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

Authors: Andreas Nord[1], Imen Chamkha[2], Eskil Elmér[2]

[1] Lund University, Department of Biology, Section for Evolutionary Ecology, Sölvegatan 37, SE-223 62 Lund, Sweden

[2] Lund University, Department of Clinical Sciences, Mitochondrial Medicine, Sölvegatan 17, SE-221 84, Lund, Sweden

* Author for correspondence: AN, e-mail: andreas.nord@biol.lu.se

ORCID numbers:
AN: 0000-0001-6170-689X
IM: 0000-0002-6919-585X
EE: 0000-0001-7901-1826

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

there is ample evidence that mitochondrial measurement in avian blood cells can provide minimally invasive insights into several key areas within the broader fields of ecology, ecological physiology, and evolutionary biology.

To the best of our knowledge, all studies on avian blood cell mitochondria to date have been performed using isolated blood cells, which are prepared using standard laboratory techniques including pipetting, vortexing and centrifugation, all of which could impact the status of the cells going into the experiment (e.g. Wiegmann *et al.* 2017). If standard handling impacts cell health, it can be speculated normal mitochondrial function in isolated hematocytes is not representative of the *in vitro* state, but this notion remains untested. Furthermore, while erythrocytes will be responsible for most of the respiration in the avian circulation (c.f. Scanes 2022), it may be difficult to reliably separate cell types by centrifugation in the small samples that are typically collected from wild birds. Thus, the exact composition of a cell isolate may, or may not, correspond to that in whole blood. Moreover, it can be argued that isolate-based measurements lack some biological value on conceptual grounds since the removal of serum before measurement effectively also removes the tissue-context of the blood. On this background, we asked whether mitochondrial measurement on avian whole blood samples would produce different results compared to when measurements were performed on the isolated hematocytes from the same blood volume. If handling of samples in conjunction with isolate production impacts mitochondrial integrity, we predicted that measurements would be indicative of leaker mitochondria with lower overall respiration compared to whole blood samples. To explore the utility of whole blood measurement, we proceeded by testing the resilience of the assay to variation in sample volume and long-term storage, and by undertaking initial validation of a protocol for permeabilising avian whole blood.

MATERIALS AND METHODS

Animals and housing

The study was performed using captive zebra finches (*Taeniopygia guttata* Vieillot) (n = 23, of which 12 were female and 11 were males). All birds were in their second calendar year, or older, and body masses ranged 12.1 to 19.4 g. The birds originated from a captive population kept in outdoor flight aviaries at the Lund University field station Stensoffa (WGS84 DD: N55.69534, E13.44739), and were brought to light- and temperature controlled indoor facilities at Lund University 5-7 d before the experiment started. Birds were kept in cages measuring 120 × 80 × 100 cm (length × depth × height) at a density of ≤ 6 birds to a cage. Room temperature was maintained at 19-20°C and photoperiod was 12:12 LD. Food (mixed millet seeds), cuttlebone, and water was provided *ad libitum*.

Blood sampling

We sterilised the ventral skin area covering the distal part of the sternum using 70% ethanol and immediately anaesthetised the birds by intraperitoneal injection of pentobarbital sodium (to a concentration of 30-40 µg per g body mass). When the bird was reactionless to an external stimulus (toe pinching), which occurred within 6-10 min after the injection, we collected a maximal blood sample (250-650 µl) from the jugular vein and then euthanised the bird by cervical dislocation. The sample was stored at 10-15°C in 2 mL K2-EDTA (ethylenediaminetetraacetic acid) tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) without agitation until analysed 15 to 45 min later.

Mitochondrial respiration measurements and experiments

Mitochondrial respiration was measured at bird body temperature (41°C) (Prinzinger, Pressmar & Schleucher 1991) using Oxygraph O2k high-resolution respirometers (Oroboros Instruments, Innsbruck, Austria). Respiration was inferred from the decline in O_2 concentration in a 2 ml suspension of sample and respirometry medium (MiR05: 0.5 mM of

EGTA, 3 mM of $MgCl_2$, 60 mM of K-lactobionate, 20 mM of taurine, 10 mM of KH_2PO_4, 20 mM of HEPES, 110 mM of sucrose, and 1 g l^{-1} free fatty acid bovine serum albumin, pH 7.1). In three of the four experiments, measurements were performed on intact cells. In the fourth experiment, we developed a protocol to permeabilise bird whole blood.

In the first experiment, we compared mitochondrial measurements on blood cell isolates and whole blood. Data were collected from 7 birds (3 males, 4 females) and all samples were run in simultaneous duplicates within 45 min of collection. We manually mixed the blood by gently tilting the sample tubes for 4-5 min. Then, we collected 100 µl whole blood samples either for immediate respiration measurement, or to isolate blood cells following Stier et al. (2017) with slight modifications according to Dawson & Salmón (2020) and Nord et al. (2021). Thus, the blood sample was first centrifuged at 3000 g for 10 min at room temperature to separate plasma from the blood cells. The pellet was then re-suspended in 500 µl cold MiR05, centrifuged at 1000 g for 5 min and the supernatant was discarded. The remaining pellet, containing all cells from the 100 µl blood sample, was collected and immediately re-suspended in 750 µl MiR05 pre-equilibrated at measurement temperature, added to 1.35 ml MiR05 contained in the respiration chamber (final volume 2 ml), and used in the assay. The sample volume used in this experiment matches that used in the original protocol describing mitochondrial measurement in bird blood cells (Stier *et al.* 2017) and is within range of that used in other recent bird studies (e.g. Dawson & Salmón 2020; Nord *et al.* 2021).

In the second experiment, we asked how blood sample volume affected respiration measurements, attempting to refine the protocol by defining the lowest possible volume yielding representative data. Blood samples were drawn and handled as above (n = 7; 3

females, 4 males). Mitochondrial respiration traits were then assayed simultaneously in 10, 25, 50 and 100 µl whole blood samples.

