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RESEARCH ARTICLE

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

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Abstract

Understanding mitochondrial biology and pathology is key to understanding the evolution of animal form and function. However, mitochondrial measurement often involves invasive, or even terminal, sampling, which can be difficult to reconcile in wild models or longitudinal studies. Non-mammal vertebrates contain mitochondria in their red blood cells, which can be exploited for minimally invasive mitochondrial measurement. Several recent bird studies have measured mitochondrial function using isolated blood cells. Isolation adds time in the laboratory and might be associated with physiological complications. We developed and validated a protocol to measure mitochondrial respiration in bird whole blood. Endogenous respiration was comparable between isolated blood cells and whole blood. However, respiration towards oxidative phosphorylation was higher in whole blood, and whole blood mitochondria were better coupled and had higher maximum working capacity. Whole blood measurement was also more reproducible than measurement on isolated cells for all traits considered. Measurements were feasible over a 10-fold range of sample volumes, although both small and large volumes were associated with changes to respiratory traits. The protocol was compatible with long-term storage: after 24 h at 5°C without agitation, all respiration traits but maximum working capacity remained unchanged, the latter decreasing by 14%. Our study suggests that whole blood measurement

Abbreviations: ATP, adenosine triphosphate; EGTA, egtazic acid; E-L coupling efficiency, flow control ratio indicating how tightly electron transport is coupled to ATP production when the mitochondria are maximally stimulated by an artificial uncoupler; E-R control efficiency, flow control ratio indicative of surplus respiratory capacity; that is, the proportion of maximum working capacity remaining during endogenous respiration; ESM, electronic supplementary material to accompany the main text; ETS, electron transport system; here in the context of its maximum respiration capacity when uncoupled from ATP synthase; FCCP, cyanide-p-trifluoro-methoxyphenyl-hydrazone; FCR, Flow control ratio; that is, a ratio between two different mitochondrial respiration states; HEPES, Hydroxyethyl piperazineethanesulfonic acid; ID, Subject identification number; K2-EDTA, Dipotassium ethylenediaminetetraacetic acid; LEAK, Mitochondrial respiration to offset proton leak across the inner mitochondrial membrane when no ATP is being produced; lmer, Linear mixed effects regression model; MiR05, Mitochondrial respiration medium 05; OXPHOS, Mitochondrial respiration driving oxidative phosphorylation during ATP synthesis; R, Repeatability; that is, a measure of the variability of successive measurements from the same blood or blood cell pool; R-L control efficiency, Flow control ratio indicating the proportion of endogenous respiration channeled toward ATP production via oxidative phosphorylation; ROS, Reactive oxygen species; ROUTINE, Baseline mitochondrial respiration with endogenous substrates.

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provides faster, more reproducible, and more biologically and physiologically relevant (mitochondrial integrity) assessment of mitochondrial respiration. We recommend future studies to take a whole blood approach unless specific circumstances require the use of isolated blood cells.

KEYWORDS

blood, blood cells, cell respiration, energy, erythrocyte, metabolism, mitochondria

1 | INTRODUCTION

The ability to convert the 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, movement, and survival, is a fundamental requirement for life. This occurs through the 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 ATP is produced during cellular respiration in the mitochondria.[1] It is therefore not surprising that mitochondrial respiration and/or volume increase when organismal demand for energy increases (e.g. Refs. [2–4]). Mitochondrial ATP is created by the transport of electrons through a series of protein complexes (the electron transport system) whilst protons are being actively pumped across the inner mitochondrial membrane to establish electrochemical potential. The energy required for oxidative phosphorylation (OXPHOS) is harnessed when protons backflow into the mitochondrial matrix at complex V, the ATP-synthase. However, some protons forego OXPHOS by leaking back into the mitochondrial matrix spontaneously or under the influence of uncoupling proteins.[1] Proton leak thus uncouples mitochondrial respiration from ATP production (LEAK) but generates heat and decreases the rate at which potentially biodegrading reactive oxygen species (ROS) are formed.[5,6] It follows that 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,[7–9] or more thermogenic to aid endothermic heat production.[10]

