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How to measure mitochondrial function in birds using red blood cells: a case study in the king penguin and perspectives in ecology and evolution

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Abstract:	1. Mitochondria are the powerhouse of animal cells. They produce through oxidative phosphorylation more than 90% of the cellular energy (ATP) required for organism's growth, reproduction and maintenance. Hence, information on mitochondrial function is expected to bring important insights in animal ecology and evolution. Unfortunately, the invasiveness of the procedures required to measure mitochondrial function (e.g. sampling of liver or muscles) has limited its study in wild vertebrate populations so far. Here, we capitalize on the fact that bird red blood cells (RBCs) possess functional mitochondria to describe a minimally-invasive approach to study mitochondrial function using blood samples. 2. In the king penguin, we present a protocol using a high-resolution respirometry system and specific agonists and antagonists enabling the assessment of mitochondrial function in RBCs. We evaluated the inter-assay repeatability of our measures of mitochondrial function, and tested the influence of sample storage and bird handling time on these measures. We also compared measures of mitochondrial function in RBCs and in the pectoral muscle obtained from the same individuals.

	3. Mitochondria from RBCs showed the expected responses to mitochondrial agonists and antagonists, and therefore the protocol presented allows computing effective measures of mitochondrial function. The different measures of RBC mitochondrial function were significantly repeatable, were not affected by the handling time of the bird prior to blood sampling (i.e. stress response), and only minimally affected by the storage time of the sample at 4°C up to 24h. Most notably, we showed that mitochondrial parameters measured in RBCs moderately correlated to those measured in the pectoral muscle. 4. The present study sheds light on the use of RBCs in birds as a valuable and minimally-invasive source of information on mitochondrial function. This approach opens new opportunities to study mitochondrial function in free-living animals and could bring knowledge gains in ecology and evolution. Fish, amphibians and reptiles also possess mitochondria in their RBCs, and the approach presented here could also be applicable to these taxa.

How to measure mitochondrial function in birds using red blood cells: a case study in the king penguin and perspectives in ecology and evolution

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Running title: Measurement of mitochondrial function in birds

Summary

1. Mitochondria are the powerhouse of animal cells. They produce through oxidative phosphorylation more than 90% of the cellular energy (ATP) required for organism's growth, reproduction and maintenance. Hence, information on mitochondrial function is expected to bring important insights in animal ecology and evolution. Unfortunately, the invasiveness of the procedures required to measure mitochondrial function (e.g. sampling of liver or muscles) has limited its study in wild vertebrate populations so far. Here, we capitalize on the fact that bird red blood cells (RBCs) possess functional mitochondria to describe a minimally-invasive approach to study mitochondrial function using blood samples.

2. In the king penguin, we present a protocol using a high-resolution respirometry system and specific agonists and antagonists enabling the assessment of mitochondrial function in RBCs. We evaluated the inter-assay repeatability of our measures of mitochondrial function, and tested the influence of sample storage and bird handling time on these measures. We also compared measures of mitochondrial function in RBCs and in the pectoral muscle obtained from the same individuals.

3. Mitochondria from RBCs showed the expected responses to mitochondrial agonists and antagonists, and therefore the protocol presented allows computing effective measures of mitochondrial function. The different measures of RBC mitochondrial function were significantly repeatable, were not affected by the handling time of the bird prior to blood sampling (*i.e.* stress response), and only minimally affected by the storage time of the sample at 4°C up to 24h. Most notably, we showed that mitochondrial parameters measured in RBCs moderately correlated to those measured in the pectoral muscle.

4. The present study sheds light on the use of RBCs in birds as a valuable and minimally-invasive source of information on mitochondrial function. This approach opens new opportunities to study mitochondrial function in free-living animals and could bring knowledge gains in ecology and evolution. Fish, amphibians and reptiles also possess mitochondria in their RBCs, and the approach presented here could also be applicable to these taxa.

Keywords: mitochondria, erythrocyte, non-invasive methodology, high-resolution respirometry, metabolism

Introduction

Life history theory (Roff 1992) and metabolic theory of ecology (Brown *et al.* 2004) suggest that metabolic rate – the rate at which organisms take up, transform and allocate energy to growth, reproduction and maintenance – is at the heart of adaptation and success of organisms to particular environments. In animals more than 90% of the cellular energy is produced as adenosine triphosphate (ATP) during mitochondrial respiration (Nicholls & Ferguson 2002). Hence, our understanding of the evolutionary success of particular individuals requires insights about the factors that shape mitochondrial function (defined here as the ability to use O_2 to oxidize substrate and produce ATP and heat) and the downstream effects that mitochondrial function can exert on life histories (Salin *et al.* 2012; Toews *et al.* 2013; Hill 2014; Stier *et al.* 2014a; Stier et al. 2014b; Stier et al. 2014c; Salin *et al.* 2015; Schwartz *et al.* 2015; Bar-Yaacov *et al.* 2015; Koch *et al.* 2016; Delhaye *et al.* 2016).

The mitochondrion consists of outer- and inner- phospholipid membranes separated by an intermembrane space, and contain mtDNA and ribosome in the mitochondrial matrix (Fig. 1). ATP is produced by the mitochondria through a process called oxidative phosphorylation (hereafter referred as OXPHOS; Nicholls & Ferguson 2002). The inner-membrane has a controlled permeability to protons and contains the five OXPHOS complexes responsible for the coupling of substrate oxidation to ATP production (Fig. 1). Complexes I to IV transport electrons from the substrates (NADH, succinate and FAD-linked substrates) toward molecular oxygen while pumping protons from the mitochondrial matrix into the inter-membrane space at the same time. This process builds up an electrochemical gradient across the

mitochondrial inner-membrane, and the energy released by the backflow of protons to the matrix through complex V (i.e. the ATP synthase) is used for the phosphorylation of ADP into ATP. Protons can also backflow to some extent to the matrix without passing by the complex V, leading to an energy released mostly as heat. This phenomenon is referred as the mitochondrial proton leak (Divakaruni & Brand 2011). The level of mitochondrial coupling between substrate oxidation and ATP production could vary both between and within species, but also within individual in response to factors such as fasting (Salin et al. 2015, Salin et al. 2016a). This is one parameter of biological interest since it determines the amount of ATP and heat generated for a given amount of O_2/substrate consumed (Brand 2005). This mitochondrial coupling between respiration and ATP production is usually estimated by the ratio between the overall mitochondrial O_2 consumption and the residual O_2 consumption linked to proton leak, a parameter also known as the respiratory control ratio (RCR). Finally, some electrons can also escape during their transport among the different complexes (especially in complex I and III), which leads to the production of reactive oxygen species (ROS) that are implicated, at least to some extent, in the ageing process (Speakman *et al.* 2015).