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

For the intact cell runs (i.e., Experiments 1-3), we allowed 10-15 min for O_2 consumption to stabilise after adding sample to the chambers, and then recorded baseline phosphorylating respiration on endogenous substrates for 2-4 min ("ROUTINE"). We then added 1 µg ml^{-1} oligomycin to inhibit F_1F_0-ATP synthase, thus preventing oxidative phosphorylation. The remaining respiration in this stage is used to offset the leak of protons that occur across the inner mitochondrial membrane ("LEAK"). Thus, the part of respiration devoted to oxidative phosphorylation ("OXPHOS") can be derived as the difference between the ROUTINE and LEAK states. This was followed by titrating the mitochondrial uncoupler carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone (FCCP) in 0.5 µl, 1 mM, aliquots until maximum respiration was reached (final concentration: 1-2 µM). At maximal non-inhibiting concentration, FCCP abolishes the proton gradient across the inner mitochondrial membrane, forcing the electron transport system to work at its maximum capacity ("ETS") to restore it. After FCCP titration, we inhibited mitochondrial complex I using 2 µM rotenone, and then added 1 µg ml^{-1} of the complex III inhibitor antimycin A to stop electron transport. Any

respiration remaining after addition of antimycin A is of non-mitochondrial origin. There was never a meaningful further reduction in respiration when antimycin A was added onto rotenone (data not shown). Thus, we adjusted respiration states for non-mitochondrial respiration using whichever was lowest of respiration on rotenone and rotenone+antimycin A, under the assumption that any absolute differences between the two were due to random noise.

In the fourth and final experiment, we developed a protocol for permeabilization of bird whole blood. Blood was drawn and processed as above (*n* = 3; 2 females, 1 male). We ran 3 samples of 25 µl per bird simultaneously, having previously established that this sample volume yielded an optimal balance between phenotypic responses and O_2 saturation during the open cell run (data not shown). Once we had collected 2-3 min of stable ROUTINE respiration, we added 0, 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 the blood cell membrane. Using different concentrations of the permeabilising agent allowed us to estimate which level of permeabilization that yielded the biologically most relevant results (details below). Four to 5 minutes later, we added 5mM malate and 5 mM pyruvate to fuel Complex I. When respiration on malate and pyruvate had remained stable for 2-3 min, we added 1 mM ADP to trigger phosphorylating respiration via Complex I. This allowed us to calculate Complex I activity without (henceforth 'State 2') and with (henceforth '$OXPHOS_{CI}$') concomitant ATP production. The additional Complex I-linked substrate glutamate (5 mM) never increased respiration above $OXPHOS_{CI}$ and so these data are not presented below. We then added 10 mM succinate, which is used by Complex II, to elicit maximum phosphorylating respiration on excess substrate (henceforth '$OXPHOS_{CI+II}$'). We subsequently induced LEAK state by inhibiting ATP synthase using 1 µg ml^{-1} oligomycin (henceforth '$LEAK_{Omy}$'), followed by uncoupling of the electron transport

system by titration of 0.5 µl aliquots of 1 mM FCCP until maximum (henceforth 'ETS_{CI+II}') (final concentration: 1-2 µM). Afterwards, we added 2 µM rotenone to measure the contribution of Complex II to ETS (henceforth 'ETS_{CII}'). Finally, we added 1 µg ml^{-1} antimycin A to gauge non-mitochondrial respiration.

Data analyses

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

Statistical analyses were performed using R 4.1.2 for Windows (R Core Team 2021). To compare the utility of blood cells and whole blood (i.e., Experiment 1), we fitted trait- and FCR-specific linear mixed effect models (lmer in the lme4 package) (Bates *et al.* 2015) with sample type as a factor and bird ID as a random intercept to account for the dependence of observations between duplicates and samples. Repeatability of traits (*R*) for each sample type was expressed as the intraclass correlation coefficient with 95% confidence intervals based on 1000 bootstrap iterations using the rptR package (Stoffel, Nakagawa & Schielzeth 2017). We used lmer models to address the effect of sample volume on respiration rates and FCRs (i.e., Experiment 2) using the same general model structure. Predicted values and their standard errors were calculated using the emmeans package (Lenth 2019) and used the pairs function in this package to perform post hoc tests for Experiment 2 data. The effects of storage time

(i.e., Experiment 3) were assessed using paired t-tests between the 0 h and 24 h samples (t.test function in R base). We did not include 72 h and 96 h data in any statistical models since only two individuals each were assayed at those time points. We restricted our evaluation of digitonin concentration in Experiment 4 to a qualitative comparison of trait values since sample size for this part of the study was low. Specifically, we assumed that the most biologically relevant level of permeabilization would be characterised by stronger relative responses to ADP (i.e., $OXPHOS_{CI}$ / State 2 larger) and greater similarity between maximal uncoupled and phosphorylating respiration (i.e., $OXPHOS_{CI+II}$ / ETS_{CI+II} smaller). ETS data were missing for one individual in the 10 µl experiment because we erroneously injected rotenone before maximal uncoupled respiration had been obtained.

RESULTS

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

Effects of sample type

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

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

Effects of blood volume

There were non-linear effects on blood sample volume on respiration traits and FCRs, with the lowest and highest sample volumes showing the most pronounced deviations. Accordingly, ROUTINE was significantly higher (by a factor 1.5 to 1.6) for all volumes 10-50 µl compared to the 100 µl sample (Fig. 3A, Table 2). OXPHOS was the highest, and similar for 25 and 50 µl, and the lowest for 10 and 100 µl (which did not differ) (Fig. 3A, Table 2). OXPHOS measured using 50 µl was 1.3-fold higher than the measurement using 10 µl, though this difference was not statistically significant. ETS was the highest on 50 µl samples (up to 1.4-fold), and significantly different from all volumes but 25 µl (despite a 1.2-fold difference; Table 2). ETS was the lowest on 100 µl samples, though this volume differed statistically only from 50 µl (Table 2). LEAK was significantly higher on 10 µl compared to all other volumes, and the lowest on 100 µl (though this volume was not statistically different from 50 µl) (Table 2).