Since mitochondria provide a functional link between energy intake and output, studies of mitochondrial function can provide key mechanistic insight into many fundamental processes within ecology and evolution (see[11] for a recent review). However, such studies are rare in comparison with 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 or anesthesia to collect biopsies, together with speedy handling of samples to ensure organelle integrity; all of which are easier to reconcile

with 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).[12,13] Studies on birds have found that this makes it possible to assess mitochondrial function even from small (25–50 μl) blood samples[14–16] that can be safely collected in organisms <5 g.[17] This is not possible when measurements are performed using other mitochondria-rich blood cells, as is necessary in mammals.[18,19] Several recent bird studies indicate that blood cell respiration varies predictably in line with organismal-level energy demand over long[16,20] and short[21] time frames, and in the contexts of seasonal adaptation[22] and metabolic senescence.[23] Moreover, blood cell mitochondrial function seems amenable to epigenetic programming during embryonic development,[24,25] much like what is a well-known fact within human medicine.[26] Thus, while much is still unknown about the function and regulation of erythrocyte mitochondria, there is ample evidence that mitochondrial measurement in blood samples can provide minimally invasive insights into many topical research questions within the broader fields of ecology, ecological physiology, and evolutionary biology.

To the best of our knowledge, all studies on avian blood cell mitochondria have been performed using isolated blood cells, prepared using standard laboratory techniques including pipetting, vortexing and centrifugation. These procedures could impact the status of the cells going into the experiment (e.g. Ref. [27]). If standard handling impacts cell health, then mitochondrial function in isolated blood cells may be less representative of the in vivo state or could introduce experimental variation, but this notion remains untested. Furthermore, while erythrocytes will be responsible for most of the respiration in the avian circulation (c.f. Ref. [28]), it is difficult to separate bird blood cell types by simple centrifugation and pipetting (e.g. Ref. [29]); likely more so in the small samples that are typically collected from wild models. Thus, the composition of a cell isolate may correspond more closely to the cell population in the original whole blood sample than what was intended, potentially complicating biological inference and comparisons between studies. On this background, we asked whether mitochondrial measurements on whole blood samples were comparable

with measurements on blood cells isolated from the same blood volume. If cell isolation impacts mitochondrial integrity or function of the electron transport chain, we predicted that isolate measurements would be associated with leakier mitochondria and/or lower overall respiration compared with whole blood samples. To explore the utility of whole blood measurement, we proceeded by testing the resilience of the assay to variations in sample volume and long-term storage. Finally, we undertook initial validation of a protocol for permeabilizing whole blood to permit studies of mitochondrial function with unlimited substrate supply.

2 | MATERIALS AND METHODS

2.1 | Animals and housing

The study was performed using captive zebra finches (*Taeniopygia guttata* Vieillot) ($n = 23$; 12 females and 11 males). All birds were in their second calendar year, or older, and body masses ranged 12.1–19.4 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 days before the experiment started. Birds were kept in cages measuring $120 \times 80 \times 100$ cm (length $\times$ depth $\times$ height) at a density of ≤ 6 birds to a cage. Room temperature was maintained at 19–20°C, and the photoperiod was 12:12 LD. Food (mixed millet seeds), cuttlebone, and water were provided ad libitum.

2.2 | Anesthesia and blood sampling

We sterilized the ventral skin area covering the distal part of the sternum using 70% ethanol and immediately anesthetized the birds by intraperitoneal injection of pentobarbital sodium (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 euthanized the bird by cervical dislocation. Samples were stored at 10–15°C in 2 ml K2-EDTA (ethylenediaminetetraacetic acid) tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) without agitation until analyzed 15 to 45 min later.

2.3 | Mitochondrial respiration measurements and experiments

Mitochondrial respiration was measured at bird body temperature (41°C[30]) 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).

All measurements were performed on intact cells. We allowed 10–15 min for O_2 consumption to stabilize after adding the 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 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. In every experiment run, 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 considered of non-mitochondrial origin. There was never a meaningful further reduction in respiration when antimycin A was added to rotenone (data not shown). Hence, we adjusted all respiratory states for non-mitochondrial respiration by subtracting whichever was the 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 first experiment, we compared mitochondrial respiration traits measured in whole blood and isolated blood cells. 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 two 100 μl whole blood aliquots to use either for immediate respiration measurement or for isolating the blood cells. We isolated the blood cells following Stier et al.[15] with slight modifications according to Dawson & Salmon[23] and Nord et al.[22] Thus, the 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 $100\,\mu l$ blood sample, was immediately re-suspended in $750\,\mu l$ MiR05 pre-equilibrated at 41°C and added to $1.35\,ml$ MiR05 contained in the respiration chamber (final volume $2\,ml$) for use in the assay. The sample volume used here is the same as in the original protocol for mitochondrial measurement in bird blood cells[15] and is within range of that used in other studies (e.g. Refs. [22,23]).

