe that this could be caused by physical damage or reduced integrity
346 of mitochondria in the isolated blood cells, because lower ETS, higher LEAK and associated
347 changes to FCRs in repeated samples of the same subject are all indicative of declining
348 mitochondrial health/viability (e.g. Sjövall *et al.* 2013a) (see also Fig. 4). It is likely that any
349 such damage resulted from the more extensive handling of the cells before the start of the
350 experiment. Studies on mammalian erythrocytes show centrifugation alone can cause time-
351 dependent structural and physiological damage to red blood cells (such as increased free and
352 corpuscular haemoglobin and increased ATP release) at spinning speeds considerably lower
353 than those used here (< 1500 *g*) (Urbina *et al.* 2016; Wiegmann *et al.* 2017; Mancuso,
354 Jayaraman & Ristenpart 2018) (but see Kiss *et al.* 2016). We are not suggesting that our whole
355 blood samples were in a 'pristine' condition, because drawing blood into a syringe, and
356 subsequent pipetting of a blood sample, may also impact the physiological and physical state
357 of the red blood cells (Wiegmann *et al.* 2017). However, our study indicates that whole blood
358 measurement may salvage more of the physiological integrity of avian blood cell mitochondria.
359
360 We found non-linearity of volume-adjusted respiration rates, manifested as inhibited or
361 enhanced respiration on the highest and lowest blood volumes (Fig. 3A). On average, optimal
362 trait combinations for coupled and uncoupled respiration was achieved using 50 µl samples,

363 but these runs were most often comparable to those on 25 µl. Flux control ratios for coupling
364 efficiency were similar in the range of 25 to 100 µl, whereas 10 µl samples were less coupled.
365 Thus, working in a range of 25 to 50 µl whole blood will provide representative results for
366 respiration traits, and a range of 25 to 100 µl will be acceptable if FCRs only are of interest.
367 Our findings contrast previous studies on pig liver homogenates where complete linearity of
368 phosphorylating respiration was demonstrated across a 3-fold range in sample volume
369 (Kuznetsov *et al.* 2002). However, studies on isolated human platelets show reduced uncoupled
370 respiration on small sample volumes (Sjövall *et al.* 2013a). This could be caused e.g. by reduced
371 accuracy on low sample concentrations acting in combination with enhanced
372 sensitivity/exposure to inhibitors. Measurement on pig skeletal muscle mitochondria also
373 suggest suppressed respiration when samples were prepared from smaller biopsies (Isner-
374 Horobeti *et al.* 2014), though this probably reflects heterogeneity in sample composition that
375 was not accounted for by weight alone. It is less clear why we observed inhibition of respiration
376 (but not FCRs) on large blood sample volumes. We do not believe that this was caused by the
377 presence of plasma in the sample for at least two reasons: 1) plasma alone does not inhibit
378 respiration neither in intact or open blood cells (REF); 2) ROUTINE was identical in whole
379 blood and isolated blood cells (Fig. 1A), which is not expected if some whole blood constituents
380 act suppressively. Nor do we consider it likely that the cells were rate-limited because O_2
381 concentration in the 100 µl runs was always well above that in our open-cell experiments where
382 no suppression of respiration was observed. The explanation for these observations should be
383 addressed in future work, including measurement of mitochondrial content in differently sized
384 samples, and using both intact and open-cell protocols. Meanwhile, we recommend
385 experimenters to use relatively constant sample volumes to the furthest extent possible.
386