FCRs, which provide rate-independent insight into mitochondrial function, were also volume-specific. $FCR_{ROUTINE/ETS}$ was similar in 50 and 100 µl and significantly lower (by up to a factor 0.7) compared to the two other volumes (Fig. 3B). $FCR_{LEAK/ROUTINE}$ (i.e., coupling efficiency under endogenous cellular conditions) was the highest in 10 µl (reflecting higher LEAK) compared to the other volumes (Table 2, Fig. 3B). $FCR_{LEAK/ETS}$ (i.e., coupling

efficiency under a stimulated cellular state) was also the highest in 10 µl followed by 25 µl, but lower in both 50 and 100 µl (which were not statistically different) (Table 2, Fig. 3B).

Effects of storage

Endogenous (ROUTINE), phosphorylating (OXPHOS) and leak (LEAK) respiration did not change significantly with 24 h storage at 5°C (all $P > 0.2$) (Fig. 4A, Table 3). However maximal respiration capacity (ETS) was 16% lower at 24 h compared to at 0 h. As a result, $FCR_{ROUTINE/ETS}$ and $FCR_{LEAK/ETS}$ was higher at 24 h (Fig. 4B, Table 3). There was no change in $FCR_{LEAK/ROUTINE}$, in keeping with stable ROUTINE and LEAK over 24 h storage. Visual inspection of Fig. 4 suggests there were relatively minor changes of respiration traits between 24 h and 72 h (though LEAK increased somewhat), but that 96-h storage induced more dramatic effects on all traits considered.

Permeabilization of whole blood

We found that digitonin can be used to reliably permeabilise avian whole blood. On average, the largest injected volume (0.4 µg per µl blood) yielded higher respiration for both maximal phosphorylating (i.e., $OXPHOS_{CI+II}$) and non-phosphorylating (i.e., Complex I) respiration, whereas phosphorylating respiration through complex 1 (i.e., $OXPHPOS_{CI}$), uncoupled (i.e., ETS_{CI}, ETS_{CI+II}) and LEAK respiration was rather similar. However, our measurements revealed that the strongest response to ADP titration was obtained on permeabilization using 0.2 µg digitonin per µl blood (i.e., 5 µl 5 mg ml^{-1}), i.e., there was a smaller net increase in respiration when ADP was added onto complex 1 substrates when the cells were permeabilised using 10 µl digitonin (Fig. 5).

DISCUSSION

In this study, we asked whether measurement on whole blood could provide a feasible and potentially more biologically contextualised approach to studying mitochondrial respiration in avian blood cells. While endogenous respiration did not differ between whole blood samples and isolated cells produced from the same blood volume, we found that both phosphorylating (i.e., OXPHOS; Fig. 1B) and maximal (i.e., ETS; Fig. 1C) respiration was significantly higher in whole blood. By contrast, blood cells displayed significantly higher LEAK than whole blood (Fig. 1D). This translated to significant differences in flux control ratios (FCR), which indicated that the isolated blood cells respired at a higher proportion of maximum (Fig. 2A) and that these samples were more uncoupled under both endogenous and excited cellular conditions (Figs 2B-C). Moreover, while our repeatability estimates for blood cell homogenate measurement were consistently high and mostly within the range of those reported for the same traits reported elsewhere (Stier et al. 2017), repeatabilities of the whole blood samples were consistently higher (> 0.93) and considerably less variable for all traits considered (Table 1). Thus, in our study whole blood measurement was more precise, and biological inference differed in several key traits depending on whether measurements were performed on the intact tissue or on a derived sample. We believe that this could be caused by physical damage or reduced integrity of mitochondria in the isolated blood cells, because lower ETS, higher LEAK and associated changes to FCRs in repeated samples of the same subject are all indicative of declining mitochondrial health/viability (e.g. Sjövall *et al.* 2013a) (see also Fig. 4). It is likely that any such damage resulted from the more extensive handling of the cells before the start of the experiment. Studies on mammalian erythrocytes show centrifugation alone can cause time-dependent structural and physiological damage to red blood cells (such as increased free and corpuscular haemoglobin and increased ATP release) at spinning speeds considerably lower than those used here (< 1500 *g*) (Urbina *et al.* 2016;

Wiegmann *et al.* 2017; Mancuso, Jayaraman & Ristenpart 2018) (but see Kiss *et al.* 2016). We are not suggesting that our whole blood samples were in a 'pristine' condition, because drawing blood into a syringe, and subsequent pipetting of a blood sample, may also impact the physiological and physical state of the red blood cells (Wiegmann *et al.* 2017). However, our study indicates that whole blood measurement may salvage more of the physiological integrity of avian blood cell mitochondria.