In the second experiment, we asked how blood sample volume affected respiration traits, 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 $100\,\mu l$ whole blood samples. The volume of MiR05 in the chambers was adjusted accordingly to maintain a total volume of $2\,ml$.

In the third experiment, we investigated the effect of storage time on whole blood respiration, 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 mitochondrial respiration in $50\,\mu l$ blood samples in $1.95\,ml$ MiR05. The tube with remaining blood was then stored without agitation

TABLE 1   Comparison of mitochondrial respiration metrics in whole blood and in isolated blood cells from the same blood volume

Model and parameter	Estimate (SEM)	df	*LRT*	*p*	Fold difference	*R* (95% CI)
ROUTINE (pmol $O_2 \times s^{-1} \times \mu l$ blood^{-1})						
Sample		1	0.4	.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 $O_2 \times s^{-1} \times \mu l$ blood^{-1})						
Sample		1	8.0	**.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 $O_2 \times s^{-1} \times \mu l$ blood^{-1})						
Sample		1	19.1	**<.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 $O_2 \times s^{-1} \times \mu l$ blood^{-1})						
Sample		1	12.7	**<.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)
E-R control efficiency						
Sample		1	19.0	**<.0001**	0.77	
Blood cells	0.365 (0.049)					0.968 (0.806–0.994)
Whole blood	0.511 (0.049)					0.982 (0.893–0.996)
R-L control efficiency						
Sample		1	12.2	**.0004**	0.71	
Blood cells	0.559 (0.034)					0.849 (0.325–0.972)
Whole blood	0.685 (0.034)					0.933 (0.691–0.988)
E-L coupling efficiency						
Sample		1	26.4	**<.0001**	0.55	
Blood cells	0.726 (0.023)					0.790 (0.202–0.950)
Whole blood	0.848 (0.023)					0.949 (0.711–0.990)

Note: This table shows model estimates, test statistics, degrees of freedom, and resultant *p*-values, when assaying mitochondrial respiration in zebra finches using either $100\,\mu l$ whole blood or all cells from $100\,\mu l$ 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., $p < .05$) effects appear in bold font.

Abbreviations: CI, confidence interval; df, degrees of freedom; *LRT*, likelihood ratio test statistic; *R*, intraclass repeatability; SEM, standard error of mean.

at $5.2 \pm 0.7°C$ (mean $\pm$ SD; range 3.9–6.5°C) and another 50 µl aliquot from the same sample was measured again (after 5 min of mixing) 24 h later (henceforth, time = "24 h"). Samples from 4 birds (2 females, 2 males) were also opportunistically measured at 72 or 96 h after collection (one female and one male sampled per time point).

Because intact cell measurement provides incomplete insight into the function of the mitochondrial complexes, we undertook initial validation of an open-cell protocol for whole blood. We found that samples were readily permeabilized over a range of digitonin concentrations, suggesting this is a fruitful avenue to explore in future work. A representative experiment, together with a full protocol and breakdown of the results, are provided in the Electronic Supplementary Materials (ESM 1).

2.4 | Data analyses

We calculated three flux control ratios (FCRs) to address the integrity of mitochondrial respiration following the method and terminology proposed by Gnaiger[31]: (1) E-R control efficiency (1 − ROUTINE/ETS), which is a measure of the proportion of maximum working capacity remaining during endogenous respiration; (2) R-L control efficiency ((ROUTINE − LEAK)/ROUTINE), which is the proportion of endogenous respiration channeled toward ATP production via oxidative phosphorylation; (3) E-L coupling efficiency (1 − LEAK/ETS), which is indicative of how "tightly" electron transport is coupled to ATP production under a stimulated cellular state.

Statistical analyses were performed using R 4.1.2 for Windows.[32] 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)[33] 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.[34] 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[35] and the pairs function in this package was used 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 and 24 h samples (t.test function in R base).

