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Sexual dimorphism in red blood cell mitochondrial respiration during breeding fasts in king penguins

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Abstract

Because of their extended fasting period on land during breeding male king penguins have been extensively studied to unravel the physiological adaptations that enables them to fast while having to find a partner, defend their territory, or brood their offspring. While the different phases of fasting and the nature of the metabolic fuels used are well characterised in male king penguins, fewer studies have focused on the efficiency of the conversion of the metabolic resources into energy at a cellular-level, through mitochondrial respiration.

Furthermore, little information is available in females in general while they experience fasting periods as well. Here, we measured mitochondrial respiration rates of red blood cells (RBCs) at the beginning (3 days) and at the end (10 days) of a natural egg-incubation fast in male and female king penguins. We tested if (1) RBC mitochondrial metabolism and its efficiency are modulated by fasting duration in free-living king penguins, but also (2) assess if this modulation is sex-specific. In response to fasting, the respiration allocated to ATP synthesis in RBCs decreased in both sexes. Interestingly, RBC mitochondrial metabolic rates were higher in females at any stage of fasting. Furthermore, RBC mitochondrial metabolism efficiency decreased in males after 10 days of fasting, while it remained constant in females. Our results demonstrate that RBC mitochondrial metabolism is context and state-dependent, differing between sexes and changing with fasting. They underline the importance of taking both sexes into account in physiological studies, where females remain under-represented.

Introduction

In nature, birds face fasting in different periods of their life. Fasting periods during breeding (i.e. breeding fasts), are of particular interest in eco-evolutionary biology, as fasting success influences not only the condition and survival of individuals, but also the survival of their offspring (Ancel et al., 1998; Ankney & MacInnes, 1978; Bertile et al., 2016; Groscolas et al., 2008; Le Maho, 1983; Olsson, 1997). Fasting physiology during breeding has been studied especially in penguins since they face particularly long breeding fast periods (Cherel et al., 1988, 1988a; Groscolas & Robin, 2001; Le Maho, 1983; Robin et al., 1988). For king penguins (*Aptenodytes patagonicus*) the longest breeding fast occurs during courtship and incubation, where males can fast up to 5 weeks (Fig.1). The female then relieves its male partner, and both sexes alternate between fasting periods on land and foraging periods at sea (Stonehouse, 1960; Weimerskirch et al., 1992). Three different and successive phases (*Phase I, Phase II, Phase III*) have been described in fasting process (Bourgeon et al., 2010; Cherel et al., 1988, 1988a; Le Maho et al., 1981; Mrosovsky & Sherry, 1980; Robin et al., 1987; Robin et al., 1988; Secor & Carey, 2016). During the king penguin breeding season both sexes enter into *phase II* of fasting, which is characterized by low and stable mass-specific body mass loss (drop by *ca* 20% of their initial body mass) (Cherel et al., 1994a, 1988a; Groscolas & Robin, 2001; Schull et al., 2016). During this phase, metabolic rate is usually reduced (*ca* 33% compared to non-fasting or re-fed birds, Cherel et al., 1988; Fahlman et al., 2005; Rey et al., 2008) and lipids are used as the main metabolic substrate (Cherel et al., 1988; Secor & Carey, 2016).

The conversion of metabolic resources into energy (and its efficiency) occurs within mitochondria in cells, where oxidative phosphorylation is the main process to transform metabolic fuel into energy (i.e. ATP synthesis). Investigating mitochondrial metabolism is a powerful tool, as it allows gaining insights into the cellular responses used by the organisms to cope with changing conditions (Cossin-Sevrin, 2024; Koch et al., 2021; Stier et al., 2019a; Thoral et al., 2024b). With regard to fasting, exploring mitochondrial metabolism provides crucial information on their ability and efficiency to convert metabolic fuel into energy under different nutritional status (Bourguignon et al., 2017; Monternier et al., 2014; Rey et al., 2008). Indeed, avian red blood cells (RBCs) have functional mitochondria, therefore blood samples can be used to measure mitochondrial metabolism repeatedly and less-invasively, enabling longitudinal sampling (Koch et al., 2021; Stier et al., 2013). This approach has been validated in many avian species, including in the king penguin (Stier et al., 2017), and is becoming more prevalent in ecophysiological studies (Cossin-Sevrin, 2024; Koch et al., 2021; Thoral et al., 2024b). Although RBCs are not actively taking part in the fasting metabolism, RBC mitochondrial metabolism was found to correlate with measures of mitochondrial traits in other tissues (e.g. muscle in king penguins; Stier et al., 2017) as well with whole-animal metabolism (Casagrande et al., 2023; Koch et al., 2021; Thoral et al., 2024a).

In king penguins, the demands in oxygen carrying capacity radically change according to the individual live stages: from foraging behaviour, including deep diving (Handrich et al. 1997; Kooyman et al., 1992; Pütz et al., 1998), to fasting periods where the whole-organism metabolism declines (e.g. *Phase II*) (Cherel et al., 1988; Fahlman et al., 2005; Rey et al., 2008) which could mean that RBC mitochondrial metabolism might change as well. In chicks, pectoral muscle mitochondrial metabolism efficiency increases in response to fasting in comparison to re-fed chicks, but also with fasting duration (*phase II vs. phase III*) (Bourguignon et al., 2017; Monternier et al., 2014). There is, however, a current gap of knowledge regarding the modulation of RBC mitochondrial metabolism during prolonged fasting in adult breeders. The only available studies concern (i) short-term fasting (3 days), showing no differences in RBC mitochondrial metabolism between sexes (Stier et al., 2019a), and, (ii) a longer fasting period (20 days, *phase II*), showing in both sexes a decrease in the abundance of uncoupling protein in mitochondria extracted from pectoralis muscle, likely contributing to a reduction in metabolism observed in fasting birds (Rey et al., 2008).

Similarly, little focus has been given to the within-individual variation in mitochondrial respiration across breeding fast in adults, including changes in RBC mitochondrial metabolism during fasting. Moreover, most of the previous studies focusing on king penguin physiology (except Stier et al., 2019a, see above) have been conducted on males or do not separate sexes. Yet, the contribution to reproduction can differ between sexes (e.g. fasting duration) (Olsson, 1996; Stonehouse, 1960; Weimerskirch et al., 1992), which can lead to strong differences in physiological requirements and how both sexes manage their energy budget (Landen et al., 2023; Varlamov et al., 2015).