We found non-linearity of volume-adjusted respiration rates, manifested as inhibited or enhanced respiration on the highest and lowest blood volumes (Fig. 3A). On average, optimal trait combinations for coupled and uncoupled respiration was achieved using 50 µl samples, but these runs were most often comparable to those on 25 µl. Flux control ratios for coupling efficiency were similar in the range of 25 to 100 µl, whereas 10 µl samples were less coupled. Thus, working in a range of 25 to 50 µl whole blood will provide representative results for respiration traits, and a range of 25 to 100 µl will be acceptable if FCRs only are of interest. Our findings contrast previous studies on pig liver homogenates where complete linearity of phosphorylating respiration was demonstrated across a 3-fold range in sample volume (Kuznetsov *et al.* 2002). However, studies on isolated human platelets show reduced uncoupled respiration on small sample volumes (Sjövall *et al.* 2013a). This could be caused e.g. by reduced accuracy on low sample concentrations acting in combination with enhanced sensitivity/exposure to inhibitors. Measurement on pig skeletal muscle mitochondria also suggest suppressed respiration when samples were prepared from smaller biopsies (Isner-Horobeti *et al.* 2014), though this probably reflects heterogeneity in sample composition that was not accounted for by weight alone. It is less clear why we observed inhibition of respiration (but not FCRs) on large blood sample volumes. We do not believe that this was caused by the presence of plasma in the sample for at least two reasons: 1) plasma alone does

not inhibit respiration neither in intact or open blood cells (REF); 2) ROUTINE was identical in whole blood and isolated blood cells (Fig. 1A), which is not expected if some whole blood constituents act suppressively. Nor do we consider it likely that the cells were rate-limited because O_2 concentration in the 100 µl runs was always well above that in our open-cell experiments where no suppression of respiration was observed. The explanation for these observations should be addressed in future work, including measurement of mitochondrial content in differently sized samples, and using both intact and open-cell protocols. Meanwhile, we recommend experimenters to use relatively constant sample volumes to the furthest extent possible.

The whole blood samples were robust to cold storage. After 24 h at 5°C without agitation, all respiration traits but ETS (which decreased by 16%) remained unaltered. This largely corroborates previous work on both human and avian blood cells (Sjövall *et al.* 2013a; Stier *et al.* 2017) and pig liver homogenates (Kuznetsov *et al.* 2002). However, neither Sjövall et al. (2013) or Stier et al. (2017) found any reduction in ETS over 24 h. These authors measured the cells 3-5 h after blood collection, whereas our first measurement was performed within 0.75 h after bleeding the birds. It is tempting to speculate that most changes to ETS occur within the first h of collection. In line with this, there were but small changes to ETS in the two samples that were opportunistically sampled after 72 h in our study (Fig. 4), whereas long-term storage (96 h) was associated with suppression of all traits. Sjövall (2013) found that respiration of permeabilised human platelets remained largely unaltered up to 48 h when the blood was stored at room temperature. This does not seem to be the case in birds: while ROUTINE, OXPHOS and LEAK remained fairly stable when the blood was stored 2-2.5 h at room temperature under 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 shown).

We provide proof of principle that whole blood samples, just like isolated hematocytes (Stier *et al.* 2019), can be permeabilised. Based on the response to ADP addition to a sample containing only Complex 1 substrates, reflecting $OXPHOS_{CI}$ capacity (Fig. 5), a digitonin dose of 0.2 µg per µl blood provided the best result. It is noteworthy that addition of substrates to intact cells yielded nearly identical values for $OXPHOS_{CI+II}$ compared to the open cells, driven mostly by Complex II activity (Fig. 5). This semi-permeability to substrates in avian blood cells could potentially be exploited in various intact cell applications but *in vitro* and *in vivo.*

CONCLUSIONS

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

volumes to minimize variation within an experiment, and suggest samples are run on the day of collection.

ACKNOWLEDGEMENTS

The authors thank Elsie Ye Xiong and Michael Tobler for providing zebra finches for use in the study. Camilla Björklöv and Agnieszka Czopek excellently assisted with care for the experimental animals.

FUNDING

This study was supported by the Birgit and Hellmuth Hertz Foundation (grant no. 2017-39034), and the Swedish Research Council (grant no. 2020-04686) (to AN).

ETHICS

All procedures were approved by the Malmö/Lund Animal Ethics Committee, acting under jurisdiction of the Swedish Board of Agriculture (permit no. 9246-19, 16977-19).

DATA AVAILABILITY

Data will be deposited in Figshare upon acceptance of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing or financial interests.

REFERENCES

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, **67,** 1-48.

Bourguignon, A., Rameau, A., Toullec, G., Romestaing, C. & Roussel, D. (2017) Increased mitochondrial energy efficiency in skeletal muscle after long-term fasting: its relevance to animal performance. *Journal of Experimental Biology*, **220,** 2445-2451.

Brand, M.D., Buckingham, J.A., Esteves, T.C., Green, K., Lambert, A.J., Miwa, S., Murphy, M.P., Pakay, J.L., Talbot, D.A. & Echtay, K.S. (2004) Mitochondrial superoxide and aging: uncoupling-protein activity and superoxide production. *Biochemical Society Symposium***,** 203-213.

Casagrande, S., Stier, A., Monaghan, P., Loveland, J.L., Boner, W., Lupi, S., Trevisi, R. & Hau, M. (2020) Increased glucocorticoid concentrations in early life cause mitochondrial inefficiency and short telomeres. *Journal of Experimental Biology*, **223,** jeb222513.

Dawson, N.J., Ivy, C.M., Alza, L., Cheek, R., York, J.M., Chua, B., Milsom, W.K., McCracken, K.G. & Scott, G.R. (2016) Mitochondrial physiology in the skeletal and cardiac muscles is altered in torrent ducks, *Merganetta armata*, from high altitudes in the Andes. *Journal of Experimental Biology*, **219,** 3719-3728.

Dawson, N.J. & Salmón, P. (2020) Age-related increase in mitochondrial quantity may mitigate a decline in mitochondrial quality in red blood cells from zebra finches (*Taeniopygia guttata*). *Experimental Gerontology*, **133,** 110883.

García-Díaz, C.C., Chamkha, I., Elmér, E., Nord, A. 2022. Plasticity of mitochondrial function restores fuel production during rest-phase hypothermia in a winter bird. *bioRxiv*, 2022.05.25.493417. doi: 10.1101/2022.05.25.493417

Gyllenhammer, L.E., Entringer, S., Buss, C. & Wadhwa, P.D. (2020) Developmental programming of mitochondrial biology: a conceptual framework and review. *Proceedings of the Royal Society of London B,* **287,** 20192713.