387 The whole blood samples were robust to cold storage. After 24 h at 5°C without agitation, all
388 respiration traits but ETS (which decreased by 16%) remained unaltered. This largely
389 corroborates previous work on both human and avian blood cells (Sjövall *et al.* 2013a; Stier *et*
390 *al.* 2017) and pig liver homogenates (Kuznetsov *et al.* 2002). However, neither Sjövall et al.
391 (2013) or Stier et al. (2017) found any reduction in ETS over 24 h. These authors measured the
392 cells 3-5 h after blood collection, whereas our first measurement was performed within 0.75 h
393 after bleeding the birds. It is tempting to speculate that most changes to ETS occur within the
394 first h of collection. In line with this, there were but small changes to ETS in the two samples
395 that were opportunistically sampled after 72 h in our study (Fig. 4), whereas long-term storage
396 (96 h) was associated with suppression of all traits. Sjövall (2013) found that respiration of
397 permeabilised human platelets remained largely unaltered up to 48 h when the blood was stored
398 at room temperature. This does not seem to be the case in birds: while ROUTINE, OXPHOS
399 and LEAK remained fairly stable when the blood was stored 2-2.5 h at room temperature under
400 constant motion, ETS at +2 h was a factor 0.3-0.9 of ETS at 0 h for samples 10-100 µl (data not
401 shown).
402
403 We provide proof of principle that whole blood samples, just like isolated hematocytes (Stier
404 *et al.* 2019), can be permeabilised. Based on the response to ADP addition to a sample
405 containing only Complex 1 substrates, reflecting $OXPHOS_{CI}$ capacity (Fig. 5), a digitonin dose
406 of 0.2 µg per µl blood provided the best result. It is noteworthy that addition of substrates to
407 intact cells yielded nearly identical values for $OXPHOS_{CI+II}$ compared to the open cells, driven
408 mostly by Complex II activity (Fig. 5). This semi-permeability to substrates in avian blood cells
409 could potentially be exploited in various intact cell applications but *in vitro* and *in vivo.*
410
411 CONCLUSIONS

412 We found that whole blood measurement provides rapid, more biologically relevant, and more
413 precise assessment of mitochondrial respiration in intact avian hematocytes compared to
414 measurement using hematocytes isolated as per standard procedures. We also give data-based
415 recommendations on sample volume ranges, show that 24 h cold-storage in the blood collection
416 tubes is possible at acceptable changes to respiration traits and that whole blood can be
417 permeabilised. Changes to respiration parameters of the intact cells were indicative of handling-
418 induced damage. It is likely that less extensive handling protocols (e.g. Stier *et al.* 2019) will
419 provide more comparable results. Even so, our study suggests that centrifugation and removal
420 of plasma are unnecessary complications that add significantly to processing time and reduce
421 biological context. Thus, we advocate use of whole blood whenever possible. When blood cell
422 isolate measurements are necessary, such as when low sample volume is not sufficient for
423 complementary analyses of plasma metabolites, care should be taken to keep mechanical stress
424 to a minimum. We also recommend use of relatively constant blood volumes to minimize
425 variation within an experiment, and suggest samples are run on the day of collection.
426

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435

436 ETHICS

437 All procedures were approved by the Malmö/Lund Animal Ethics Committee, acting under
438 jurisdiction of the Swedish Board of Agriculture (permit no. 9246-19, 16977-19).
439
440 DATA AVAILABILITY
441 Data will be deposited in Figshare upon acceptance of the manuscript.
442
443 CONFLICT OF INTEREST
444 The authors declare that they have no competing or financial interests.
445
446
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565 TABLES

566

567 **Table 1. Comparison of mitochondrial respiration metrics in whole blood and in isolated**
568 **blood cells from the same blood volume.** The table shows model estimates, test statistics,
569 degrees of freedom and resultant *P*-values, when assaying mitochondrial respiration in zebra
570 finches using either 100 µl whole blood or all cells from 100 µl blood. All samples were run in
571 duplicate. *R* refers to intraclass repeatability of these samples and is presented with 95%
572 confidence bands inferred from 1000 bootstrap iterations. Significant (i.e., $P < 0.05$) effects are
573 printed in bold font. Abbreviations: CI = Confidence Interval; d.f. = Degrees of Freedom; FCR
574 = Flux Control Ratio; *LRT* = Likelihood Ratio Test statistic; *R* = Intraclass Repeatability; s.e.m.
575 = Standard Error of Mean.