Given that studying mitochondrial function could provide important insights in ecology and evolution it might be surprising that very few ecologists and evolutionary biologists have embarked on this path (e.g. Salin *et al.* 2012; Toews *et al.* 2013; Monternier *et al.* 2014). A lack of communication and transfer of knowledge between mitochondrial biologists and ecologists/evolutionary biologists can probably explain in part this phenomenon. However, we strongly believe that methodological considerations have been a main limiting factor for the study of

mitochondrial function in natural populations. Indeed, the classical approach to investigate mitochondrial biology is to obtain a tissue sample (typically from the liver or muscles) and then work with isolated mitochondria, permeabilized cells or homogenate samples (Brand & Nicholls 2011). Consequently, studying mitochondrial function usually involves terminal sampling in small animals (e.g. Toews *et al.* 2013) or laborious surgical procedures in larger animals (e.g. Monternier *et al.* 2014). Those invasive procedures are nevertheless rarely compatible with the research aims of most ecologists and evolutionary biologists, eager to collect information in natural populations while keeping the disturbance to their study system as low as possible and/or to perform repeated measurements of the same individual over time (*i.e.* longitudinal design; Stier *et al.* 2015). In this context, our aim was to develop a minimally-invasive method to study mitochondrial function in non-mammalian vertebrates, and in particular bird species. Blood sampling is frequently performed in natural populations of birds and well accepted as a minimally invasive procedure (Sheldon *et al.* 2008). RBCs are by far the most abundant cell type in the blood, and interestingly RBCs of birds (as well as other non-mammalian vertebrate species) possess not only a nucleus but also functional mitochondria (Stier *et al.* 2013; Stier *et al.* 2015). In the present study, our aim is to validate the use of RBCs to study mitochondrial function in birds.

We describe a standard protocol that allows measuring mitochondrial function in bird RBCs using a high-resolution respirometry system. We investigated the response of intact RBCs to well-known mitochondrial agonists and antagonists that allow the dissection of mitochondrial function in different parameters of interest (Gnaiger 2009; Brand & Nicholls 2011). We conducted our study in a natural

population of king penguins (*Aptenodytes patagonica*), which is a large bird species frequently used to assess mitochondrial function in the wild using pectoral muscle biopsies (e.g. Rey *et al.* 2008; Monternier *et al.* 2014). We used this opportunity to measure mitochondrial function in RBCs and to compare our results with findings from the skeletal muscle, which is a tissue commonly used to assess mitochondrial function. To evaluate the robustness of measures of mitochondrial function in RBCs, we tested the sensitivity of our mitochondrial parameters to the effect of handling stress (*i.e.* the time elapsed between capture and blood sampling) and of storage time (i.e. the time elapsed between blood sampling in the field and mitochondrial analysis in the laboratory; 4h to 24h). We also report on the repeatability of our measures and on two different ways of normalizing mitochondrial respiration.

Material and Methods

STUDY SITE AND ANIMALS

This study took place in the king penguin colony of "La Grande Manchotière" (*ca.* 24,000 breeding pairs) on Possession Island in the Crozet Archipelago (46° 25'S; 51° 52'E). Adult king penguins were caught either during courtship on the beach near the research facility (N = 9 females and 9 males) or during incubation 3 days after the start of their incubation shift (N = 46 females in incubation shift 2 and 29 males in incubation shift 3).

SAMPLING PROCEDURES

Birds caught during courtship were immediately transferred to the nearby research facility (< 2min walking distance). A blood sample (c.a. 2mL) was then collected from the marginal flipper vein using a heparinised syringe and stored on crushed ice until further processing. A 200 mg muscle biopsy was taken under isoflurane-induced anesthesia from the superficial pectoralis muscle as described previously (Rey *et al.* 2008). Fifty mg of muscle were immediately immersed in ice-cold BIOPS solution (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) until further processing.

Birds caught during incubation were blood sampled in the colony within 4 min after capture. For 23 of these birds, a second blood sample was taken after 30 min of standardized handling (see Viblanc *et al.* 2015 for details on capture and handling protocol) to test the effect of handling time (i.e. stress response) on mitochondrial measurements.

All blood samples were kept on crushed ice (< 2 hours) prior to centrifugation at 3000*g* for 10 min to separate plasma from RBCs. The plasma fraction was then removed and 100 µL of RBCs was transferred into a new tube containing 1 mL of ice-cold phosphate buffer saline (PBS). White blood cells and thrombocytes are located on top of the blood cell pellet after centrifugation (Samour 2006). Therefore, RBCs were pipetted from the bottom part of the cell pellet in our experiments to limit contamination by other cell types. After gentle homogenisation, RBCs were washed a first time by centrifuging the samples at 600 *g* for 5 min to pellet the cells and discarding the supernatant. RBCs were then re-suspended in 1 mL of ice-cold PBS and stored at 4°C until being used for mitochondria measurements.

MITOCHONDRIAL MEASUREMENTS IN INTACT RBCs

We choose to work with intact RBCs, since our preliminary observations revealed difficulties to properly permeabilize avian RBCs. Immediately before the start of the mitochondrial measurements, samples were washed a second time as described above and re-suspended in 1mL of respiratory buffer MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L), pH 7.1). We then added 1 mL of RBC suspension to 1mL of MiR05 buffer already equilibrated at 38°C in the respirometry chamber of one Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). This system allows measuring small changes in O_2 concentration in a closed chamber, and thereby provides a good opportunity to measure mitochondrial respiration using minimum amount of biological samples.

We applied a protocol involving serial additions of various mitochondrial agonists/antagonists to our RBC suspension in order to get a comprehensive assessment of mitochondrial function, as illustrated in Fig. 2A, and in the corresponding stepwise description below:

1. Baseline O_2 consumption is recorded after approximately 5 min of stabilization following the addition of the sample to the chamber ($R_{baseline}$).
2. ATP-dependent O_2 consumption is inhibited by adding oligomycin (1 µg.mL^{-1}), an inhibitor of ATP synthase ($R_{oligomycin}$). The residual oxygen consumption at this stage is mostly linked to mitochondrial proton leak.

3. Maximal uncoupled O_2 consumption is then obtained by the addition of the mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone) at a final concentration of 1 µM (R_{FCCP}). At this concentration, FCCP abolishes the proton gradient, thereby forcing the OXPHOS system to work at its maximum capacity to compensate for proton leakage. The maximal uncoupled respiration is limited by the capacity of the electron transport system (ETS) to oxidize the available substrate. Because FCCP can inhibit mitochondrial respiration above a certain threshold, we performed a preliminary stepwise titration study to determine the optimal concentration of FCCP that leads to maximum mitochondrial O_2 consumption.
4. Mitochondrial O_2 consumption is then abolished by adding antimycin A (5 µM), an inhibitor of mitochondrial complex III ($R_{antimycinA}$). The residual oxygen consumption after antimycin A inhibition reflects non-mitochondrial oxygen consumption.