Isner-Horobeti, M.E., Charton, A., Daussin, F., Geny, B., Dufour, S.P. & Richard, R. (2014) Microbiopsies versus Bergström needle for skeletal muscle sampling: impact on maximal mitochondrial respiration rate. *European Journal of Applied Physiology,* **114,** 885-889.

Kiss, F., Toth, E., Miszti-Blasius, K. & Nemeth, N. (2016) The effect of centrifugation at various g force levels on rheological properties of rat, dog, pig and human red blood cells. *Clinical Hemorheology and Microcirculation,* **62,** 215-227.

Koch, R.E., Buchanan, K.L., Casagrande, S., Crino, O., Dowling, D.K., Hill, G.E., Hood, W.R., McKenzie, M., Mariette, M.M., Noble, D.W.A., Pavlova, A., Seebacher, F., Sunnucks, P., Udino, E., White, C.R., Salin, K. & Stier, A. (2021) Integrating Mitochondrial Aerobic Metabolism into Ecology and Evolution. *Trends in Ecology & Evolution,* **36,** 321-332.

Kramer, M.H. & Harris, D.J. (2010) Avian Blood Collection. *Journal of Exotic Pet Medicine,* **19,** 82-86.

Kuznetsov, A.V., Strobl, D., Ruttmann, E., Königsrainer, A., Margreiter, R. & Gnaiger, E. (2002) Evaluation of mitochondrial respiratory function in small biopsies of liver. *Annals of Biochemistry,* **305,** 186-194.

Lenth, R.V. (2019) emmeans: Estimated marginal means, aka least-squares means. R package version 1.3.3. https://CRAN.R-project.org/package=emmeans.

Liknes, E.T. & Swanson, D.L. (2011) Phenotypic flexibility in passerine birds: Seasonal variation of aerobic enzyme activities in skeletal muscle. *Journal of Thermal Biology,* **36,** 430-436.

Malkoc, K., Casagrande, S. & Hau, M. (2021) Inferring Whole-Organism Metabolic Rate From Red Blood Cells in Birds. *Frontiers in Physiology,* **12,** 691633-691633.

Mancuso, J.E., Jayaraman, A. & Ristenpart, W.D. (2018) Centrifugation-induced release of ATP from red blood cells. *PLoS ONE,* **13,** e0203270.

Moras, M., Lefevre, S.D. & Ostuni, M.A. (2017) From Erythroblasts to Mature Red Blood Cells: Organelle Clearance in Mammals. *Frontiers in Physiology,* **8,** 201701076.

Moritz, K.M., Lim, G.B. & Wintour, E.M. (1997) Developmental regulation of erythropoietin and erythropoiesis. *American Journal of Physiology,* **273,** R1829-R1844.

Nicholls, D.G. & Ferguson, S.J. (2013) *Bioenergetics,* 4th edn. Academic Press, Cambridge, MA, USA.

Nord, A., Metcalfe, N.B., Page, J.L., Huxtable, A., McCafferty, D.J. & Dawson, N.J. (2021) Avian red blood cell mitochondria produce more heat in winter than in autumn. *The FASEB Journal,* **35,** e21490.

Prinzinger, R., Pressmar, A. & Schleucher, E. (1991) Body temperature in birds. *Comparative Biochemistry and Physiology A,* **99,** 499-506.

R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Roussel, D., Marmillot, V., Monternier, P.-A., Bourguignon, A., Toullec, G., Romestaing, C. & Duchamp, C. (2020) Skeletal muscle metabolism in sea-acclimatized king penguins. II. Improved efficiency of mitochondrial bioenergetics. *Journal of Experimental Biology,* **223,** jeb233684.

Salin, K., Auer Sonya, K., Rey, B., Selman, C. & Metcalfe Neil, B. (2015) Variation in the link between oxygen consumption and ATP production, and its relevance for animal performance. *Proceedings of the Royal Society of London B,* **282,** 20151028.

Scanes, C.G. (2022) Blood. *Sturkie's Avian Phyysiology* (ed. C.G. Scanes, Dridi, S). Academic Press, Cambridge, Massachusetts.

Sjövall, F., Ehinger, J.K.H., Marelsson, S.E., Morota, S., Åsander Frostner, E., Uchino, H., Lundgren, J., Arnbjörnsson, E., Hansson, M.J., Fellman, V. & Elmér, E. (2013a) Mitochondrial respiration in human viable platelets—Methodology and influence of gender, age and storage. *Mitochondrion,* **13,** 7-14.

Sjövall, F., Morota, S., Persson, J., Hansson, M.J. & Elmér, E. (2013b) Patients with sepsis exhibit increased mitochondrial respiratory capacity in peripheral blood immune cells. *Critical Care,* **17,** R152.

Speakman, J.R., Talbot, D.A., Selman, C., Snart, S., McLaren, J.S., Redman, P., Krol, E., Jackson, D.M., Johnson, M.S. & Brand, M.D. (2004) Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell,* **3,** 87-95.

Stier, A., Bize, P., Hsu, B.-Y. & Ruuskanen, S. (2019) Plastic but repeatable: rapid adjustments of mitochondrial function and density during reproduction in a wild bird species. *Biology Letters,* **15,** 20190536.

Stier, A., Bize, P., Schull, Q., Zoll, J., Singh, F., Geny, B., Gros, F., Royer, C., Massemin, S. & Criscuolo, F. (2013) Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. *Frontiers in Zoology,* **10,** 33.

Stier, A., Monaghan, P. & Metcalfe, N.B. (2022) Experimental demonstration of prenatal programming of mitochondrial aerobic metabolism lasting until adulthood. *Proceedings of the Royal Society of London B,* **289,** 20212679.