Model and parameter	Estimate (s.e.m.)	d.f.	*LRT*	*P*	Fold difference	*R* (95% CI)
ROUTINE (pmol O_2 × s^{-1} × µl $blood^{-1}$)						
Sample		1	0.4	0.5328	0.98	
Blood cells	0.397 (0.035)					0.911 (0.606-0.982)
Whole blood	0.391 (0.035)					0.985 (0.921-0.997)
OXPHOS (pmol O_2 × s^{-1} × µl $blood^{-1}$)						
Sample		1	8.0	**0.0046**	1.17	
Blood cells	0.225 (0.023)					0.808 (0.269-0.957)
Whole blood	0.264 (0.023)					0.948 (0.741-0.989)
ETS (pmol O_2 × s^{-1} × µl $blood^{-1}$)						
Sample		1	19.1	**< 0.0001**	1.26	
Blood cells	0.648 (0.058)					0.886 (0.507-0.977)
Whole blood	0.815 (0.058)					0.965 (0.775-0.993)
LEAK (pmol O_2 × s^{-1} × µl $blood^{-1}$)						
Sample		1	12.7	**< 0.0001**	0.74	
Blood cells	0.172 (0.019)					0.908 (0.548-0.981)
Whole blood	0.128 (0.019)					0.967 (0.827-0.992)
$FCR_{ROUTINE/ETS}$						
Sample		1	19.0	**< 0.0001**	0.77	
Blood cells	0.635 (0.049)					0.968 (0.806-0.994)
Whole blood	0.489 (0.049)					0.982 (0.893-0.996)
$FCR_{LEAK/ROUTINE}$						
Sample		1	12.2	**0.0004**	0.71	
Blood cells	0.441 (0.034)					0.849 (0.325-0.972)
Whole blood	0.315 (0.034)					0.933 (0.691-0.988)
$FCR_{LEAK/ETS}$						
Sample		1	26.4	**< 0.0001**	0.55	
Blood cells	0.274 (0.022)					0.790 (0.202-0.950)
Whole blood	0.152 (0.022)					0.949 (0.711-0.990)

576

577 **Table 2. Effects of sample volume when measuring mitochondrial respiration in zebra**
578 **finch whole blood.** The table shows model estimates (± standard error; s.e.m.), test statistics,
579 degrees of freedom and corresponding *P*-values when comparing the effect of sample volume
580 when measuring mitochondrial respiration in zebra finch whole blood. Significant (i.e., $P <$
581 0.05) effects are printed in bold font. Different letters with brackets denote statistically
582 significant pairwise differences. Abbreviations: d.f. = Degrees of Freedom; FCR = Flux Control
583 Ratio; *LRT* = Likelihood Ratio Test statistic; s.e.m. = Standard Error of Mean. $*P_{10–25\ \mu l} = 0.053$.