3 | RESULTS

A representative trace of oxygen consumption in a whole blood experiment is provided in the online supplements (Figure S1 in ESM 1).

3.1 | Effects of sample type—blood cells versus whole blood

ROUTINE respiration did not differ between isolated blood cells and whole blood samples (Table 1, Figure 1A). However, OXPHOS and ETS were significantly higher when measured on whole blood samples compared with on blood cells (1.2 and 1.3-fold, respectively) (Figure 1B,C, Table 1). In contrast, LEAK was 30% lower in the whole blood samples (Figure 1D, Table 1). Consequently, the E-R control efficiency (which measures proportionally
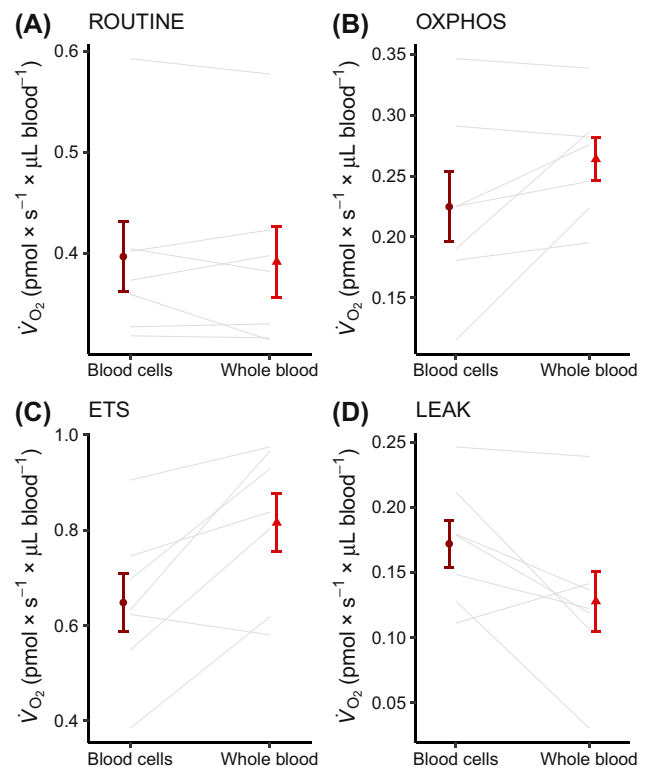

FIGURE 1 Difference in mitochondrial respiration traits as assayed in either isolated blood cells or whole blood from zebra finches. (A) baseline ("ROUTINE") oxygen consumption in the endogenous cellular state; (B) oxygen consumption devoted to oxidative phosphorylation alone, where ATP is produced ("OXPHOS"); (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"). Colored plotting symbols show raw data means ± 1 standard error. Gray lines show the averaged individual responses. Statistics are reported in Table 1.

how much respiration can be increased from ROUTINE) was significantly higher in the whole blood samples (0.511, compared with 0.365 in isolated cells; Figure 2A, Table 1). Whole blood samples also had significantly higher phosphorylating efficiency and higher coupling efficiency during maximal FCCP-stimulated respiration (i.e., R-L control and E-L coupling efficiencies were higher) (Figure 2B,C, Table 1).