We determined mitochondrial O_2 consumption by subtracting residual non-mitochondrial O_2 consumption ($R_{antimycinA}$) from O_2 consumption measured in response to the other conditions. We computed four measures of mitochondrial respiration and three different flux control ratios (FCR) to evaluate the degree of mitochondrial coupling between O_2 consumption and ATP synthesis, but also the proportion of mitochondrial capacity being used under endogenous conditions (Gnaiger 2009). The seven measures of mitochondrial function derived from our protocol are described in Table 1.

NORMALIZATION OF MITOCHONDRIAL RESPIRATION

Pipetting an exact volume of RBCs (*i.e.* 100 µL) might be challenging considering the viscosity of the cell pellet after centrifugation. Consequently, the volume of cells might not be as accurate as desired and biased our estimates of mitochondrial parameters. Hence, we tested two methods of *post-measurement* normalization using 18 samples collected in courtship birds. We either weighed the amount of RBCs pipetted before the start of the analyses using a high-precision electronic balance (± 0.1mg, Sartorius AC211S®), or we quantified the total protein content in the remaining RBCs samples at the end of the analyses using the Pierce BCA protein assay (ThermoScientific). We calculated standardised respiration rates by dividing respiration rates either by the fresh mass of RBCs or by their protein content.

REPEATABILITY OF RBC MITOCHONDRIAL MEASUREMENTS

We evaluated the repeatability of our mitochondrial measurements by assaying 15 samples in duplicate, coming from both courtship and incubating birds. We evaluated the repeatability both on raw data and on data normalized by the fresh mass of RBCs.

EFFECTS OF STORAGE TIME

To evaluate the effect of storage time on mitochondrial measurements, we used two approaches. First, we measured 8 samples twice, a first time after 4h of storage at 4°C and a second time after 24h of storage. Second, we used single measurements collected from the 75 incubating birds, in which the time elapsed

between blood sampling and mitochondrial measurements varied between 2 and 10 hours. Samples were always stored at 4°C in 1mL of PBS in closed 1.5 mL eppendorf tubes without agitation.

MITOCHONDRIAL MEASUREMENTS IN SKELETAL MUSCLE

Mitochondrial respiratory function of pectoral muscle was determined in permeabilized muscle fibers using a method described previously by Pesta & Gnaiger (2011). Structurally sound fiber bundles were selected from biopsies maintained in ice-cold BIOPS, and mechanically separated, removing any visible adipose and connective tissue. Fiber bundles were transferred in BIOPS solution containing saponin (50µg/ml) for permeabilization and mixed gently at 4°C for 30 min. Then, permeabilized fibers were washed 10 min at 4°C in the Mir05 buffer. Permeabilized fibers were carefully blotted on Whatman filter paper for 2-3s, weighed and placed in the Oxygraph chamber containing 2mL of MiR05 at 38°C. Respiration was fuelled using either pyruvate/malate (5/2.5mM) or succinate (5mM) as respiratory substrates, and *LEAK* respiration was recorded in the presence of substrate but absence of ADP. Phosphorylating state of respiration (*i.e.* classical state III) was determined in the presence of ADP (1 mM), and we define here this state as '*ROUTINE*' to facilitate comparison with intact RBCs since it encompasses respiration linked both to ATP production and mitochondrial proton leak. The respiration linked to ATP synthesis (*OXPHOS*) was calculated as the difference between *LEAK* and '*ROUTINE*' respiration, as done for RBCs. Then, cytochrome-c (10 µM) was added in order to check the integrity of mitochondria within permeabilized fibers by the absence of stimulation of respiration. Mitochondrial preparations exhibiting an

increase in O_2 uptake greater than 15% in response to cytochrome-c were excluded from subsequent analysis (Kuznetsov *et al.* 2008). Thereafter, the maximal capacity of the electron transport system (*ETS*) was measured by sequential addition of 1µM of FCCP. Finally, antimycin A (20 µM) was added to allow the measurement of non-mitochondrial oxygen consumption rate. To determine mitochondrial O_2 consumption, we subtracted residual O_2 consumption measured after antimycin A inhibition, from O_2 consumption measured in response to the other conditions. Mitochondrial respiration rates of permeabilized fibers were expressed as pmol $O_2.s^{-1}.mg^{-1}$ wet weight.

STATISTICS

We used Generalized Estimating Equations (GEE) with *bird identity* as individual factor and *state* as the repeated effect to evaluate 1) differences in O_2 consumption in response to the different experimental conditions (i.e. baseline, oligomycin, FCCP and antimycin A) and 2) differences between RBC mitochondrial parameters (i.e. *ROUTINE*, *OXPHOS, LEAK and ETS*). Paired-comparisons involving two groups (*i.e.* effects of storage and sampling times) were performed using non-parametric exact Wilcoxon paired-tests considering the relatively small sample sizes ($N \leq 23$). To evaluate the relevance of normalizing RBC mitochondrial respiration, we ran multivariate analyses (MANOVAs) with either fresh RBC mass or total protein content as explanatory factors of mitochondrial O_2 consumption rates. To evaluate the repeatability of mitochondrial parameters, we calculated the intraclass coefficients of correlations (ICC), but also the coefficients of variation (CV) expressed in % for the 14 samples assessed in duplicates. Correlation tests were performed

using either non-parametric Spearman correlations (for N ≤ 18) or parametric Pearson correlations (for N ≥ 40). Means are always quoted ± SE and p-values ≤ 0.05 were considered as significant.

ETHICAL STATEMENT

The present study used samples collected as part of two on-going scientific programs of the French Polar Institute (IPEV 119 ECONERGY and IPEV 131 PHYSIONERGY). All experiments were approved by an independent ethics committee (Comité d'éthique Midi-Pyrénées pour l'expérimentation animale) commissioned by the French Polar Institute, and comply with the current laws of France. Authorizations to enter the breeding colony and handle the birds were provided by the "Terres Australes and Antarctiques Françaises" (permit n°2013-72 issued on 29 October 2013).

Results

OXYGEN CONSUMPTION IN RESPONSE TO MITOCHONDRIAL AGONISTS AND ANTAGONISTS

Using the 18 birds caught in courtship, we found a significant effect of mitochondrial agonists/antagonists on O_2 consumption (Fig 2A & 2B; GEE model: χ^2 = 606.6, $p < 0.001$). Post-hoc comparisons revealed that inhibition of ATP synthase significantly decreased O_2 consumption by 61.8 ± 1.4 % ($p < 0.001$), while mitochondrial uncoupling with FCCP significantly increased O_2 consumption by 18.5 ± 5.8 % compared to baseline ($p = 0.001$). Inhibition of mitochondrial respiration with antimycin A decreased O_2 consumption by 85.7 ± 1.1 % compared to baseline ($p < 0.001$).