In this study, we measured RBC mitochondrial metabolism in both female and male king penguins during the incubation period, after 3 and 10 days of breeding fast (Fig.1). We tested (1) if RBC mitochondrial metabolism was modulated by fasting duration in free-living conditions, and (2) if this modulation was sex-specific. We also (3) investigated if the variation in RBC mitochondrial metabolism was predicted by the body condition at the beginning of fasting (i.e. initial body condition). We predicted a decrease in RBC mitochondrial metabolism in response to fasting (in both sexes), as prior experimental work has shown a reduction of the whole-animal metabolism during fasting (Cherel et al., 1988; Fahlman et al., 2004; Froget et al., 2001; Groscolas et al., 2010). We also predicted to observe sex-specific differences in RBC mitochondrial metabolism in king penguins, since each sex experiences different fasting duration and faces distinct energetic costs associated with reproduction (e.g. physiological costs of egg formation and laying) (Adams, 1992; Olsson, 1996; Stonehouse, 1960; Weimerskirch et al., 1992). Moreover, evidence in the literature (mostly on humans and laboratory-models) suggests that mitochondrial metabolism can vary between sexes; the females showing higher mitochondrial metabolism or using different metabolic substrates (Khalifa et al., 2017; Sultanova et al., 2020; Ventura-Clapier et al., 2017). We further expected individuals with a lower initial body condition and body mass to express a reduced RBC mitochondrial metabolism as the whole-organism oxygen consumption is positively associated with the organism's body mass and size (Burness et al., 1998; Cherel et al., 1988; Glazier, 2022; Naya et al., 2018).

Methods

Study area and ecology of king penguins

This study was conducted on Possession Island in the Crozet Archipelago (46°25′S; 51°52′E). Data were collected during the breeding season 2021-2022 (from November to March) in the king penguin colony “La Grande Manchotière, Baie du Marin”, which gathers around 20,000 breeding pairs (Barbraud et al., 2020). Breeding pairs were randomly selected in the border of the colony during courtship to minimize disturbance in the colony. Breeding pairs were marked with a non-permanent animal dye (Porcimark, Kruuse, Langeskov, Denmark). After marking, breeding pairs (n = 40) were monitored daily to record egg-laying and hatching dates (± 24h), but also the dates of parental exchange for incubation and brooding tasks. Immediately after laying, females depart to forage at sea, while males start incubating the egg by extending the fasting period that had already started during courtship (Fig.1: parental shift 1, male). Partners will then switch and the female will provide parental care, while the male will start his foraging trip (Fig.1: parental shift 2, female). Such parental alternations between foraging trips at sea and fasting periods in the colony will persist during the whole incubation (raw data average ± SD: 54.5 ± 1.2 days) and chick brooding (Stonehouse, 1960; Weimerskirch et al., 1992).

Females and males were visually sexed according to which partner assumed the first incubation shift, and according to morphometrics and vocalisations (Jouventin, 1982; Kriesell et al., 2018; Weimerskirch et al., 1992). We investigated variation in RBC mitochondrial aerobic metabolism around 3 days (mean ± SD [range]: 3.0 ± 0.3 [2-4] days) and 10 days after fasting (mean ± SD [range]: 9.4 ± 0.7 [8-11] days) in both sexes: females during shift 2 and males during shift 3 (Fig.1). The day when one partner was seen on the egg was counted as “day 1”. We used females during shift 2 and males during shift 3 to ensure that measures were done at a similar stage of fasting: during these shifts, both sexes came back from their first foraging trip after the egg laying to resume incubation, and had thus undergone similar fasting durations on land. Adults were captured for blood sampling and morphometric measurements (see below) in the colony while incubating (Table A1). During the procedure, the egg was carefully placed in a warm cloth in a box and the adult was provided with a dummy egg, while having its eyes covered by a hood to reduce handling-stress. Flipper length (index of structural size) was measured using a solid metal ruler (± 1mm). Body girth

was obtained (± 1mm) by measuring the circumference of the body beneath the flippers with the animal in an upright position; see protocol in (Viblanc et al., 2012).

Red blood cell mitochondrial aerobic metabolism

Mitochondrial metabolic rates were measured in fresh red blood cells (RBCs) using high-resolution respirometry (2 *Oroboros* Instruments, Innsbruck, Austria). Blood samples (2 mL max) were collected from the marginal flipper-vein with G23 needles fitted to a 2.5 mL heparinized syringe at day 3 and day 10 of the incubation shift. Mitochondrial aerobic respiration was measured within 2 to 12.5 hours (average: whole dataset = 6h05min, females = 5h48min, males = 6h25min) after sampling at 38°C on permeabilized RBCs (see description of the protocol in Appendix 1).

Three respiration rates were analysed: (1) *ROUTINE*: the endogenous cellular respiration rate before RBC permeabilization; (2) *LEAK:* the respiration rate contributing to proton leak. *LEAK* was measured with the addition of oligomycin following the stimulation of both complexes I and II respiration with exogenous substrates (pyruvate, malate, ADP, followed by succinate). (3) *OXPHOS[CI+II]*: the respiration rate supporting ATP synthesis through oxidative phosphorylation. *OXPHOS[CI+II]* was measured as the maximal respiration rate of both complexes I and II, from which *LEAK* was subtracted. Non-mitochondrial respiration was measured after the addition of antimycin A (raw data average ± SD: 0.91 ± 1.47 pmol.s^{-1}) and was subtracted from respiration rates. We also calculated 2 mitochondrial flux control ratios (FCR): (1) *OXPHOS* coupling efficiency [*OxCE = OXPHOS[CI+II] / (OXPHOS[CI+II] + LEAK)*], which provides an indication on the proportion of mitochondrial respiration dedicated to ATP synthesis (i.e. oxidative phosphorylation) compared with the maximal respiration rates, constituting a proxy of RBC mitochondrial metabolic efficiency; (2) *FCR R/OL,* calculated as *ROUTINE / (OXPHOS[CI+II] + LEAK)*, which provides indication on the proportion of maximal respiration rates (contributing to the oxidative phosphorylation, but also to the proton leak) being used under endogenous cellular conditions. Respiration rates were standardised by the volume of RBCs (50 µL). Total protein levels were quantified in each sample (retrieved from the respirometric chamber), using a Pierce™ BCA Protein Assay Kit (the protein content of MIR05 respiration buffer was subtracted from the total protein level of each sample, thus giving result of the protein content of the RBCs used in assay). Repeatability of the quantification of total protein (based on sample-duplicate) was: R [CI 95%] = 0.980 [0.976, 0.984]. Technical repeatability of

mitochondrial metabolic rates was: *ROUTINE:* R [CI 95%] = 0.769 [0.326, 0.970]; Stimulation of complexes I and II respiration: R = 0.956 [0.861, 0.995]; *LEAK*: R = 0.786 [0.458, 0.975] based on 9 duplicates.