Respiration traits measured on both whole blood and isolated blood cells were significantly and highly reproducible (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 reproducible, and the calculated repeatabilities had markedly narrower confidence intervals, for all respiration traits and FCRs investigated (Table 1).
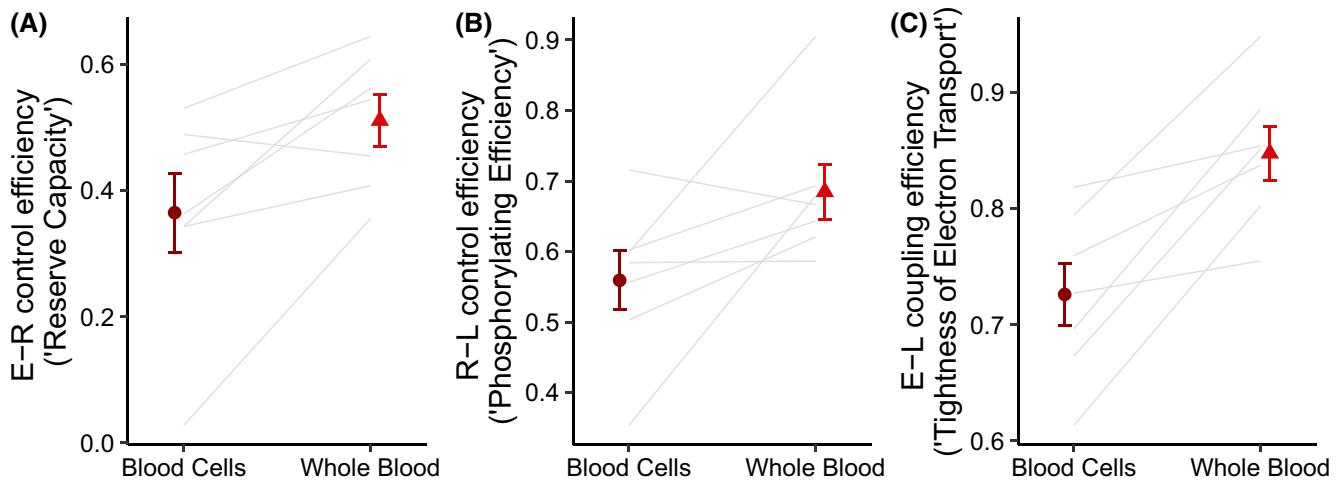

FIGURE 2 Flux control ratios for mitochondrial respiration traits measured in isolated blood cells or whole blood. (A) E-R control efficiency indicates the proportion of maximum working capacity (i.e., ETS) remaining during endogenous respiration (i.e., ROUTINE); (B) R-L control efficiency is a measure of the efficacy of ATP-producing respiration (i.e., OXPHOS) under endogenous cellular conditions; (C) E-L coupling efficiency measures the coupling of electron transport in a maximally FCCP-stimulated cellular state (i.e, ETS). Colored plotting symbols show raw data means ±1 standard error. Gray lines show averaged individual responses. Duplicate samples were averaged before means and standard errors were calculated. Details on the calculation of FCRs are provided in the main text, and statistics appear in Table 1.
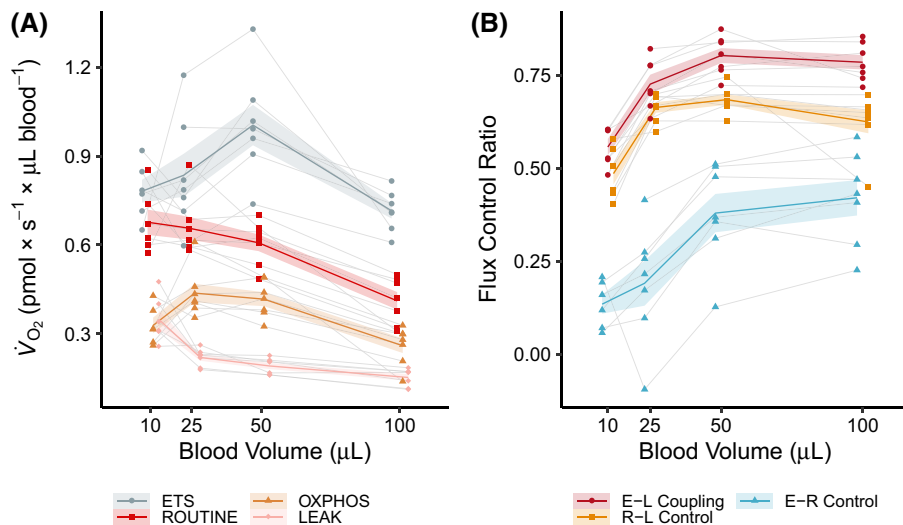

FIGURE 3 Effects of sample volume when assaying mitochondrial function in zebra finch whole blood. The panels show mitochondrial respiration traits (A) and flux control ratios (FCR) (B) in relation to blood sample volume. Derivation of traits and FCRs is detailed in the main text. Colored lines connect volume-specific means, and the shaded areas denote ±1 standard error. Colored plotting symbols show raw data and colored lines show the averaged response. Gray lines show individual responses. Note that data are jittered in the *x*-space for clarity. Statistics are reported in Table 2.