This protocol allowed to compute seven measures of mitochondrial function (see Table 1) derived from the changes in mitochondrial respiration rates in response to mitochondrial agonists/antagonists (Fig 2C).

NORMALIZATION OF RBC MITOCHONDRIAL RESPIRATION RATES

Multivariate analyses revealed that mitochondrial respiration rates were significantly influenced by the fresh mass of cells used (MANOVA: $F_{3,14} = 11.06$, $p = 0.001$, effect size (partial η^2) = 0.70), or to a slightly lesser extent by the total protein content of RBC samples (MANOVA: $F_{3,14} = 5.7$, $p = 0.002$, effect size (partial η^2) = 0.64).

REPEATABILITY OF RBC MITOCHONDRIAL MEASUREMENTS

Mitochondrial parameters were overall significantly repeatable as shown in Table 2 (all ICC > 0.87). Normalizing mitochondrial parameters by the fresh mass of RBCs increased the repeatability of mitochondrial measurements in all cases (all ICC > 0.91).

EFFECT OF STORAGE TIME ON RBC MITOCHONDRIAL PARAMETERS

The comparison of mitochondrial parameters measured 4h after sampling or 24h after sampling did not reveal significant differences (Wilcoxon paired exact tests, all p > 0.14, Fig 3), except for a slight increase with time in $FCR_{R/ETS}$ (p = 0.039, Fig 3B). In addition, we found overall no significant correlations between storage time and mitochondrial parameters using the 75 samples collected from incubating birds

(Pearson correlations, all p > 0.21), except for a weak but significant negative correlation with $FCR_{L/ETS}$ (r = 0.23, p = 0.049).

EFFECT OF HANDLING TIME ON RBC MITOCHONDRIAL PARAMETERS

We found overall no significant impact of handling time on mitochondrial parameters (Wilcoxon paired exact tests, all p > 0.21, Fig 4).

RELATIONSHIPS BETWEEN MITOCHONDRIAL PARAMETERS IN RBCs AND PECTORAL MUSCLE

Table 3 reports the coefficients of correlation between RBC mitochondrial respiration values and muscle mitochondrial respiration values measured using two different respiration substrates, pyruvate-malate or succinate. When using pyruvate-malate as substrate for the muscle, we found significant positive relationships between RBC and muscle for *ROUTINE* and *OXPHOS* values (Fig. 5A & B). When using succinate as substrate, we found marginally significant positive relationships for *OXPHOS* and *LEAK* values (Fig. 5C & D) and significant positive relationships between RBC and muscle for *ETS* and $FCR_{L/ETS}$ values (Fig. 5E & F). Other relationships were not significant (Table 3).

Discussion

In this study, we present and validate the use of a novel approach where intact RBCs are used to measure mitochondrial function in a minimally invasive manner. We illustrate this approach in birds, but such methodology could likely be adapted to other non-mammalian vertebrates (i.e. fish, amphibians and reptiles) since they also have functional mitochondria in their RBCs (Stier *et al.* 2015). Despite

the fact that mammalian RBCs are lacking mitochondria, blood sampling might nonetheless also be used in this taxon to measure mitochondrial function, though using other blood cell types (e.g. platelets or white blood cells), as recently demonstrated in humans (Sjövall *et al.* 2013; Pecina *et al.* 2014). However, larger blood volume and isolation of specific blood cells will be required in mammals to have access to sufficient amount of mitochondria. The use of blood samples to assess mitochondrial function should open new opportunities to study mitochondrial function in free-living vertebrates and address fundamental roles of mitochondria in ecology and evolution (Dowling *et al.* 2008; Ballard & Pichaud 2013; Hill 2014; 2015; Salin *et al.* 2015; Koch *et al.* 2016). This method should also facilitate future studies looking at the functional importance of mitochondria in the RBCs of non-mammalian vertebrates. It may in turn also bring new and important insight in the evolution of mammalian erythrocytes that lack both nucleus and mitochondria.

CHARACTERIZING MITOCHONDRIAL FUNCTION IN INTACT RBCs

Mitochondria from intact RBCs of king penguins exhibited the expected responses to classical mitochondrial agonists and antagonists. It confirms previous finding in zebra finches about the presence of functional mitochondria in bird RBCs (Stier *et al.* 2013). We show how mitochondrial drugs can be used to extract 7 parameters of interests reflecting various aspects of mitochondrial function (Table 1). *ROUTINE* respiration reflects the natural activity of mitochondria under the current physiological and cellular state (*i.e.* substrate and ADP availability, ATP turnover, proton leak) and is an intermediary state between the classical state III (unlimited availability of substrate and ADP) and state IV (unlimited availability of

substrate but zero ATP synthesis) classically measured in isolated mitochondria or permeabilized tissues/cells (Brand & Nicholls 2011). We decomposed RBCs' *ROUTINE* mitochondrial respiration into two components, the ATP-dependent respiration (*OXPHOS)* and the leak respiration (*LEAK)*. The first one reflects the ability of mitochondria to produce ATP via OXPHOS and the second one reflects the proton leakiness of the mitochondria. Both components have different but equally important biological implications. ATP is important to fulfil most cellular activities. Therefore, variation in *OXPHOS* respiration may account for variation in cell replication and growth, and by extension for variation in organismal growth, maturation and reproduction. On the other hand, proton leak is known to be important to produce heat and/or regulate ROS production (Brand 2000). Thus, variation in *LEAK* respiration may account for variation in cell (and organismal) maintenance and lifespan. The values we observe here for RBCs (72.7% of mitochondrial respiration linked to ATP synthesis *vs.* 27.3% linked to proton leak) are close to those found in various cell types of mammals and birds, since in those cells approximately 60-80% of the mitochondrial respiration is used to synthesize ATP, and 20-40% is linked to the proton leak (Porter & Brand 1995; Else *et al.* 2004; Jimenez *et al.* 2014). *ETS* represents the maximal mitochondrial activity under current physiological conditions; this respiration rate can be constrained by substrate availability and oxidation but is independent of ATP turnover. Importantly, the optimum FCCP concentration is likely to vary across species and conditions, and thus it should be determined on a case-by-case basis. Some caution is also needed when interpreting *ETS* respiration since the use of uncoupling agents in intact cells

411 can have deleterious side effects, such as an intra-cellular acidification (see Brand &
412 Nicholls 2011 for details).