Statistical analyses

After discarding 3 samples for total protein level measurements (i.e. outliers detected using Interquartile Criterion Method, most probably linked to dilution error), our final sample-size was lower than expected due to missing data (because of logistical constraints, such as bad weather and limited access to the colony). Sample-sizes can vary between traits and models due to missing information either for RBC mitochondrial metabolism (25 samples) and/or total protein level (9 samples). Our final sample-size includes a total of 135 measurements (n day 3 = 70, n day 10 = 65) from 79 individuals (40 females and 39 males). Statistical analyses were conducted on R v.3.6.3 (R Core Team, 2020), using generalized linear mixed models (GLMMs) with *lme4* package (Bates et al., 2015). RBC mitochondrial metabolic rates and FCRs did not visually fulfil the criteria of normality and this was confirmed with Cullen and Frey plots from *fitdistrplus* package (Delignette-Muller & Dutang, 2015). We therefore analysed RBC mitochondrial metabolic rates and FCRs using GLMMs with a gamma error distribution (log link). For *LEAK*, we used the *bobyqa* optimizer in the model because of convergence issues (Bates et al., 2015). To test whether the impact of fasting duration on RBC mitochondrial metabolic rates and FCRs (all tested as response variables) differed between sexes and according to fasting day at the population-level, we built models with the duration of fasting (2-levels: day 3 *vs.* day 10) and sex (2-levels: female *vs.* male) as fixed effect factors, as well as the interaction between these two factors (interaction removed if non-significant). Total protein level ($mg.mL^{-1}$) was included as covariate in the model. We controlled for the non-independence of measures from the same individual by including the bird ID as a random intercept. Additionally, to test if the variation in mitochondrial metabolism could be modulated and predicted by the body condition at the beginning of the fasting period (i.e. a proxy of their foraging success at sea), we conducted an additional set of statistical analysis where the response was the difference in mitochondrial metabolic rates within individual between 3 and 10 days of fasting (i.e. day 10 minus day 3). For this, we used GLMs with day-3 mitochondrial metabolic rate (i.e. used as a baseline), the body condition (day-3 scale mass index, see below), sex (2-levels: female *vs.* male), and the total protein level ($mg.mL^{-1}$) at day 10 were included in the model as fixed effects. We included

the interaction between the individual sex and scale mass index (see below) in this set of models and removed the interaction if non-significant. Because data were missing for some individuals (i.e. flipper length and/or body girth), these additional analyses were performed on a sub-sample (27 females and 22 males). Body girth is highly correlated with body mass in incubating king penguins ($P<.001$, $R^2=.83$, see more details in Viblanc et al., 2012). Here, we calculated the scale mass index as described in Peig & Green (2009) (referred hereafter as body condition) using body girth as a proxy of body mass in the formula. Preliminary analysis showed that the scale mass index and sex were not strongly associated (LM: $P > 0.76$, see Table A1 for morphometric measurements in females and males), and thus we used both variables in our analyses.

For GLMMs, results from type III ANOVA are presented in the text (F and P values estimated based on F-statistics), while results from type II ANOVA (χ^2 and P values estimated based on Wald chi-square tests) are presented in the text for GLMs. Multiple *post hoc* comparisons with Tukey Honest Significant Difference (HSD) correction were performed using *emmeans* package (Lenth, 2021; Searle et al., 1980). Effect-sizes (Cohen's D) were estimated with the *effectsize* package (Ben-Shachar et al., 2020). Pseudo-R^2 were estimated with a variance-function-based method for GLMMs (using *rsq* package) and based on the proportion of variation in the responses explained by the available predictors for GLMs (Zhang, 2017). Values were considered as statistically significant for $P < 0.05$.

Results

OXPHOS[CI+II] was significantly lower at day 10 compared to day 3 of fasting in both sexes ($F = 3.02$, $P = 0.008$, Table 1). *ROUTINE*, *LEAK* and *FCR R/OL* did not significantly differ between fasting days (all $F < 2.70$, all $P > 0.06$, Table 1). For all metabolic traits, confidence intervals (95%) of predicted estimates obtained with non-parametric bootstrap method did not remain different from zero for the effect of fasting day (Table A2.B).

Independently of the fasting stage, *ROUTINE*, *OXPHOS[CI+II]* and *LEAK* were significantly higher in females than in males (all Cohen's $D > 0.51$, all $P < 0.04$ for the main effect of sex, see Table 1, Figs. 2 and 3). *FCR R/OL* was not statistically different between sexes (all $F < 5.32$, all $P > 0.08$). To confirm these results, we also used a non-parametric bootstrap method (n bootstraps = 100), and confidence intervals (95%) of predicted estimates for the effect of sex remained different from zero for all metabolic traits (Table A2.A).

OXPHOS coupling efficiency was significantly affected by the interaction between fasting duration and sex (see Table 2). *OXPHOS* coupling efficiency significantly decreased in males at the end of fasting (-9.5% on predicted averages at 10 days, Tukey HSD *post hoc* comparison: estimate ± SE = 0.019 ± 0.006, P = 0.006, Table 2, Fig. 4), while it remained similar in females independantly of the fasting stage (Tukey HSD *post hoc* comparison: P = 0.98, Table 2, Fig. 4).