Stier, A., Romestaing, C., Schull, Q., Lefol, E., Robin, J.-P., Roussel, D. & Bize, P. (2017) How to measure mitochondrial function in birds using red blood cells: a case study in

the king penguin and perspectives in ecology and evolution. *Methods in Ecology and Evolution,* **8,** 1172-1182.

Stoffel, M.A., Nakagawa, S. & Schielzeth, H. (2017) rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods in Ecology and Evolution,* **8,** 1639-1644.

Trzcionka, M., Withers, K.W., Klingenspor, M. & Jastroch, M. (2008) The effects of fasting and cold exposure on metabolic rate and mitochondrial proton leak in liver and skeletal muscle of an amphibian, the cane toad *Bufo marinus*. *Journal of Experimental Biology,* **211,** 1911-1918.

Udino, E., George, J.M., McKenzie, M., Pessato, A., Crino, L., Buchanan, K.L. & Mariette, M.M. (2021) Prenatal acoustic programming of mitochondrial function for high temperatures in an arid-adapted bird. *Proceedings of the Royal Society of London B,* **288,** 20211893.

Urbina, A., Godoy-Silva, R., Hoyos, M. & Camacho, M. (2016) Acute hydrodynamic damage induced by SPLITT fractionation and centrifugation in red blood cells. *Journal of Chromatography B,* **1020,** 53-61.

Walter, I. & Seebacher, F. (2009) Endothermy in birds: underlying molecular mechanisms. *Journal of Experimental Biology,* **212,** 2328-2336.

Wiegmann, L., de Zélicourt, D.A., Speer, O., Muller, A., Goede, J.S., Seifert, B. & Kurtcuoglu, V. (2017) Influence of Standard Laboratory Procedures on Measures of Erythrocyte Damage. *Frontiers in Physiology,* **8**.

Zheng, W.H., Li, M., Liu, J.-S., Shao, S.-L. & Xu, X.-J. (2014) Seasonal variation of metabolic thermogenesis in Eurasian tree Sparrows (*Passer montanus*) over a latitudinal gradient. *Physiological and Biochemical Zoology,* **87,** 704-718.

TABLES

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

Model and parameter	**Estimate (s.e.m.)**	**d.f.**	***LRT***	***P***	**Fold difference**	***R* (95% CI)**
ROUTINE (pmol $O_2 \times s^{-1} \times$ 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 $O_2 \times s^{-1} \times$ 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 $O_2 \times s^{-1} \times$ 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 $O_2 \times s^{-1} \times$ 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					0.949 (0.711-0.990)

	(0.022)					
592						

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

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)			

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

Model and parameter	Mean (s.e.m.)	d.f.	*t*	*P*
ROUTINE (pmol O_2 × s^{-1} × µl $blood^{-1}$)				
Sample		5	-1.40	0.2214
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 × s^{-1} × µl $blood^{-1}$)				
Sample		5	-1.47	0.2015
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 × s^{-1} × µl $blood^{-1}$)				
Sample		5	3.23	**0.0233**
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 × s^{-1} × µl $blood^{-1}$)				
Sample		5	-0.94	0.3905
0 h	0.175 (0.008)			
24 h	0.188 (0.019)			
72 h	0.243 (0.042)			
96 h	0.190 (0.102)			
$FCR_{ROUTINE/ETS}$				
Sample		5	-4.16	**0.0089**

0 h 24 h 72 h 96 h	0.595 (0.066) 0.745 (0.049) 0.751 (0.057) 0.871 (0.010)			
$FCR_{LEAK/ROUTINE}$ Sample 0 h 24 h 72 h 96 h	 0.264 (0.019) 0.256 (0.016) 0.325 (0.008) 0.357 (0.017)	5	0.74	0.4904
$FCR_{LEAK/ETS}$ Sample 0 h 24 h 72 h 96 h	 0.155 (0.017) 0.190 (0.016) 0.244 (0.012) 0.311 (0.018)	5	-3.29	**0.0217**