413 Cell flux control ratios (FCRs) are often viewed as the most useful general
414 tests of mitochondrial function in intact cells (Gnaiger 2009; Brand & Nicholls 2011).
415 Notably, since FCRs are ratios, they are internally normalized, which facilitates their
416 interpretation (see below for a discussion on normalization). $FCR_{L/R}$ provides
417 information on the coupling efficiency of the mitochondria between O_2 consumption
418 and ATP production under the current physiological state, while $FCR_{L/ETS}$ provides
419 information on coupling state under stimulated conditions. $FCR_{R/ETS}$ provides
420 information about the proportion of the capacity of the electron transport system
421 being used under current physiological conditions. FCR values are usually
422 characteristic of a specific method in a specific tissue and in a specific group of
423 organisms. Consequently, there are currently no standard FCR values in RBCs that
424 can be used for comparison. Still, $FCR_{L/R}$ in king penguin RBCs are close to those
425 found in cultured myoblasts/fibroblasts of Japanese quails ($FCR_{L/R}$ ≈ 0.25 to 0.42;
426 Jimenez *et al.* 2014).

427 One important consideration when working with RBCs is that they contain a
428 high amount of intra-cellular O_2 bound to haemoglobin. Thus, haemoglobin may act
429 as a reservoir that releases O_2 in the medium in response to a decrease in extra-
430 cellular O_2 caused by mitochondrial respiration, which would lead to an
431 underestimation of the different measures of mitochondrial respiration. If true, this
432 release of O_2 by haemoglobin should become visible (and measurable) when the
433 mitochondrial respiration is blocked; that is after the addition of antimycin A. We
434 never detected a release of O_2 by king penguin RBCs after antimycin A inhibition (see

Fig 2A; minimum $R_{antimycinA}$ = 2.0 pmol.s^{-1}.mL^{-1}, N = 93). Comparable findings were found in zebra finch (*Taenopygia guttata*) and Japanese quail (*Coturnix japonica*) (Stier, unpublished results), suggesting that disruption of measures due to haemoglobin is unlikely to be widespread. Still, studies must check for this potentially important source of error when applying the proposed methodology in a new species.

ROBUSTNESS OF RBC MITOCHONDRIAL MEASURES

Our results indicate that our different measures of mitochondrial function were significantly repeatable, that RBCs could be stored at 4°C up to 24h without major effects on measures of mitochondrial function (with the exception of minor but significant effects on $FCR_{L/ETS}$ and $FCR_{R/ETS}$), and that the handling time of birds before blood sampling did not alter measures of mitochondrial function. Those findings are in agreement with a recent study in human platelets pointing out that mitochondrial function in those blood cells is relatively well conserved at 4°C up to 48h after blood collection (Sjövall *et al.* 2013). The possibility of conserving samples at 4°C for several hours before measurement would undoubtedly be a real asset for field studies since field sites and laboratory facilities are often not located at the same place. The absence of effect of handling time on mitochondrial parameters is another asset for field studies, since capturing and blood sampling wild animals under controlled time condition can be very laborious. It is nonetheless important that future studies test for repeatability and investigate the importance of storage time and handling time on measures of mitochondrial parameters before generalities on the robustness of RBC measures can be drawn.

459

460 CORRELATIONS BETWEEN MITOCHONDRIAL PARAMETERS IN RBCS AND PECTORAL
461 MUSCLES

462 Although demonstrating the presence of functional mitochondria in RBCs is
463 an important first step, the next logical question is whether measures of
464 mitochondrial function in RBCs do reflect to some extent what is happening in other
465 tissues of the same individual, and as such can provide some general information at
466 the scale of the organism. Although we were unable to compare RBCs and pectoral
467 muscle in the exact same experimental conditions (*i.e.* permeabilized RBCs vs.
468 permeabilized muscle; our attempts to permeabilize RBCs were not successful), we
469 showed that mitochondrial parameters measured in intact RBCs moderately
470 correlated to those measured in permeabilized pectoral muscle fibers (see Table 3
471 for details). Interestingly, similar findings have been found in humans between blood
472 mononuclear cells and organs such as kidney and heart (Karamercan *et al.* 2013).
473 Altogether, it indicates that mitochondrial parameters are to some extent correlated
474 among tissues, including blood. However, this is unlikely to be true for every
475 experimental condition and study species, as exemplified by the lack of significant
476 correlation reported in brown trout between liver and muscle mitochondrial
477 respiration rates (Salin et al. 2016b). This opens new opportunities to ecologists and
478 evolutionary biologists eager to investigate links between mitochondrial function
479 and organismal performance using minimally invasive sampling techniques (i.e.
480 blood sampling). Having access to such minimally-invasive methodology is a pre-
481 requisite when it comes to make links with fitness traits such as reproductive success
482 and survival, but also when working with protected species.

FURTHER IMPROVEMENTS IN CHARACTERIZING MITOCHONDRIAL FUNCTION USING RBCS

We see at least three methodological improvements to be addressed in future studies. First, while working with intact cells has several advantages (e.g. working in an undisturbed cellular environment and lack of artefacts due to mitochondrial preparation; Brand & Nicholls 2011), it may be beneficial for some studies to better control the environment in which mitochondrial function is measured (i.e. substrate and ADP availability). This could be achieved either by isolating mitochondria or by being able to permeabilize RBCs properly and artificially providing substrates and ADP (Brand & Nicholls 2011). Such methodological development will undoubtedly broaden the scope of questions that could be answered using mitochondria coming from non-mammalian RBCs.

Second, normalizing mitochondrial respiration is not an easy task, and has several implications for data interpretation (Brand & Nicholls 2011). We have shown that normalizing measurement by the fresh mass of cells used or by their protein content improve the repeatability of the measurement. However, it would also be possible to normalize mitochondrial respiration by the number of cells or by the mitochondrial content of these cells.

Finally, as stated in the introduction, mitochondrial function is not only reflected in terms of O_2 consumed and ATP produced, but also in terms of ROS produced. Assessing ROS production is challenging, but has already being done using fluorescent probes in non-mammalian vertebrate RBCs (e.g. Olsson *et al.* 2008; Stier *et al.* 2014a; Delhaye *et al.* 2016). Interestingly, it is now possible to simultaneously

record O_2 consumption and fluorescence signal using the O2k-fluorescence module (Oroboros Instruments, Innsbruck, Austria) that could be added to the O2k-Oroboros device that we used in this study. Other fluorescent probes may also help to collect additional information on mitochondrial function, such as mitochondrial membrane potential, ATP synthesis (Salin et al. 2016a), or calcium flux, and in turn help to broaden the scope of questions that can be addressed in ecology and evolution.