For *ROUTINE*, *OXPHOS[CI+II]*, *LEAK* and *OXPHOS* coupling efficiency, changes in mitochondrial metabolic rates were not predicted by the initial body condition of the individual (scale mass index measured at day 3 of fasting) (all F < 0.32, all P > 0.58, Table 3). *FCR R/OL* was significantly affected by the interaction between the scale mass index and the sex of the individuals (F = 5.68, P = 0.02), although these differences were not statistically significant in *post hoc* analyses (Tukey HSD *post hoc*: P = 0.13). Changes in mitochondrial metabolic rates between 3 and 10 days of fasting were all negatively associated with the initial metabolic rate measured at day 3 (all F > 5.96, all P < 0.02, Table 3). Changes in *OXPHOS[CI+II]* and *OXPHOS* coupling efficiency between day 3 and day 10 were significantly smaller in males (all F > 4.31, all P < 0.04, Table 3, Fig. A1).

Discussion

Our first aim was to investigate whether RBC mitochondrial metabolism could be modulated in response to fasting, and secondly if such response differs between sexes. Both sexes showed a decrease in the respiration allocated to ATP synthesis (i.e. oxidative phosphorylation, *OXPHOS[CI+II]*) after 10 days of fasting. In other words: when providing all the necessary metabolic substrates to sustain RBC mitochondrial metabolism, the oxygen consumption associated with ATP synthesis decreased with fasting. However, the endogenous RBC respiration (oxygen consumption before permeabilization, *ROUTINE*), the ratio between *ROUTINE* and the maximal respiration rates (*FCR R/OL*) and the respiration associated with proton leak (*LEAK*) were not affected by fasting duration. Thus, it seems that reduction of whole-animal metabolic rate with fasting (Cherel et al., 1988; Fahlman et al., 2004; Froget et al., 2001; Groscolas et al., 2010), does not align with a reduction of metabolic rate in mitochondria, at least in red blood cells, but rather fasting reduces the ATP production capacity of RBC mitochondria. Such an explanation seems reasonable in light with the fact that during fasting the need for the ATP production capacity of RBC is probably lowered, due

to an overall low whole-animal metabolism. In contrast, such ATP production capacities are more needed during foraging, especially for diving species where the oxygen carrying capacity of RBC is crucial (Handrich et al., 1997; Signore et al., 2021). It however, needs to be pointed out that first, we did not measure the whole-animal metabolism in the current study and second, our results represent the average response to fasting for our study sample, but we found a high inter-individual variation (Fig.A1).

In line with our predictions, we found some sex-specific responses: RBC mitochondrial metabolism efficiency was maintained in females across fasting while it declined in males. In king penguins, 3 and 10 days of fasting correspond to *phase II* of fasting, where metabolic needs are mostly covered by lipid oxidation (Cherel et al., 1988a; Groscolas & Robin, 2001; Robin et al., 1988; Secor & Carey, 2016). Thus, sex-specific differences in RBC metabolism efficiency could be linked to a more efficient lipid mobilisation and conversion into energy in females, similarly as found in short-term (12-48h) fasting in Japanese quails (*Coturnix japonica*), and humans (Hedrington & Davis, 2015; Lamosová et al., 2004; Montero et al., 2018; Tarnopolsky, 2008). Second, sex-steroid hormones are increasingly thought to be involved in metabolic processes (Dai et al., 2013; Gaignard et al., 2017, 2018; Price & Dai, 2015; Rosa-Caldwell & Greene, 2019). Indeed, oestrogen has been shown to increase mitochondrial metabolic efficiency by for instance modulating the expression of electron transport chain proteins in laboratory conditions (rodents, human cell lines) (Chen et al., 2005; Klinge, 2008; Stirone et al., 2005; Velarde, 2013, 2014). On the other hand, progesterone can increase mitochondrial membrane potential, concomitantly increasing ATP production, through a receptor within mitochondria (Dai et al., 2013; Price & Dai, 2015; Rosa-Caldwell & Greene, 2019; Velarde, 2014). In king penguins, estradiol and progesterone levels are higher in females than in males during courtship, but the lack of data on oestrogen and progesterone levels in males during the fasting process makes any hypothesis based on hormonal levels difficult to validate (Cherel et al., 1994b; Jouventin & Mauget, 1996; Mauget et al., 1994). Thus, further studies are needed to reveal why the females were able to keep the efficiency high during fasting (despite having a higher proton leak, see below).

Interestingly, all RBC mitochondrial metabolic rates were higher in king penguin females compared to males, independently of their fasting stage. The proportion of maximal respiration rates being used under endogenous cellular conditions (before RBC

permeabilization, *FCR R/OL*) was similar in both sexes. It is worth noting that lower mitochondrial metabolic rates and efficiency across fasting for males could be linked to a lower number of mitochondria per cell, and further studies are needed to assess whether RBC mitochondrial content differs between sexes in and out of the fasting context.

Whereas both sexes fulfil similar reproductive tasks (parental care, protection against predators, territory defence) (Olsson, 1996), it is most likely that physiological needs and metabolic experiences differ between sexes. As males experience a first longer breeding fast (up to a month; Fig.1) (Stonehouse, 1960; Weimerskirch et al., 1992), we cannot exclude that this prolonged fasting period may have metabolic and physiological consequences on a longer-term (see Schull et al., 2016). Further research focusing on metabolic responses in both sexes is crucially needed to comprehensively investigate sex-specific variation and potential differences in physiological adaptations between sexes (Ah-King et al., 2014; Arnegard et al., 2020; Garcia-Sifuentes & Maney, 2021; Orbach, 2022). With regard to sex-specific response, our results are in contrast with Stier et al. (2019a), who found no differences in mitochondrial metabolism between sexes. Differences in sample-sizes and protocol (intact cells *vs.* permeabilized cells here) might explain the discrepancy in results.

At the whole-organism level, oxygen consumption is positively associated with the organism's body mass and size (Burness et al., 1998; Glazier, 2022; Naya et al., 2018), thus as our third hypothesis, we expected to explain inter-individual variations in RBC mitochondrial metabolism by differences in the initial body condition at the beginning of fasting. However, except for *FCR R/OL*, changes in mitochondrial metabolic rates between 3 and 10 days of fasting were not influenced by the initial body condition. While we found a significant effect of the interaction between sex and body condition on *FCR R/OL*, *post hoc* analysis revealed that most of the variance was explained by variation in scale mass index rather than sex, thus these results should be interpreted with caution. Interindividual variation in RBC mitochondrial respiration could be associated with intrinsic factors (e.g. genetic background, cost of maintaining vital function), but also extrinsic factors (e.g. activity level associated with territorial defence, immune response to pathogens).