TABLE 2 Effects of sample volume when measuring mitochondrial respiration in zebra finch whole blood

Model and parameter	Estimate (SEM)	df	LRT	p
ROUTINE (pmol $O_2 \times s^{-1} \times \mu l$ blood^{-1})				
Blood volume		1	27.5	**<.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	**<.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	**.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	**<.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)			
E-R control efficiency				
Blood volume		1	29.6	**<.0001**
10 µl [A]	0.159 (0.051)			
25 µl [A]	0.191 (0.049)			
50 µl [B]	0.389 (0.049)			
100 µl [B]	0.421 (0.049)			
R-L control efficiency				
Blood volume		1	28.5	**<.0001**
10 µl [A]	0.486 (0.025)			
25 µl [B]	0.664 (0.022)			
50 µl [B]	0.685 (0.022)			
100 µl [B]	0.625 (0.022)			
E-L coupling efficiency				
Blood volume		1	49.7	**<.0001**
10 µl [A]	0.566 (0.023)			
25 µl [B]	0.726 (0.022)			

(Continues)

TABLE 2 (Continued)

Model and parameter	Estimate (SEM)	df	LRT	p
50 µl [C]	0.803 (0.022)			
100 µl [C]	0.785 (0.022)			

Note: This table shows model estimates (±standard error; SEM), 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* < .05) effects appear in bold font. Different letters within brackets denote statistically significant pairwise differences.

Abbreviations: df, degrees of freedom; *LRT*, likelihood ratio test statistic; SEM, standard error of mean.

*$p_{10-25\,\mu l}$ = .053.

3.2 | Effects of blood volume

There were non-linear effects of 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 of 1.5 to 1.6) for all volumes 10–50 µl compared with the 100 µl sample (Figure 3A, Table 2). OXPHOS was the highest for 25 and 50 µl, and the lowest for 10 and 100 µl (Figure 3A, Table 2), though the 50 µl sample was significantly different only from the 100 µl sample. 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, although this volume differed statistically only from 50 µl (Table 2). LEAK was significantly higher on 10 µl compared with all other volumes, and the lowest on 100 µl (but this volume was not statistically different from 50 µl) (Table 2).

FCRs, which provide rate-independent insight into mitochondrial function, were also volume-specific. Specifically, E-R control efficiency was similar in 50 and 100 µl and significantly higher (reflecting more surplus capacity) compared with the two other volumes (Figure 3B). R-L control efficiency (i.e., the efficiency with which O_2 consumption is channeled toward oxidative phosphorylation) was the lowest in 10 µl (reflecting higher LEAK) compared with the other volumes (Table 2, Figure 3B). E-L coupling efficiency was also the lowest in 10 µl followed by 25 µl but higher in both 50 and 100 µl (which were not statistically different) (Table 2, Figure 3B).

3.3 | Effects of storage

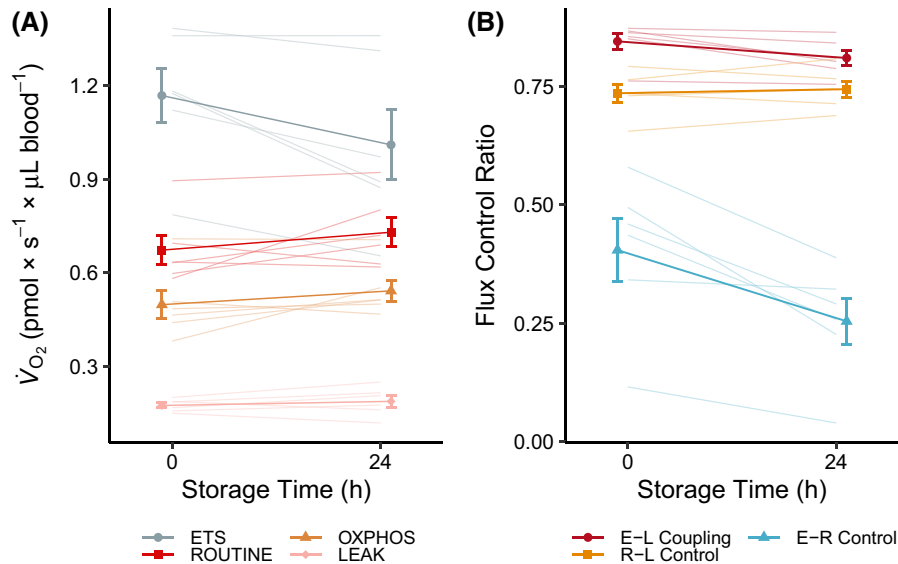Endogenous (ROUTINE), phosphorylating (OXPHOS), and leak (LEAK) respiration did not change significantly