PERSPECTIVES IN ECOLOGY & EVOLUTION

The applications of our methodology in ecology and evolution are likely to be broad in terms of scientific questions that could be addressed. Indeed, subtle variations at the cellular level in mitochondrial function are likely to have profound consequences at the organismal level (Salin *et al.* 2015), and we believe that the links between mitochondrial function and organismal phenotype deserves now more attention than ever. Hereafter, we highlight four promising avenues where measures of mitochondrial function in RBCs could help to gain knowledge.

First of all, whole organism metabolic rate, which is the result of oxygen consumption by mitochondria at the cellular level, has been a trait under great scrutiny in ecology and evolution in the last decades (Brown *et al.* 2004). However, metabolic rate is in the vast majority of cases measured in terms of O_2 consumption, while the true energetic currency is ATP, and the relationships between O_2 consumption and ATP production are not constant (Brand 2005; Salin *et al.* 2015). Since the fractions of O_2 consumption used for ATP synthesis and mitochondrial proton leak have very different biological implications, gaining insight about mitochondrial function at the cellular level should further improve our

understanding of metabolic rate acting as a factor driving ecological and evolutionary processes.

Secondly, mitochondrial function requires a close collaboration between the nuclear and the mitochondrial genomes (*i.e.* named mito-nuclear interactions) since more than 90% of the proteins required for mitochondrial function are encoded in the nucleus and imported into the mitochondria (Wolff *et al.* 2014). While the mitochondrial genome was thought to be an evolutionary bystander for a long time, we have now evidence arguing for the existence of evolutionary adaptations at the mtDNA level (e.g. Pavlova *et al.* 2013; Ballard & Pichaud 2013). Such phenomena might also give rise to mito-nuclear incompatibilities between individuals/populations (i.e. decreased fitness of hybrids), and such incompatibilities are believed to be one potential driver of reproductive isolation and speciation (Bar-Yaacov *et al.* 2015). Characterizing mitochondrial function of mtDNA variants appears essential to evaluate their adaptive value, and characterizing mitochondrial function of potential mito-nuclear hybrids appears essential to shed light on the mechanisms underlying mito-nuclear incompatibilities. However, characterizing mitochondrial function in these two contexts has rarely been done to date (but see Toews *et al.* 2013).

Thirdly, mitochondria are inherited only from the mother, even if inheritance patterns could be slightly more complex in some cases (White *et al.* 2008). This gives rise to evolutionary constraints for males, since mitochondrial mutations benefitting females could spread even if they harm males, a phenomenon known as the "mother's curse" (Gemmell *et al.* 2004). In vertebrate species, we often lack

554 information about differences in mitochondrial function arising from such
555 constraints, and even more surprisingly, we have little information to date about the
556 inheritance and heritability patterns of mitochondrial function *per se*.

557 Finally, mitochondrial function undoubtedly contributes to animal
558 performance and fitness, probably in an environment-dependent manner (Stier *et al.*
559 2014a; Stier et al. 2014b; Salin *et al.* 2015; Conley 2016). Indeed, mitochondrial
560 function will condition the amount of nutrients and O_2 used, as well as the amount
561 of ATP and ROS produced. Decreasing mitochondrial efficiency to produce ATP might
562 seem counter-productive at a first glance. However, such mitochondrial
563 "uncoupling" between O_2 and ATP production could be useful for endotherms to
564 produce heat such as in the brown fat of mammals, but also to slow-down ageing by
565 reducing ROS production (Brand 2000). In contrast, increasing mitochondrial
566 efficiency might be beneficial when resources are limited or to optimize physical
567 performances (Monternier *et al.* 2014; Conley 2016), while it might incur some costs
568 in terms of ROS production.

569

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577

578 **Author's contribution**

579 A.S. designed the study, did the fieldwork, conducted laboratory analyses on RBCs,
580 analyzed the data and wrote the paper. P.B. provided guidance on data analysis, and
581 wrote the paper. D.R. provided guidance on the experiments and helped to draft the
582 manuscript. Q.S., E.L. and JP.R. contributed to the realization of the project, the
583 collection of samples in the field, and commented on the manuscript. C.R. provided
584 invaluable technical advice on mitochondrial measurements, and conducted
585 laboratory analyses on muscle samples.

586

587 **Data accessibility**

588 Data will be loaded on Dryad after the manuscript has been accepted.

589

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751 **Table 1.** Calculation and meaning of mitochondrial parameters measured in intact
752 RBCs. Mitochondrial parameters are derived from changes in O_2 consumption in
753 response to specific mitochondrial agonists/antagonists (i.e. respiration rates
754 $R_{agonists/antagonists}$, see also Fig. 2A).

Parameter	Calculation	Information
ROUTINE	$R_{baseline}$ - $R_{antimycinA}$	Mitochondrial O_2 consumption under endogenous cellular conditions
OXPHOS	$R_{baseline}$ - $R_{oligomycin}$	O_2 consumption used for ATP synthesis: ability of mitochondria to produce ATP via oxidative phosphorylation (OXPHOS)
LEAK	$R_{oligomycin}$ - $R_{antimycinA}$	O_2 consumed by mitochondrial proton leak: proton leakiness of the mitochondria
ETS	R_{FCCP} - $R_{antimycinA}$	Maximum capacity of the electron transport system (ETS) under the current cellular state (i.e. availability/oxidation of substrates)
$FCR_{L/R}$	*LEAK* ÷ *ROUTINE*	Fraction of *ROUTINE* respiration being linked to mitochondrial proton *LEAK*: coupling efficiency of the mitochondria between O_2 consumption and ATP production under endogenous cellular conditions
$FCR_{L/ETS}$	*LEAK* ÷ *ETS*	Fraction of *ETS* maximum capacity being linked to mitochondrial proton *LEAK*: coupling efficiency of the mitochondria between O_2 consumption and ATP production under stimulated conditions
$FCR_{R/ETS}$	*ROUTINE* ÷ *ETS*	Fraction of *ETS* maximum capacity used by the cell under endogenous cellular conditions

Table 2. Repeatability of measures of mitochondrial function based on 14 samples ran in duplicates. Two indicators of repeatability are shown: the intra-class coefficients of correlations (ICC) and the coefficient of variation (CV). *P*-values associated with ICC are given between brackets. Repeatability estimates are reported both for uncorrected parameters and for parameters corrected by the fresh mass of red blood cells, except for FCRs since they are ratios (*NA* = not attributed).