Overall, our study revealed that the fasting process can modulate RBC mitochondrial metabolism, as measured here in king penguins, by decreasing the respiration allocated to

energy production. An adjustment in RBC energy budget and management could potentially be linked to a decrease in physical activity and metabolic requirements during fasting on land. Our study also emphasises the importance of studying physiological responses in females as well, which can express a different metabolic strategy. Further research is needed to address the origins of a different RBC energetic between sexes, in king penguins, but to assess if such sexual dimorphism is present in other species.

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Ethics

All the procedures were approved by the French Ethical Committee (APAFIS#16465-2018080111195526 v4) and the Terres Australes et Antarctiques Françaises (Arrêté TAAF A-2021-49).

Authors contribution

N.C-S and V-A.V designed the study. C.B, C.L, N.C-S, T.F, M.L and V-A.V conducted the fieldwork and collected the samples. C.B, N.C-S and N.G conducted the mitochondrial respirometry measurements. N.C-S performed statistical analysis under the supervision of K.A and S.R. N.C-S wrote the first version of the manuscript under the supervision of K.A and S.R. All co-authors revised the manuscript.

Data available statement

Data are available on Figshare DOI: 10.6084/m9.figshare.27901875.

Competing interests

We declare we have no competing interests.

Appendix 1

Blood samples were kept on ice and centrifuged (+4°C, 5min at 800g). 50 µL of red blood cells (RBCs) were resuspended in PBS for storage and kept at +4°C until mitochondrial metabolism measurements. RBC mitochondrial metabolic rates were measured using High-Resolution Respirometry on fresh blood samples. RBCs were centrifuged a second time (+4°C, 5min at 800g) to remove PBS and resuspended in MIR05 buffer before the High-Resolution Respirometry measurements. Mitochondrial metabolic rates were measured using *Oroboros* Instruments (Innsbruck, Austria) at 38°C adapted from a protocol described in (Stier et al., 2019b) on permeabilized RBC: digitonin (20µg/mL), pyruvate (5mM), malate (2mM), ADP (1.25mM), succinate (10mM), oligomycin (2.5µM), antimycin A (2.5 µM).

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Table 1. Results of generalized linear mixed models (GLMMs) testing the effects of the fasting day (3 *vs.* 10) and sex (Female *vs.* Male) on red blood cell mitochondrial metabolic rates (n measurements: day 3 = 70 and day 10 = 65 in 40 females and 39 males) in breeding king penguins. GLMMs estimates are reported with their standard error. Total protein level ($mg.mL^{-1}$) was included as covariate in the models. The interaction between fasting day and sex was preliminary tested in the models below and removed because non-significant. The individual ID was included as random intercepts in the models. σ: standard deviation. Values are considered as statistically significant for $P < 0.05$ (presented in bold).

	ROUTINE			*OXPHOS[CI+II]*			*LEAK*		
	Estimate	Std. error	P	Estimate	Std. error	P	Estimate	Std. error	P
Intercept	-2.381	0.122	< **0.001**	-0.953	0.098	< **0.001**	-2.640	0.101	< **0.001**
Fasting day (day 10)	-0.011	0.028	0.703	-0.057	0.021	**0.008**	0.001	0.025	0.959
Sex (M)	-0.139	0.058	**0.017**	-0.270	0.054	< **0.001**	-0.078	0.039	**0.042**
Total protein levels	0.248	0.021	< **0.001**	0.145	0.017	< **0.001**	0.133	0.018	< **0.001**
Random effects									
σ (Bird ID)	0.150			0.142			0.090		
σ (Residuals)	0.176			0.141			0.153		
N (individuals)	79			79			79		
n (observations)	135			135			135		
Pseudo R^2	0.710			0.764			0.493		

Table 2. Results of generalized linear mixed models (GLMMs) testing the effects of the interaction between the fasting day (3 *vs.* 10) and sex (Female *vs.* Male), on red blood cell mitochondrial metabolic efficiency (*OXPHOS* coupling efficiency: OxCE) (n measurements: day 3 = 70 and day 10 = 65 in 40 females and 39 males) in breeding king penguins. *Post hoc* tests on the interaction between the fasting day and sex are reported and estimated using Tukey HSD correction (see Methods). GLMMs estimates are reported with their standard error. As OxCE is a ratio, total protein level was not included as fixed effect in this model. The individual ID was included as random intercepts in the model. σ: standard deviation. Values were considered as statistically significant for $P < 0.05$ (presented in bold).

	OxCE		
	Estimates	Std. error	*P*
Intercept	-0.165	0.008	**< 0.001**
Fasting stage (day 10)	-0.002	0.005	0.676
Sex (M)	-0.025	0.011	**0.029**
Fasting stage * Sex:	-0.016	0.008	**0.039**
***Post-hoc* tests:**			
day 3 (M) *vs.* day 10 (M)	0.019	0.006	**0.006**
day 3 (F) *vs.* day 10 (F)	0.002	0.006	0.975
day 3 (F) *vs.* day 10 (M)	0.043	0.011	**< 0.001**
day 10 (F) *vs.* day 10 (M)	0.041	0.011	**0.002**
day 3 (F) *vs.* day 3 (M)	0.025	0.011	0.129
day 10 (F) *vs.* day 3 (M)	0.023	0.012	0.204
Random effects			
σ (Bird ID)	0.028		
σ (Residuals)	0.027		
N (individuals)	79		
Observations	135		
Pseudo R^2	0.759		

Table 3. Results of linear mixed models (LMMs) testing changes in red blood cell mitochondrial metabolic rates (i.e. day 10 minus day 3) according to the initial metabolic rate (measured at day 3), the sex (Female *vs.* Male), the initial body condition (scale mass index measured at day 3), and the total protein levels ($mg.mL^{-1}$) measured at day 10. Models outcomes are based on a subsample of 49 individuals (27 females and 22 males), including i) individuals from which mitochondrial aerobic metabolism was measured both at day 3 and day 10 of fasting; but also ii) individuals with an estimation of the body condition (scale mass index based on flipper length and body girth measurements, see Methods). LMMs estimates are reported with their standard error. σ: standard deviation. Values were considered as statistically significant for $P < 0.05$ (presented in bold).

