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## RESEARCH ARTICLE

# Threshold effect in the H<sub>2</sub>O<sub>2</sub> production of skeletal muscle mitochondria during fasting and refeeding

Damien Roussel\*, Mélanie Boël, Mathieu Mortz, Caroline Romestaing, Claude Duchamp and Yann Voituron

## ABSTRACT

Under nutritional deprivation, the energetic benefits of reducing mitochondrial metabolism are often associated with enhanced harmful pro-oxidant effects and a subsequent long-term negative impact on cellular integrity. However, the flexibility of mitochondrial functioning under stress has rarely been explored during the transition from basal non-phosphorylating to maximal phosphorylating oxygen consumption. Here, we experimentally tested whether ducklings (*Cairina moschata*), fasted for 6 days and subsequently refed for 3 days, exhibited modifications to their mitochondrial fluxes, i.e. oxygen consumption, ATP synthesis, reactive oxygen species generation (ROS) and associated ratios, such as the electron leak (% ROS/O) and the oxidative cost of ATP production (% ROS/ATP). This was carried out at different steady-state rates of oxidative phosphorylation in both pectoralis (glycolytic) and gastrocnemius (oxidative) muscles. Fasting induced a decrease in the rates of oxidative phosphorylation and maximal ROS release. These changes were completely reversed by 3 days of refeeding. Yet, the fundamental finding of the present study was the existence of a clear threshold in ROS release and associated ratios, which remained low until a low level of mitochondrial activity was reached (30–40% of maximal oxidative phosphorylation activity).

**KEY WORDS:** Free electron leak, ROS/ATP ratio, Oxidative phosphorylation, Reactive oxygen species, Skeletal muscle, Starvation, Bird

## INTRODUCTION

Many physiological mechanisms, such as modulation of lipid mobilization and oxidation and reduction of energy expenditure, are involved in enhancing energy conservation and survival during fasting (McCue, 2010). One of the common adjustments often reported is an overall reduction in the mitochondrial activity of oxidative tissues, such as the liver and skeletal muscles (Dumas et al., 2004; Ramsey and Hagopian, 2006; Sorensen et al., 2006; Brown and Staples, 2011; Bourguignon et al., 2017; Monternier et al., 2017). Mitochondria are also a source of reactive oxygen species (ROS), harmful byproducts of mitochondrial respiration which, in turn, may be causally involved in aging processes, as suggested by the mitochondrial free radical theory of aging (Lambert et al., 2007; Barja, 2013). Studies have shown that moderate energy restriction enhances resistance to oxidative stress and increases the mean and maximum healthy lifespan in many

organisms (Sohal et al., 1994; López-Torres et al., 2002; Bevilacqua et al., 2004; Bevilacqua et al., 2005; Fontana et al., 2010; Walsh et al., 2014). In contrast, an extreme reduction in available energy such as that during fasting may induce pro-oxidant effects including, in some incidents, enhanced mitochondrial ROS production or free electron leak (ROS/O), and their subsequent negative impact on cellular integrity and life span (Sorensen et al., 2006; Fontana et al., 2010; Brown and Staples, 2011; Salin et al., 2018).