FIGURES

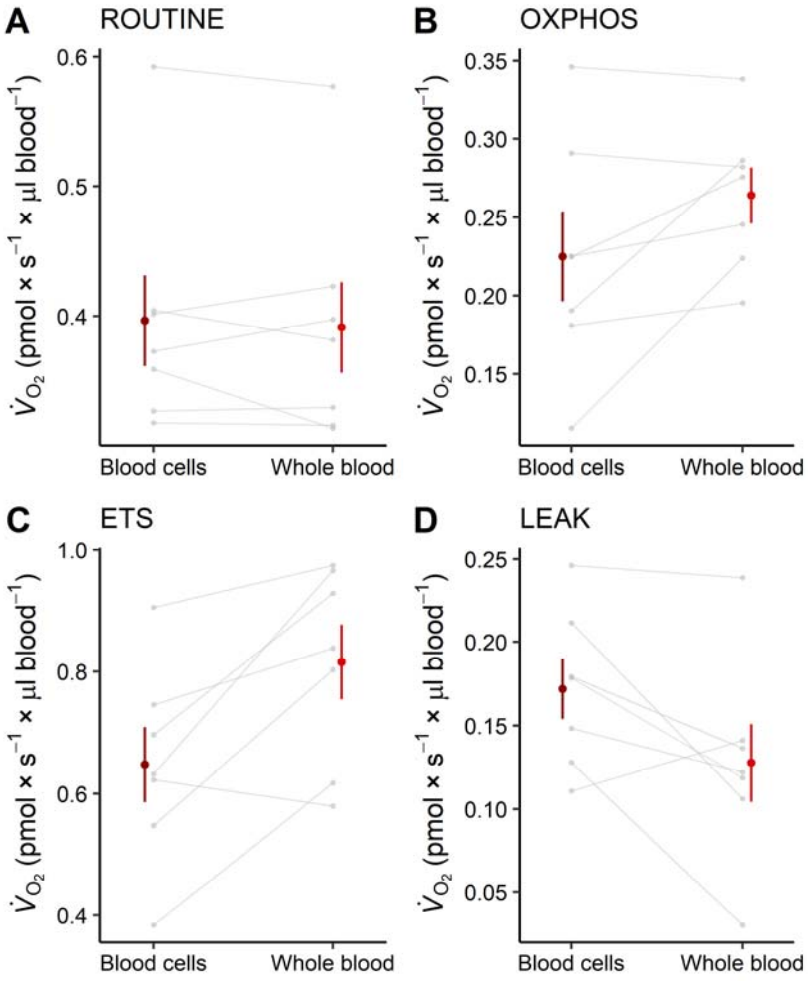

Figure 1. Difference in mitochondrial respiration traits as assayed in either isolated blood cells or whole blood from zebra finches. A) baseline ("ROUTINE") oxygen consumption in the endogenous cellular state; B) oxygen consumption devoted to oxidative phosphorylation alone, where ATP is produced ("OXPHOS);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

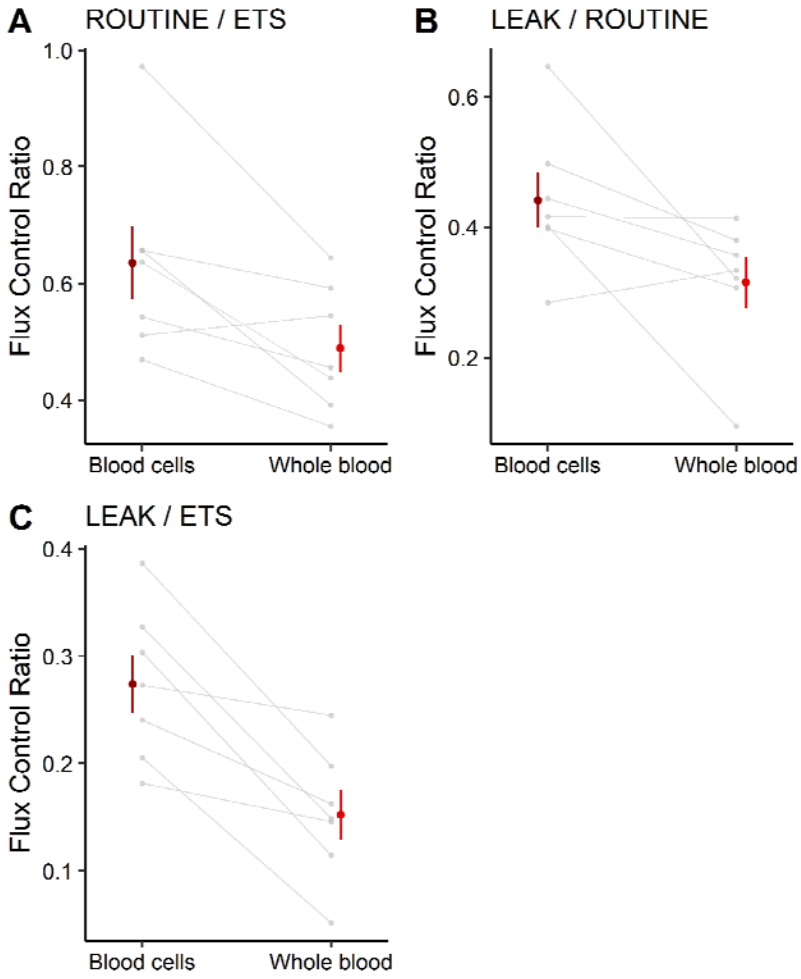

Figure 2. Flux Control Ratios for mitochondrial respiration traits measured in isolated blood cells or whole blood. A) The intensity of endogenous respiration (ROUTINE) relative to maximum working capacity of the electron transport system (ETS); B) Coupling efficiency of the electron transport system (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.