584
585
586

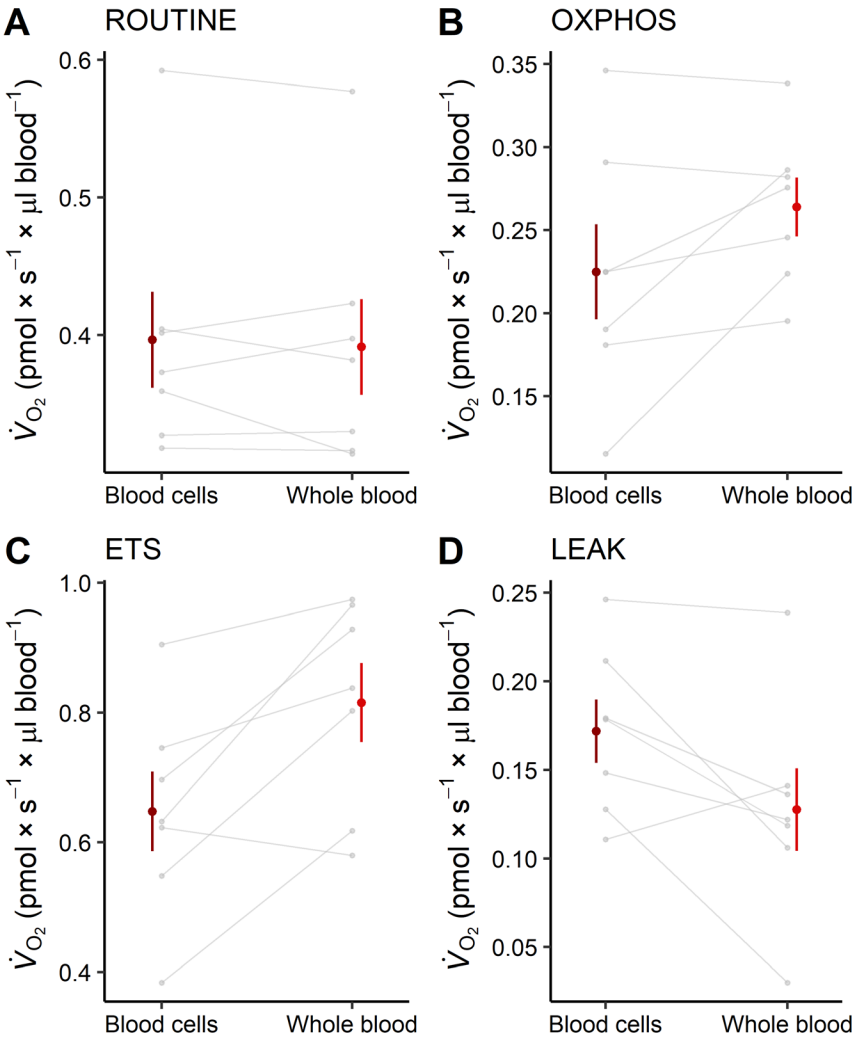
Model and parameter	Estimate (s.e.m.)	d.f.	*LRT*	*P*
ROUTINE (pmol $O_2 \times s^{-1} \times \mu l\ blood^{-1}$)				
Blood volume		1	27.5	**< 0.0001**
10 µl [A]	0.669 (0.037)			
25 µl [A]	0.656 (0.034)			
50 µl [A]	0.607 (0.034)			
100 µl [B]	0.411 (0.034)			
OXPHOS (pmol $O_2 \times s^{-1} \times \mu l\ blood^{-1}$)				
Blood volume		1	22.2	**< 0.0001**
10 µl [AB]	0.328 (0.029)			
25 µl [C]*	0.437 (0.026)			
50 µl [BC]	0.416 (0.026)			
100 µl [A]	0.259 (0.026)			
ETS (pmol $O_2 \times s^{-1} \times \mu l\ blood^{-1}$)				
Blood volume		1	13.9	**0.0031**
10 µl [A]	0.784 (0.060)			
25 µl [AB]	0.835 (0.055)			
50 µl [B]	1.005 (0.055)			
100 µl [A]	0.715 (0.055)			
LEAK (pmol $O_2 \times s^{-1} \times \mu l\ blood^{-1}$)				
Blood volume		1	39.9	**< 0.0001**
10 µl [A]	0.345 (0.018)			
25 µl [B]	0.219 (0.017)			
50 µl [BC]	0.191 (0.017)			
100 µl [C]	0.152 (0.017)			
$FCR_{ROUTINE/ETS}$				
Blood volume		1	29.6	**< 0.0001**
10 µl [A]	0.841 (0.051)			
25 µl [A]	0.809 (0.049)			
50 µl [B]	0.620 (0.049)			
100 µl [B]	0.579 (0.049)			
$FCR_{LEAK/ROUTINE}$				
Blood volume		1	28.5	**< 0.0001**
10 µl [A]	0.514 (0.025)			
25 µl [B]	0.336 (0.022)			
50 µl [B]	0.315 (0.022)			
100 µl [B]	0.375 (0.022)			
$FCR_{LEAK/ETS}$				
Blood volume		1	49.7	**< 0.0001**
10 µl [A]	0.434 (0.023)			
25 µl [B]	0.274 (0.022)			
50 µl [C]	0.197 (0.022)			
100 µl [C]	0.215 (0.022)			

587 **Table 3. Effects of storage time on mitochondrial respiration metrics in zebra finch whole**
588 **blood.** The table shows mean (± standard error; s.e.m.) trait values at 0 h and after 24 h storage
589 at 5°C, as well as test statistics, degrees of freedom and corresponding *P*-values for paired *t*-
590 tests of the effects of storage time on respiration traits. Significant (i.e., $P < 0.05$) effects are
591 printed in bold font. Each of two individuals were opportunistically sampled after 72 and 96 h
592 storage. These data are presented in grey font for comparison but were not included in any
593 statistical tests. Abbreviations: d.f. = Degrees of Freedom; FCR = Flux Control Ratio; s.e.m. =
594 Standard Error of Mean; *t* = *t*-test statistic.