Parameter	Raw value ICC	CV ± SE (%)	Mass corrected ICC	CV ± SE (%)
ROUTINE	***0.884*** (< 0.001)	6.6 ± 1.1	***0.934*** (< 0.001)	6.2 ±1.0
OXPHOS	***0.915*** (< 0.001)	6.3 ±1.1	***0.947*** (< 0.001)	6.3 ± 1.2
LEAK	***0.873*** (< 0.001)	10.4 ± 1.9	***0.912*** (< 0.001)	9.3 ± 1.5
ETS	***0.876*** (< 0.001)	9.2 ± 2.1	***0.930*** (< 0.001)	7.4 ± 1.3
$FCR_{L/R}$	***0.924*** (< 0.001)	5.8 ± 1.2	*NA*	*NA*
$FCR_{L/ETS}$	***0.915*** (< 0.001)	7.6 ± 1.6	*NA*	*NA*
$FCR_{R/ETS}$	***0.943*** (< 0.001)	6.2 ± 1.4	*NA*	*NA*

Table 3. Correlations between mitochondrial parameters measured in intact RBCs and in permeabilized pectoral muscle fibers fuelled either with pyruvate-malate (complex I substrate) or succinate (complex II substrate). Spearman non-parametric coefficients of correlation (ρ) are reported along with their associated p-values (N = 13 for pyruvate-malate and N = 14 for succinate). Significant ($p \leq 0.05$) and marginal effects ($p \leq 0.10$) are shown in bold and plotted in figure 5.

RBCs \ Muscle	Pyruvate-Malate	Succinate
ROUTINE	***ρ = 0.709* (p = 0.007)**	*ρ = 0.248* (p = 0.392)
OXPHOS	***ρ = 0.615* (p = 0.025)**	***ρ = 0.503* (p = 0.067)**
LEAK	*ρ = 0.341* (p = 0.255)	***ρ = 0.512* (p = 0.061)**
ETS	*ρ = 0.071* (p = 0.817)	***ρ = 0.727* (p = 0.003)**
$FCR_{L/R}$	*ρ = 0.390* (p = 0.188)	*ρ = 0.231* (p = 0.427)
$FCR_{L/ETS}$	*ρ = -0.258* (p = 0.394)	***ρ = 0.789* (p = 0.001)**
$FCR_{R/ETS}$	*ρ = 0.148* (p = 0.629)	*ρ = 0.103* (p = 0.725)

Fig. 1. The mitochondrion and OXPHOS system.

Fig. 2. Bioenergetics assessment of intact red blood cells: **(A) typical mitochondrial measurement run** showing O_2 concentration (blue line) and O_2 consumption (red line) in response to the injection of oligomycin (inhibitor of ATP synthase), FCCP (protonophore stimulating mitochondrial respiration by abolishing proton gradient) and Antimycin A (inhibitor of mitochondrial respiration). Mitochondrial parameters of interest: *ROUTINE, LEAK, OXPHOS* and *ETS* are also shown (see Table 1 for definitions). **(B) average responses to the mitochondrial agonists/antagonists** in terms of O_2 consumption (N = 18). **(C) mitochondrial parameters of interest** (N = 18, see Table 1 for definitions). Means are quoted ± SE and different letters indicate significant differences according to GEE models and associated post-hoc tests.

Fig 3. Mitochondrial parameters of the same samples measured 4 or 24h after collection: (A) mitochondrial respiration rates, and (B) mitochondrial flux control ratios. Means are quoted ± SE (N = 8) and non-significant effects (*ns*) and significant effects (*) are indicated; see Table 1 for the calculation and definition of the different parameters.

Fig. 4: Mitochondrial parameters of the same individuals sampled once before 4 minutes of handling, and once after 30 minutes of standardized handling: (A) mitochondrial respiration rates and (B) mitochondrial flux control ratios. Means are quoted ± SE (N = 23) and non-significant effects (*ns*) are indicated; see Table 1 for the calculation and definition of the different parameters.

790 **Fig 5. Correlations between mitochondrial parameters measured in intact RBCs and in**
791 **permeabilized pectoral muscle fibres** fuelled either with pyruvate-malate (complex I
792 substrate, N = 13) or succinate (complex II substrate, N = 14). Significant correlations ($p < 0.05$) are indicated by a solid line and marginally significant correlations ($p < 0.10$) by a
793 dashed line; see Table 1 for the calculation and definition of the different parameters and
794 Table 3 for statistical values.
795
796