	ROUTINE			*OXPHOS[CI+II]*			*LEAK*		
	Estimate	Std.error	P	Estimate	Std.error	P	Estimate	Std.error	P
Intercept	-0.150	0.348	0.668	-0.343	0.639	0.594	0.005	0.146	0.754
Metabolic rate measured at day 3	-0.860	0.118	**<0.001**	-0.687	0.132	**<0.001**	-1.074	0.189	**<0.001**
Sex (M)	-0.040	0.024	0.103	-0.164	0.048	**0.001**	-0.005	0.009	0.640
Scale mass index measured at day 3	$2.2e10^{-4}$	0.001	0.839	0.001	0.002	0.578	$7.4e10^{-5}$	$4.5e10^{-4}$	0.868
Total protein levels at day 10	0.072	0.014	**<0.001**	0.101	0.024	**<0.001**	0.016	0.006	**0.006**
N (individuals)	49			49			49		
Pseudo R^2	0.689			0.523			0.491		

Figures

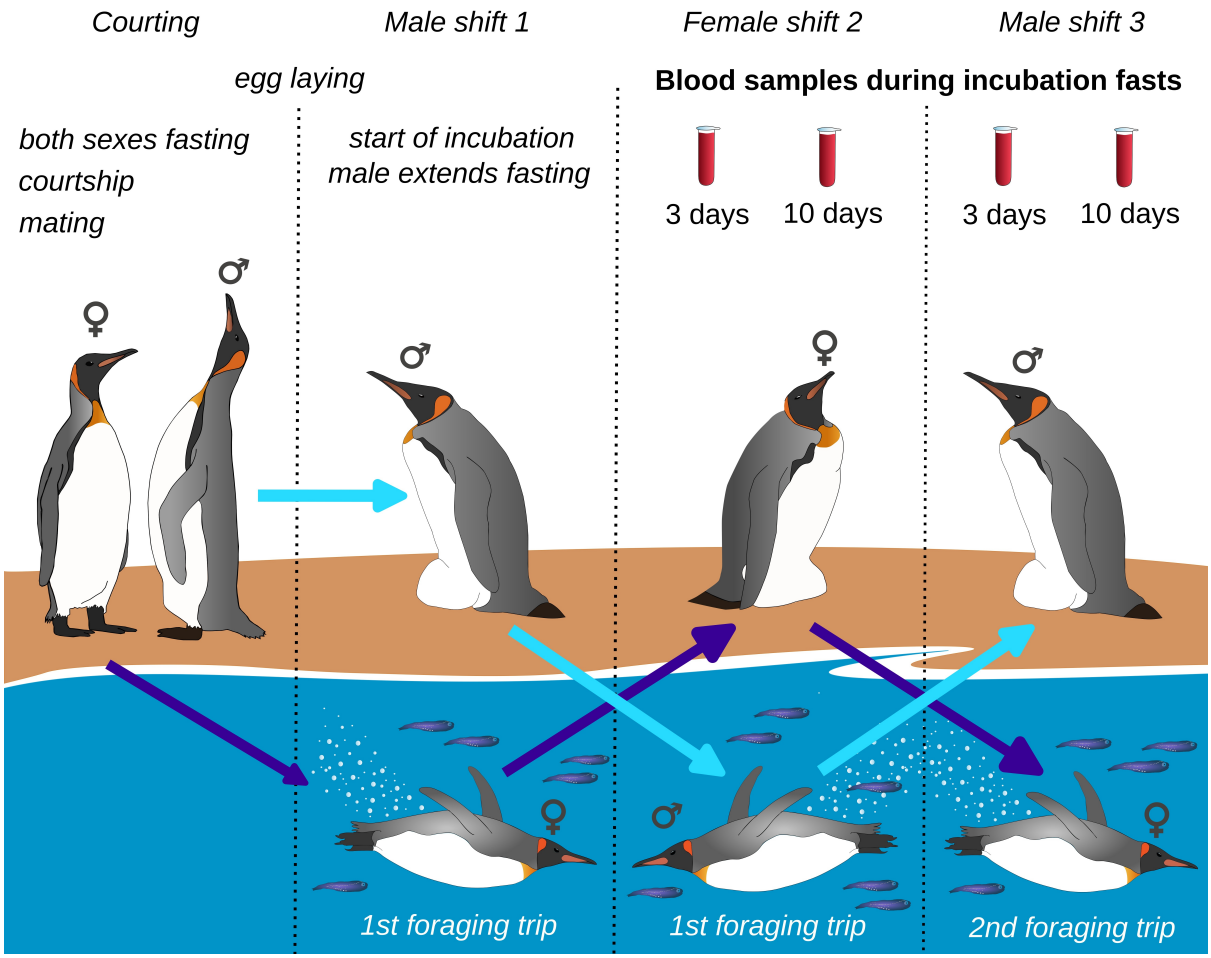


Fig.1: Schema of the king penguin breeding cycle (i.e. start of the egg incubation period) and blood sample collection.