595

Model and parameter	Mean (s.e.m.)	d.f.	*t*	*P*
ROUTINE (pmol $O_2 \times s^{-1} \times \mu l\ blood^{-1}$)		5	-1.40	0.2214
Sample				
0 h	0.673 (0.047)			
24 h	0.731 (0.047)			
72 h	0.746 (0.112)			
96 h	0.538 (0.083)			
OXPHOS (pmol $O_2 \times s^{-1} \times \mu l\ blood^{-1}$)		5	-1.47	0.2015
Sample				
0 h	0.498 (0.046)			
24 h	0.542 (0.035)			
72 h	0.503 (0.070)			
96 h	0.348 (0.062)			
ETS (pmol $O_2 \times s^{-1} \times \mu l\ blood^{-1}$)		5	3.23	**0.0233**
Sample				
0 h	1.169 (0.088)			
24 h	1.011 (0.111)			
72 h	1.011 (0.225)			
96 h	0.619 (0.102)			
LEAK (pmol $O_2 \times s^{-1} \times \mu l\ blood^{-1}$)		5	-0.94	0.3905
Sample				
0 h	0.175 (0.008)			
24 h	0.188 (0.019)			
72 h	0.243 (0.042)			
96 h	0.190 (0.102)			
$FCR_{ROUTINE/ETS}$		5	-4.16	**0.0089**
Sample				
0 h	0.595 (0.066)			
24 h	0.745 (0.049)			
72 h	0.751 (0.057)			
96 h	0.871 (0.010)			
$FCR_{LEAK/ROUTINE}$		5	0.74	0.4904
Sample				
0 h	0.264 (0.019)			
24 h	0.256 (0.016)			
72 h	0.325 (0.008)			
96 h	0.357 (0.017)			
$FCR_{LEAK/ETS}$		5	-3.29	**0.0217**
Sample				
0 h	0.155 (0.017)			
24 h	0.190 (0.016)			
72 h	0.244 (0.012)			
96 h	0.311 (0.018)			

596 FIGURES



597

598 **Figure 1. Difference in mitochondrial respiration traits as assayed in either isolated blood**
599 **cells or whole blood from zebra finches**. A) baseline ("ROUTINE") oxygen consumption in
600 the endogenous cellular state; B) oxygen consumption devoted to oxidative phosphorylation
601 alone, where ATP is produced ("OXPHOS); C) maximum working capacity of the electron
602 transport system when uncoupled from ATP production ("ETS"); D) the part of ROUTINE
603 used to offset the leak of protons across the inner mitochondrial membrane ("LEAK). Coloured
604 plotting symbols show raw data means ± 1 standard error. Gray points and lines show individual
605 responses. Significances are reported in Table 1.