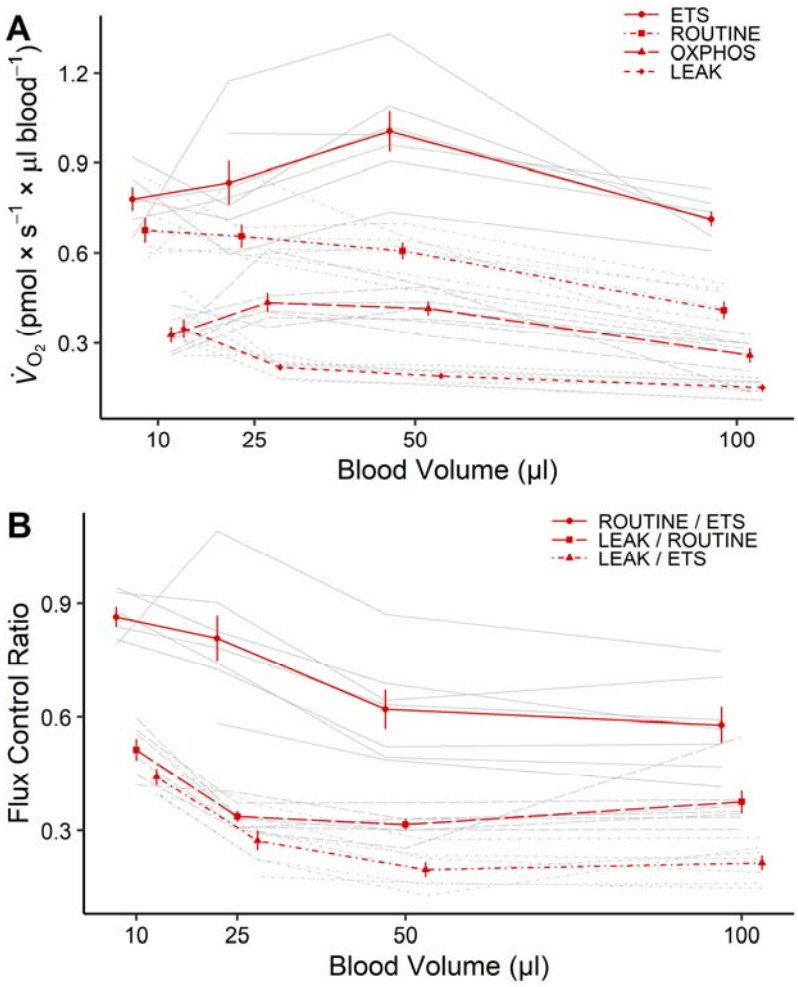

Figure 3. Effects of sample volume when assaying mitochondrial function in zebra finch whole blood. The panels show mitochondrial respiration traits (A) and Flux Control Ratios (FCR) (B) in relation to blood sample volume. Derivation of traits and FCRs are detailed in the main text and in the legends 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.

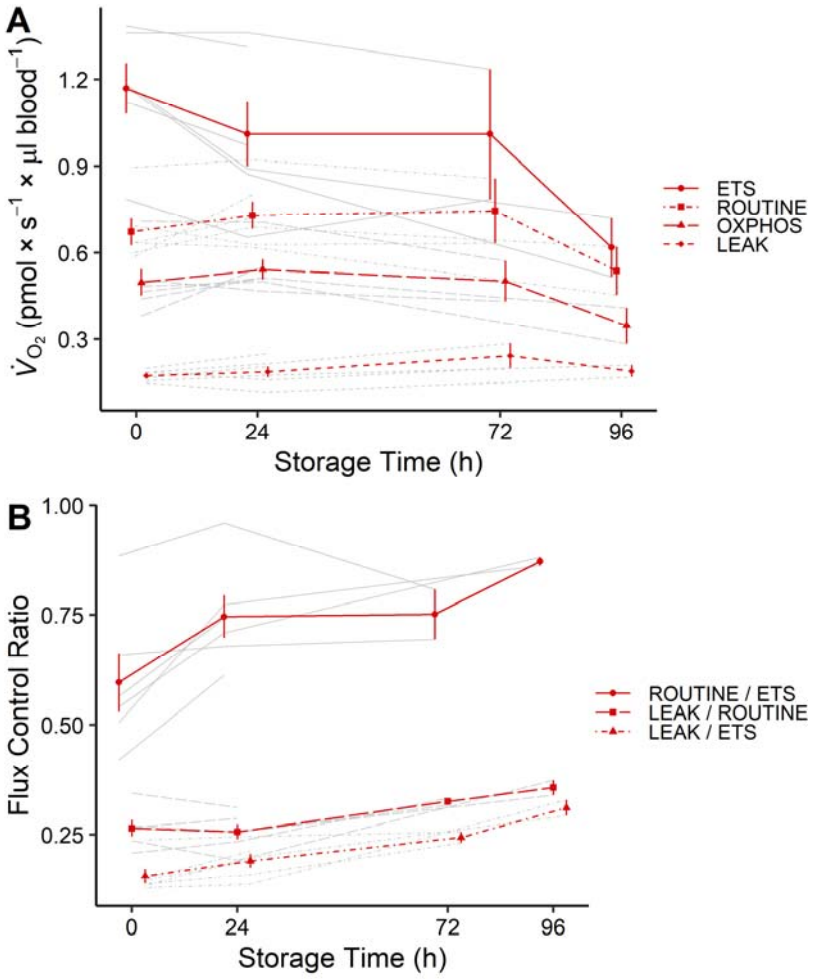

Figure 4. Effects of storage time when assaying mitochondrial function in zebra finch whole blood. The panels show mitochondrial respiration traits (A) and Flux Control Ratios (FCR) (B) in relation to blood sample volume. All individuals were measured at 0 h and after storage without agitation for 24 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.

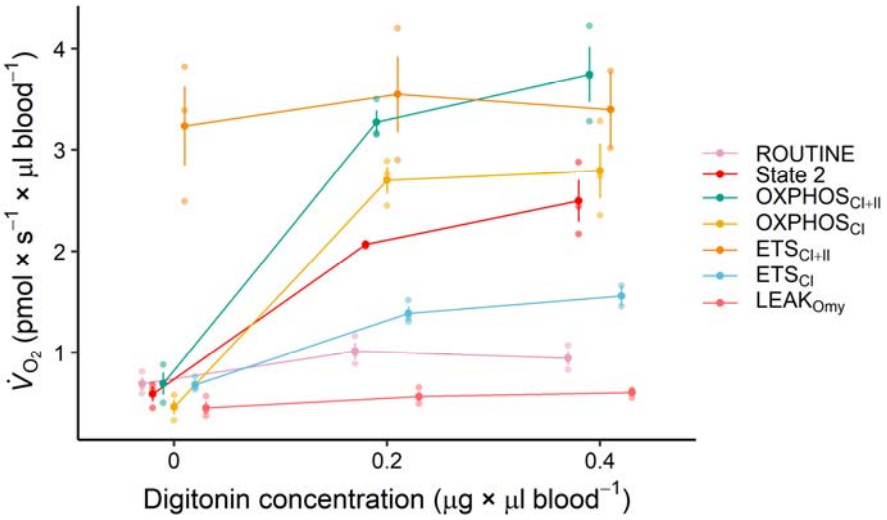

Figure 5. Effect of extent of permeabilization on mitochondrial respiration traits in zebra finch whole blood. The figure shows how variation in concentration of the permeabilising detergent digitonin (5 mg ml^{-1}) affected mitochondrial respiration traits in 25 µl whole blood samples collected from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