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. Solid plotting symbols show raw data means ±1 standard error, with means interconnected by solid lines. The semi-transparent lines show individual responses, color-coded by respiration trait or FCR. Statistics are reported in Table 3.

with 24 h storage at 5°C (all $p > .2$) (Figure 4A, Table 3). However, maximal respiration capacity (ETS) was 14% lower at 24 h compared with 0 h. As a result, E–R control efficiency and E–L coupling efficiency were significantly lower at 24 h (Figure 4B, Table 3). However, there was no change in R–L control efficiency, in keeping with stable ROUTINE and LEAK over 24 h storage. By 72 h, ROUTINE, OXPHOS, and ETS had decreased somewhat from the 24 h level and LEAK had increased, with corresponding changes to FCRs (Figure S3 in ESM 2). These changes became more exaggerated with 96 h storage (Figure S3).

4 | DISCUSSION

In this study, we asked whether the measurement of whole blood could provide a feasible and potentially more biologically contextualized approach to studying mitochondrial respiration in intact 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; Figure 1B) and maximal (i.e., ETS; Figure 1C) respiration was significantly higher in whole blood and that mitochondria in isolated blood cells displayed significantly more LEAK (i.e., they were more uncoupled) than whole blood (Figure 1D). This translated to significant differences in FCRs, which indicated that the isolated blood cells had lower surplus respiratory capacity (Figure 2A), lowered phosphorylating efficiency (Figure 2B), and

decreased coupling efficiency when stimulated to work at maximum (Figure 2C). Moreover, while our repeatability estimates for isolated erythrocytes were consistently high and mostly within the range for the same traits reported elsewhere,[15] the whole blood samples were consistently more reproducible (all $R > 0.93$) and 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 a derived sample. We do not believe that these differences were caused mainly by heterogeneity of sample types for at least two reasons. Firstly, complete separation of avian blood cell populations by centrifugation alone likely requires more diligence than applied here (c.f.[29]). Secondly, the concentration of other mitochondria-rich blood cells such as leukocytes and thrombocytes in the circulation is some 100–200 times lower than that of erythrocytes.[28] This suggests that any differential contribution by such cells to overall respiration would be minuscule in comparison with the differences between treatments; as supported by the similarity of ROUTINE between whole blood and blood cells (Figure 1A). We therefore believe that the results could be caused by physical damage or otherwise compromised integrity of mitochondria in the isolated blood cells caused by more handling, because lower ETS, higher LEAK, and associated changes to FCRs are all indicative of declining mitochondrial health/viability (e.g. Ref. [18]) (see also Figure 4). Studies on mammalian erythrocytes show centrifugation alone can cause time-dependent structural and physiological damage to red

TABLE 3 Effects of storage time on mitochondrial respiration metrics in zebra finch whole blood

Model and parameter	Mean (SEM)	df	t	p
ROUTINE (pmol $O_2 \times s^{-1} \times \mu l$ blood^{-1})				
Sample		5	−1.40	.2214
0 h	0.673 (0.047)			
24 h	0.731 (0.047)			
OXPHOS (pmol $O_2 \times s^{-1} \times \mu l$ blood^{-1})				
Sample		5	−1.47	.2015
0 h	0.498 (0.046)			
24 h	0.542 (0.035)			
ETS (pmol $O_2 \times s^{-1} \times \mu l$ blood^{-1})				
Sample		5	3.23	**.0233**
0 h	1.169 (0.088)			
24 h	1.011 (0.111)			
LEAK (pmol $O_2 \times s^{-1} \times \mu l$ blood^{-1})				
Sample		5	−0.94	.3905
0 h	0.175 (0.008)			
24 h	0.188 (0.019)			
E-R control efficiency				
Sample		5	4.16	**.0089**
0 h	0.404 (0.066)			
24 h	0.255 (0.049)			
R-L control efficiency				
Sample		5	−0.74	.4904
0 h	0.736 (0.019)			
24 h	0.744 (0.016)			
E-L coupling efficiency				
Sample		5	3.29	**.0217**
0 h	0.845 (0.017)			
24 h	0.809 (0.016)			

Note: This table shows mean (±standard error; SEM) 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* < .05) effects appear in bold font.