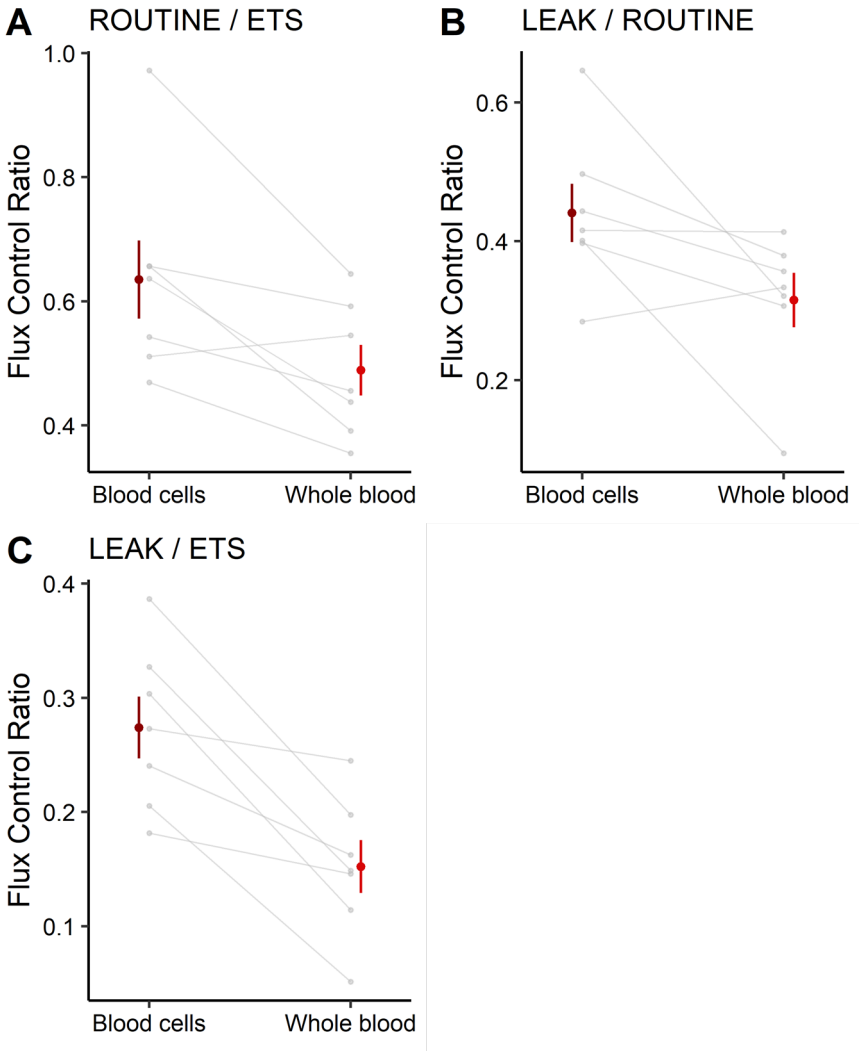


Figure 2. Flux Control Ratios for mitochondrial respiration traits measured in isolated blood cells or whole blood. A) The intensity of endogenous respiration (ROUTINE) relative to maximum working capacity of the electron transport system (ETS); B) Coupling efficiency of the electron transport system (i.e., the ratio of non-phosphorylating to phosphorylating respiration) under endogenous cellular conditions (i.e., LEAK / ROUTINE); C) coupling efficiency of electron transport during an excited cellular state (i.e., LEAK / ETS). Coloured plotting symbols show raw data means ± 1 standard error. Gray points and lines show individual responses. Significances are reported in Table 1.