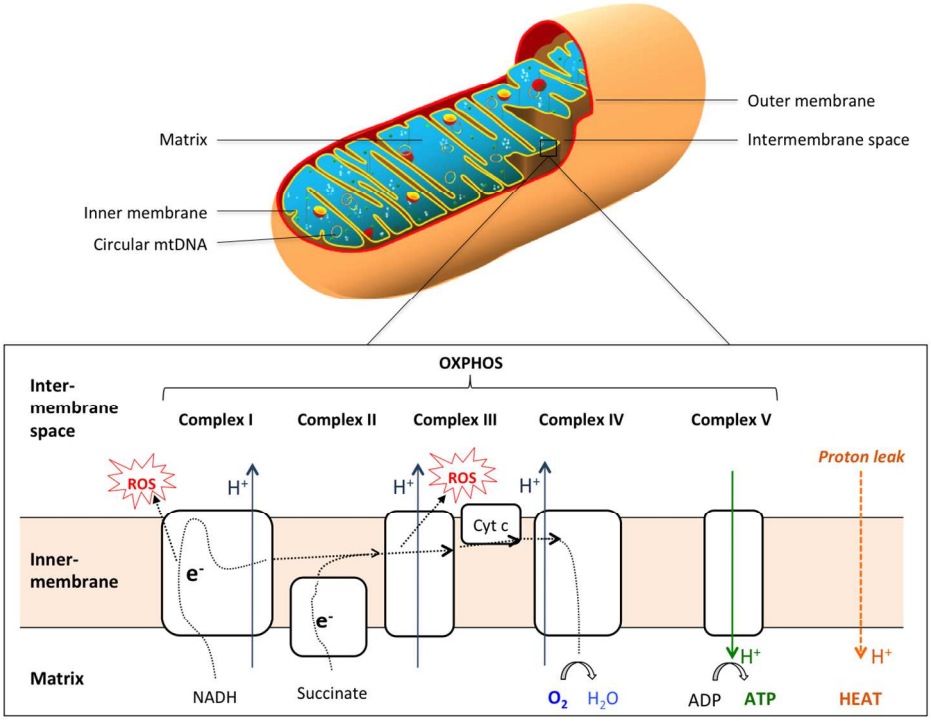

Fig. 1. The mitochondrion and OXPHOS system.
Fig. 1

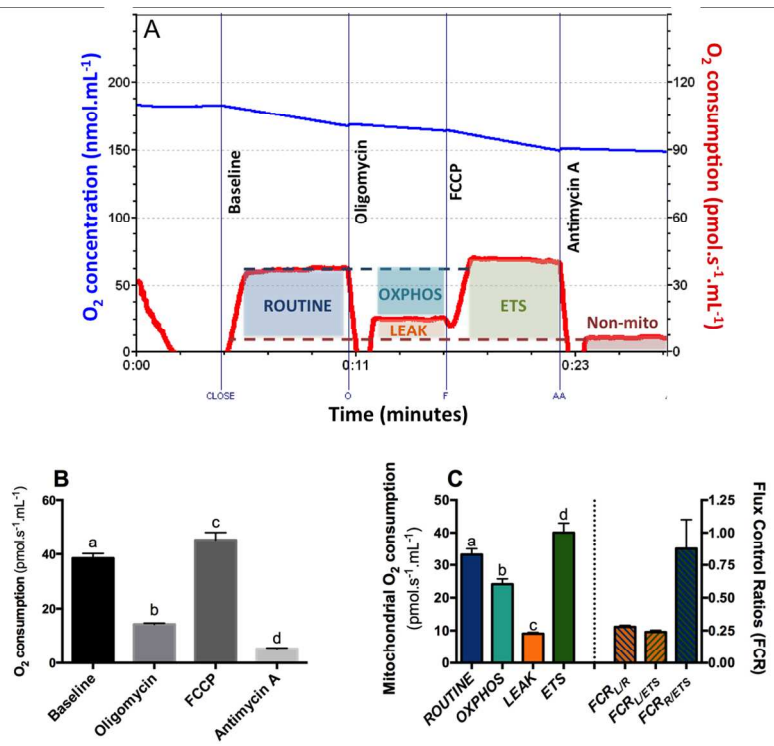

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Fig 2

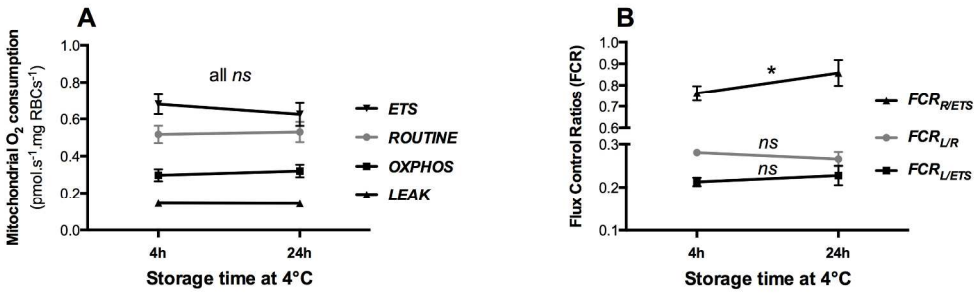

Fig 3. Mitochondrial parameters of the same samples measured 4 or 24h after collection: (A) mitochondrial respiration rates, and (B) mitochondrial flux control ratios. Means are quoted ± SE (N = 8) and non-significant effects (ns) and significant effects (*) are indicated; see Table 1 for the calculation and definition of the different parameters.

Fig 3
228x71mm (300 x 300 DPI)

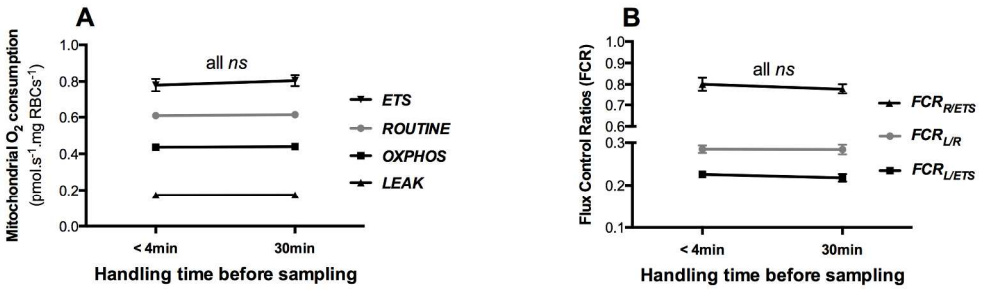

Fig. 4: Mitochondrial parameters of the same individuals sampled once before 4 minutes of handling, and once after 30 minutes of standardized handling: (A) mitochondrial respiration rates and (B) mitochondrial flux control ratios. Means are quoted ± SE (N = 23) and non-significant effects (ns) are indicated; see Table 1 for the calculation and definition of the different parameters.

Fig 4
232x72mm (300 x 300 DPI)

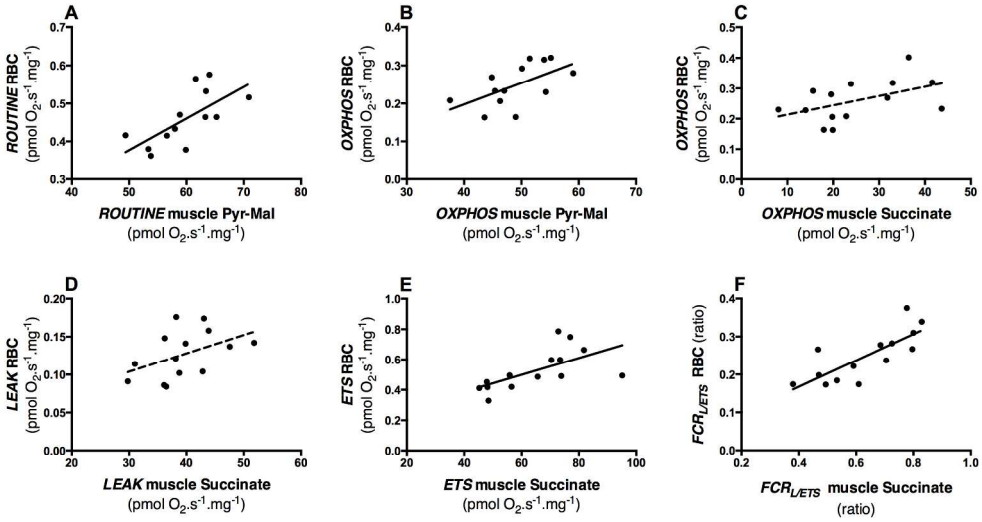

Fig 5. Correlations between mitochondrial parameters measured in intact RBCs and in permeabilized pectoral muscle fibres fuelled either with pyruvate-malate (complex I substrate, N = 13) or succinate (complex II substrate, N = 14). Significant correlations ($p < 0.05$) are indicated by a solid line and marginally significant correlations ($p < 0.10$) by a dashed line; see Table 1 for the calculation and definition of the different parameters and Table 3 for statistical values.

Fig 5
241x135mm (300 x 300 DPI)