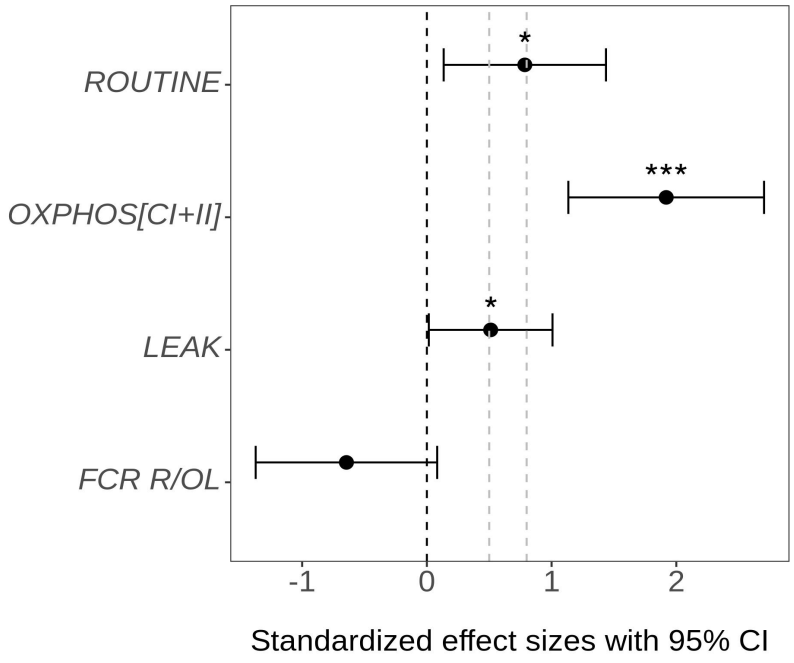
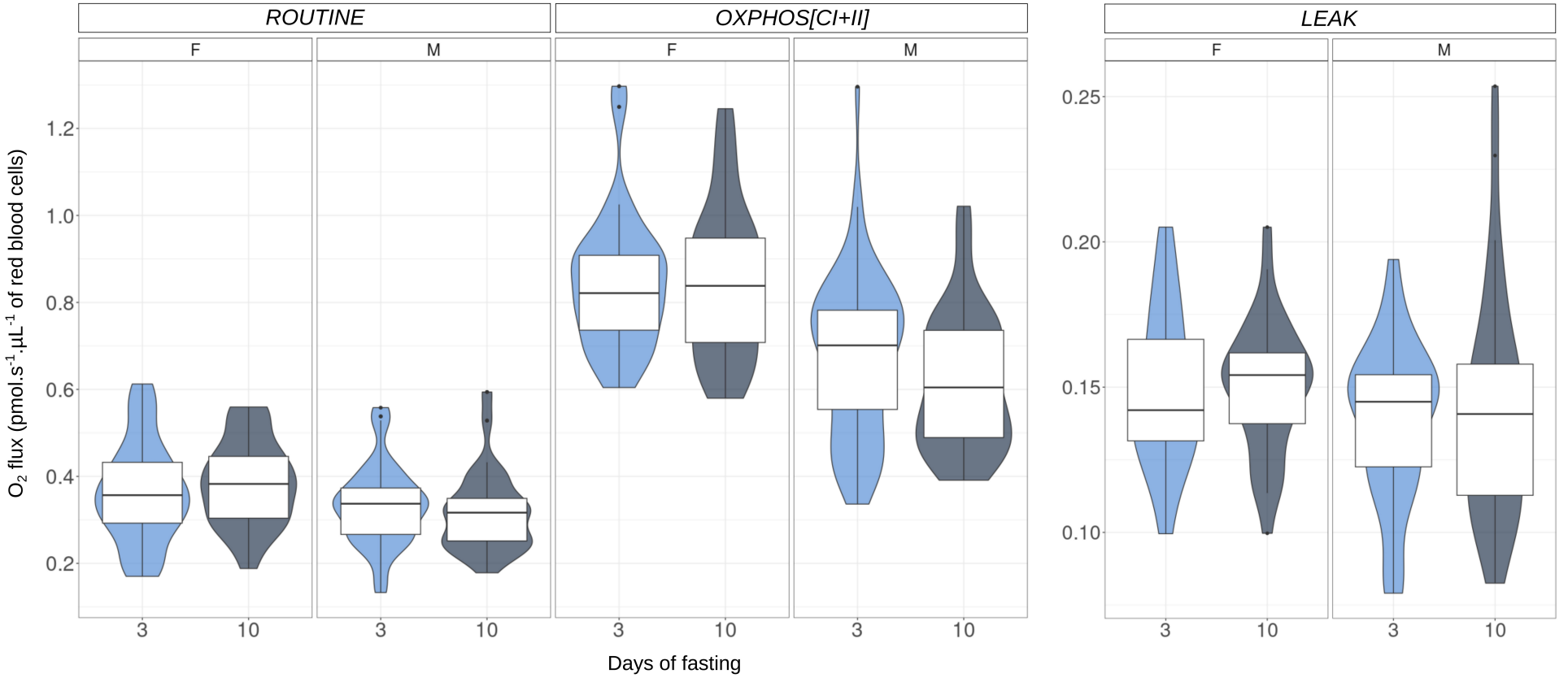
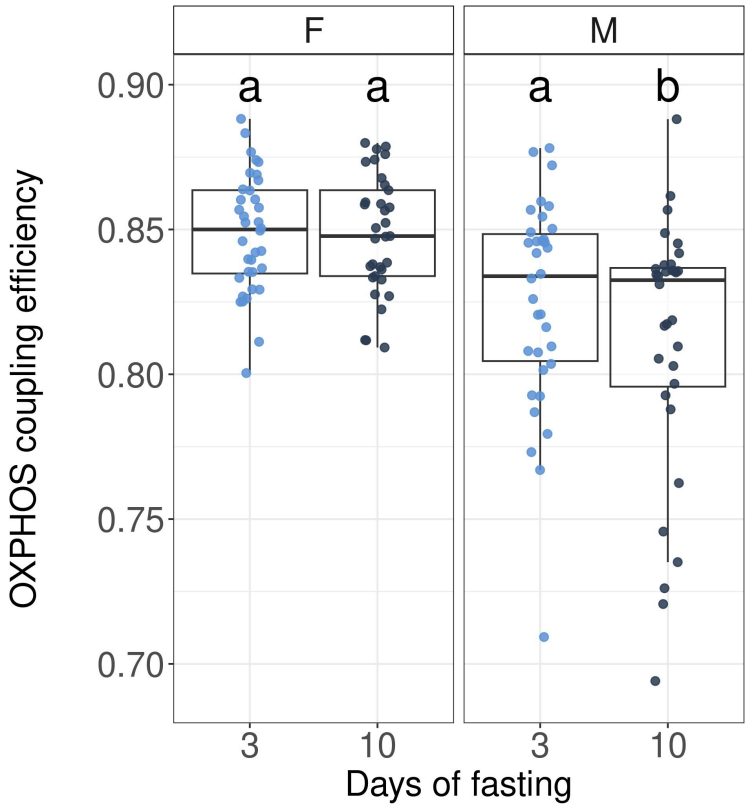


Fig.2: Differences between sexes in red blood cell mitochondrial metabolic rates and the flux control ratio *FCR R/OL* during breeding fast in king penguins. Effect-sizes are presented. Females are compared to males (males used as reference values here). A positive value indicates a higher mitochondrial metabolic rate in females than males. Red blood cell mitochondrial aerobic metabolism was measured on fasting days 3 and 10 (n day 3 = 70, n day 10 = 65 in 40 females and 39 males). Except for FCR, total protein level measured in samples retrieved from High-Resolution Respirometry assay was included as a covariate in the models. The bird ID was included as a random intercept in the models to control for the non-independence of measures from the same individual. Standardized effect sizes are based on predicted values of the model and reported with their 95% CI. See results in Tables 1 & 2.