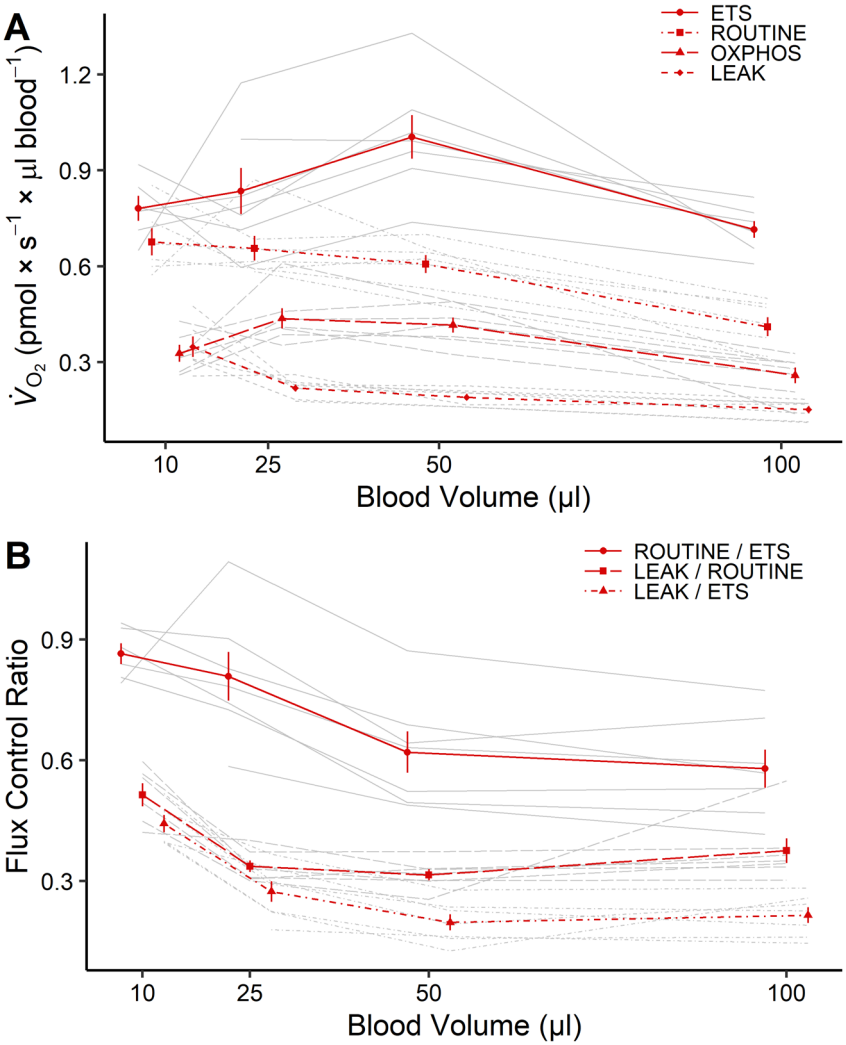


Figure 3. Effects of sample volume when assaying mitochondrial function in zebra finch whole blood. The panels show mitochondrial respiration traits (A) and Flux Control Ratios (FCR) (B) in relation to blood sample volume. Derivation of traits and FCRs are detailed in the main text and in the legends to Figs. 1, 2. Coloured plotting symbols show raw data means ± 1 standard error, and coloured lines show the averaged response. Gray lines show individual responses. Significances are reported in Table 2.