Abbreviations: df, degrees of freedom; SEM, standard error of mean; *t*, *t*-test statistic.

blood cells (such as increased free and corpuscular hemoglobin and increased ATP release) at spinning speeds considerably lower than those used here (<1500 *g*).[36–38] 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.[27] However, our study indicates that whole blood measurement may salvage more of the physiological integrity of the mitochondria.

We found non-linearity of volume-adjusted respiration rates, manifested as inhibited or enhanced respiration on the highest and lowest blood volumes (Figure 3A). On average, optimal trait combinations for coupled and uncoupled respiration were achieved using 50 μl samples, but these runs were most often similar to those on 25 μl. FCR for phosphorylating and coupling efficiencies were similar in the range of 25–100 μl, whereas 10 μl samples were less well coupled. Thus, working in a range of 25–50 μl whole blood provided representative results for respiration traits, whereas a sample volume range of 25–100 μl was acceptable if FCRs only were considered. Our findings contrast previous studies on pig liver homogenates where complete linearity of phosphorylating respiration was demonstrated across a 3-fold range in the sample volume.[39] However, studies on isolated human platelets show lower uncoupled respiration on small sample volumes.[18] This could be caused, for example, by reduced accuracy on low sample concentrations

acting in combination with enhanced sensitivity/exposure to inhibitors. Measurements on pig skeletal muscle mitochondria also suggest suppressed respiration when samples were prepared from smaller biopsies,[40] although 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) both intact and open blood cells can be measured using plasma as medium without inhibition of respiration[41,42] and (2) ROUTINE was identical in whole blood and isolated blood cells (Figure 1A), which is not expected if some whole blood constituents act suppressively on respiration. It is possible that the higher concentration of hemoglobin in larger samples sequestered enough O_2 to suppress respiration beyond some level. In line with this, maximal and minimal O_2 concentration decreased approximately linearly with sample volume between 10 and 50 μl, but nonlinearly between 50 and 100 μl (Figure S4 in ESM 3). Accordingly, the reduction in O_2 concentration during an experiment was below expectations for 100 μl samples (Figure S4). While O_2 levels were consistently above what is considered suppressive for other cell types, future studies should address the O_2-limitation hypothesis for volume-dependent blood cell respiration by measuring differently sized samples in environments ranging from hypoxic to hyperoxic. Such studies would also benefit from including measurement of mitochondrial content in differently sized samples, 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 14%) remained unaltered. This corroborates previous work on both human and avian blood cells[15,18] and pig liver homogenates.[39] However, neither Sjövall et al.[18] nor Stier et al.[15] 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 45 min of bleeding the birds. It is therefore tempting to speculate that most changes to ETS occur within the first hour of collection. Furthermore, Sjövall et al.[18] found that respiration of permeabilized 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 relatively stable when the blood was stored 2–2.5 h at room temperature under constant motion, ETS dropped by 30% to 90% over the same period (data not shown).

5 | CONCLUSIONS

We found that whole blood measurement provides a more rapid, more biologically contextualized, and more precise and reproducible assessment of mitochondrial respiration in intact avian blood cells compared with measurement on isolated blood cells. We also give data-based recommendations on sample volume ranges and show that 24 h cold-storage in the blood collection tubes is possible at mostly acceptable changes to respiration traits, and that whole blood can be permeabilized. Changes to respiration parameters of the intact cells were indicative of handling-induced damage. However, it is possible that less extensive handling protocols (see e.g. Ref. [16]) will provide more comparable results. Even so, our study suggests that centrifugation and removal of plasma are unnecessary complications that add processing time and reduce biological context. Thus, we advocate the use of whole blood whenever possible. When measurements on isolated cells 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 the use of relatively constant blood volumes to minimize variation within an experiment, and suggest samples are run on the day of collection.

DISCLOSURES

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

DATA AVAILABILITY STATEMENT

Data are deposited in Figshare: https://doi.org/10.6084/m9.figshare.21740993.v1.

ETHICS STATEMENT

All procedures were approved by the Malmö/Lund Animal Ethics Committee, acting under the authority of the Swedish Board of Agriculture (permit no. 9246-19, 16 977-19).

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