696 **Fig. 3**: Red blood cell mitochondrial metabolic rates on days 3 and 10 of breeding fast in female and male king penguins. The distribution of raw data
697 is shown using violin boxplots. Classical boxplots indicate raw data median, first and third quartiles. Raw data encompass 135 measurements (n day 3
698 = 70, n day 10 = 65 in 40 females and 39 males).



699 **Fig.4**: *OXPHOS* coupling efficiency according to fasting duration (day 3 or day 10) and sex.
700 Raw data are presented (points). Raw data distribution is presented using boxplots. The
701 interaction between the fasting duration and sex was statistically significant. Different letters
702 indicate significant differences according to statistical results (Table 2).

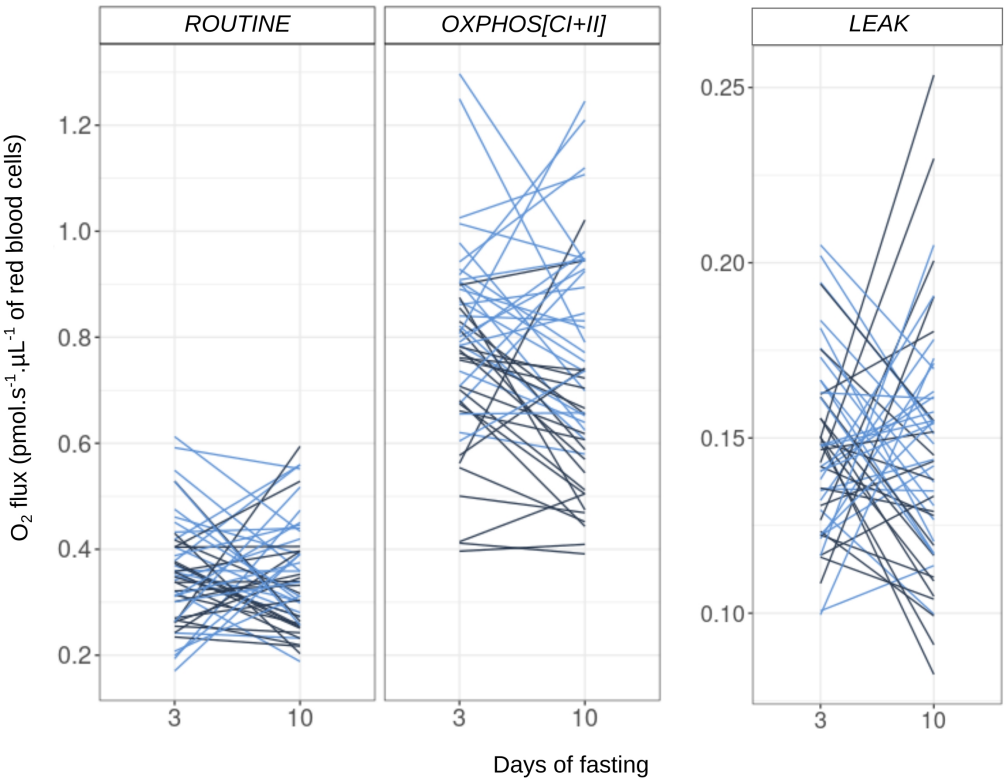
Supplementary Materials

Table A1: Morphometric measurements in both females and males king penguins that participated in this study (raw data average ± SEM [range]). Scale mass index was calculated using the formula described in Peig & Green (2009), where body girth was used as a proxy of the body mass (Viblanc et al., 2012). Body girth and flipper length were measured at day 3 of fasting (see Methods). This table encompasses the sub-samples of individuals, for which flipper length and body girth was measured.

Measurements	Females	Males
N (individuals)	27	22
Body girth (mm)	639.7 ± 5.4 [588; 695]	661.5 ± 5.7 [617; 722]
Flipper length (mm)	322.0 ± 1.7 [304; 339]	329.2 ± 2.4 [310; 360]
Scale mass index	327.1 ± 2.4 [304.0; 348.9]	326.1 ± 2.1 [313.1; 348.2]

Table A2: Distribution of the predicted estimates (mean ± SD [interquartile range 95%]) for the effect of sex (Female *vs.* Male) (A) and the effect of fasting day (day 3 *vs.* day 10) (B) on mitochondrial metabolic rates (response variables), using a non-parametric bootstrap method (n bootstraps = 100). Interquartile ranges not crossing 0 are presented in bold.

Response variable	A. Distribution of predicted estimates for **SEX**	B. Distribution of predicted estimates for **FASTING DAY**
ROUTINE	**-0.145 ± 0.034 [-0.213 – -0.081]**	-0.016 ± 0.047 [-0.097 – 0.064]
OXPHOS[CI+II]	**-0.250 ± 0.025 [-0.313 – -0.226]**	-0.060 ± 0.035 [-0.128 – 0.001]
LEAK	**-0.083 ± 0.025 [-0.132 – -0.041]**	$7e10^{-4}$ ± 0.040 [-0.078 – 0.072]
FCR R/OL	**0.096 ± 0.031 [0.030 – 0.157]**	0.042 ± 0.037 [-0.032 – 0.109]



714 **Fig. A1**: Red blood cell mitochondrial metabolic rates according to fasting day (3 *vs.* 10
715 days). Individual responses are plotted (raw data, sample size: 27 females and 22 males).
716 Purple refers to female measurements and orange refers to male measurements.