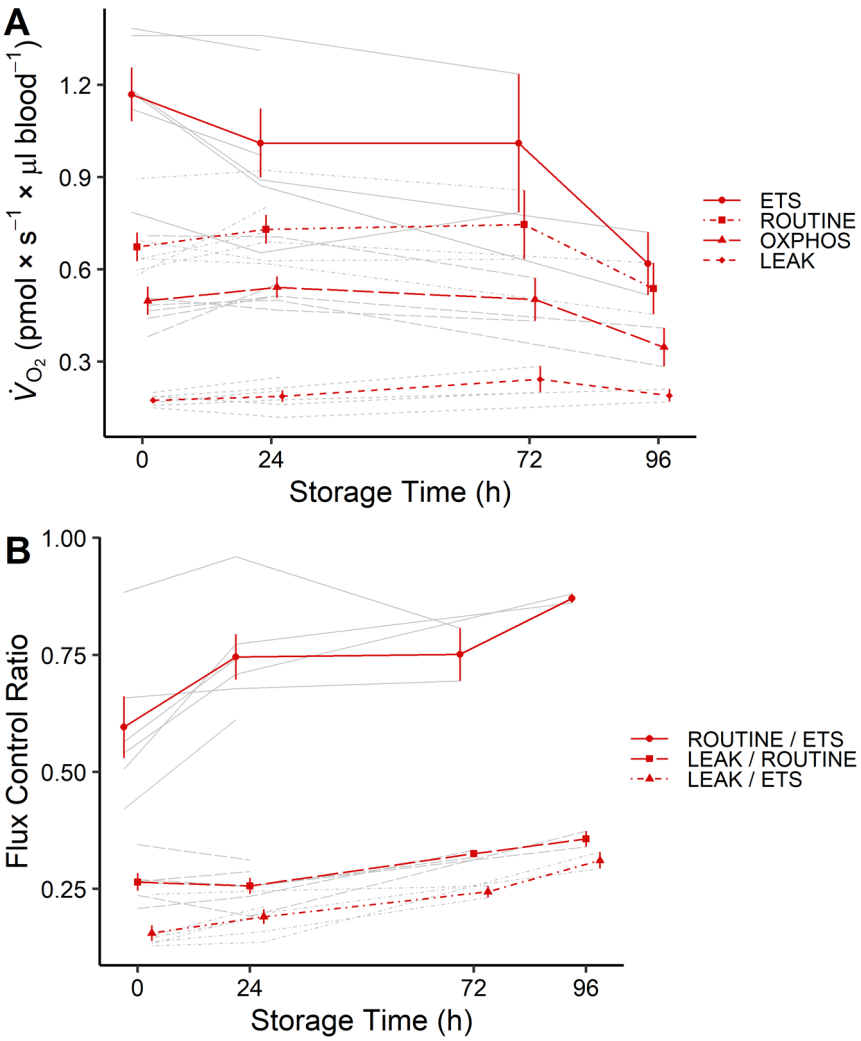
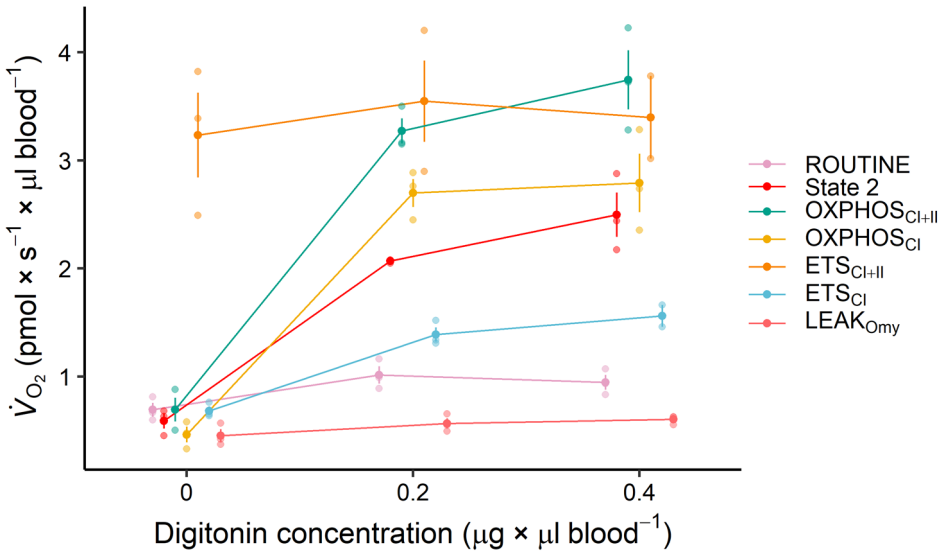


Figure 4. Effects of storage time when assaying mitochondrial function in zebra finch whole blood. The panels show mitochondrial respiration traits (A) and Flux Control Ratios (FCR) (B) in relation to blood sample volume. All individuals were measured at 0 h and after storage without agitation for 24 h at 5°C. Two individuals each were opportunistically sampled after 72 h and 96 h storage. These data are plotted for reference but were not included in any statistical tests. Derivation of traits and FCRs are detailed in the main text and in the legends to Figs. 1, 2. Coloured plotting symbols show raw data means ± 1 standard error, and coloured lines show the averaged response. Gray lines show individual responses. Significances are reported in Table 3.

632



633

634 **Figure 5. Effect of extent of permeabilization on mitochondrial respiration traits in zebra**
635 **finch whole blood.** The figure shows how variation in concentration of the permeabilising
636 detergent digitonin (5 mg ml^{-1}) affected mitochondrial respiration traits in 25 µl whole blood
637 samples collected from captive zebra finches. Calculation of respiratory states is explained in
638 the main text. Solid plotting symbols lines show raw data means ± 1 standard error, and
639 coloured lines show the averaged response. The smaller, semi-transparent plotting symbols
640 show raw data.