Several studies have related rates of mitochondrial ROS production to basal non-phosphorylating and maximal phosphorylating oxygen consumption rates. Between basal non-phosphorylating and maximal ATP synthesis rates, mitochondrial ROS production is highly sensitive to proton motive force and the redox state of the respiratory chain (Korshunov et al., 1997; Starkov and Fiskum, 2003; Quinlan et al., 2012; Kikusato and Toyomizu, 2013; Treberg et al., 2018). Typically, a small (10%) decrease in membrane potential, along with a more oxidized chain, triggers a 10-fold reduction in ROS generation (Korshunov et al., 1997; Kikusato and Toyomizu, 2013; Goncalves et al., 2015). How ROS generation behaves when mitochondria work at submaximal activity remains an important question. In addition to oxidative phosphorylation and ROS fluxes, ratios of these different parameters are also informative because they provide elemental information on (i) free electron leakage (ROS/O) and (ii) the oxidative cost of ATP molecule production (ROS/ATP), for example. The ROS/O ratio has been studied in fasting animals but contradictory results were obtained. Indeed, free electron leak has been shown to increase in rats subjected to 72 h fasting (Sorensen et al., 2006) but to decrease in dwarf Siberian hamsters fasted for 1 day (Brown and Staples, 2011). In addition, only scarce information is available regarding the effect of fasting on the ROS/ATP ratio, which represents the two sides of the ‘oxygen’ coin. For instance, this parameter was shown to decrease in aortic endothelial cells when mitochondrial pyruvate transport was inhibited and it has been proposed as an explanatory parameter of the higher longevity and fecundity of the INDY (I’m not dead yet) *Drosophila melanogaster* mutant (Brooks et al., 2007; Neretti et al., 2009). A decrease in the ROS/ATP ratio (% ROS/ATP) in which cells reduce oxidative stress per amount of ATP synthesized would carry with it potential beneficial effects on cellular integrity (López-Lluch et al., 2006; Neretti et al., 2009). At the level of the mitochondria, this can be achieved with a decrease in electron transport chain activity, which would drive a linear reduction of ATP synthesis (Beavis and Lehninger, 1986; Nogueira et al., 2001; Teulier et al., 2010; Roussel et al., 2018) and at the same time a nearly exponential reduction in ROS generation (Korshunov et al., 1997; Kikusato and Toyomizu, 2013; Goncalves et al., 2015). Consequently, a description of the specific dynamics of these ratios during the transition from basal to maximal phosphorylating oxygen consumption rates will undoubtedly complete our understanding of the close link between bioenergetics and the oxidative stress response (Picard et al., 2014).

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In the present study, we experimentally tested at different activation states, from basal to maximal oxidative phosphorylation activity, whether ducklings exhibit modification of mitochondrial fluxes and associated ratios in both pectoralis (glycolytic) and gastrocnemius (oxidative) muscles during fasting and after a short period of refeeding. Ducklings were fasted for 6 days, because previous studies have shown that 1 week of fasting triggers a down-regulation of the activity of the mitochondrial substrate oxidation system, leading to an improvement of coupling efficiency (Monternier et al., 2017; Roussel et al., 2018). But how fasting affects ROS generation in these two metabolically contrasted skeletal muscles and whether mitochondrial adjustments to fasting drive a change in % ROS/ATP remain unknown. Thus, we asked (1) whether the skeletal muscle phenotype impacts mitochondrial responses to fasting, (2) how ratios of mitochondrial fluxes, namely the free electron leak (% ROS/O) and the oxidative cost of ATP production (% ROS/ATP), are affected by fasting, and (3) whether these variations can be reversed by refeeding.

## MATERIALS AND METHODS

### Animals and tissue sampling

All experiments were conducted in accordance with animal care guidelines and were approved by the Ethics Committee of Lyon University and the Ministère de la Recherche et de l'Enseignement Supérieur. Male Muscovy ducklings, *Cairina moschata* (Linnaeus 1758), were obtained from a commercial stockbreeder (Eclousson Grimaud Frères, Roussay, France). Ducklings were reared at 25°C for 4 weeks in a constant photoperiod (8 h:16 h light:dark). Thereafter, ducklings were randomly assigned to one of the three experimental groups: fed *ad libitum* (fed group), fasted for 6 days (fasted group) or fasted for 6 days and then re-fed for 3 days (refed group). Previously published data and discussion on this species indicate that ducklings fasted for 6 days are in the 'economical' phase II of fasting (Monternier et al., 2017). Briefly, this is mainly supported by a constant daily change in body mass, an increase in circulating fatty acids and ketone bodies, and a low level of circulating uric acid (Cherel et al., 1988).

### Isolation of mitochondria

Eighteen ducklings were used to study mitochondrial bioenergetics (fed group,  $N=6$ ; fasted group,  $N=6$ ; refed group,  $N=6$ ). At the end of the nutritional protocol, animals were stunned by cranial percussion and killed by decapitation, then the pectoralis and gastrocnemius muscles were rapidly removed and placed in an ice-cold isolation buffer (100 mmol l<sup>-1</sup> sucrose, 50 mmol l<sup>-1</sup> KCl, 5 mmol l<sup>-1</sup> EDTA and 50 mmol l<sup>-1</sup> Tris-base, pH 7.4 at 4°C). Skeletal muscle mitochondria were isolated from either the pectoralis or the red part of the gastrocnemius muscle in the ice-cold isolation buffer as previously described (Teulier et al., 2010; Monternier et al., 2017). Briefly, the mitochondrial isolation procedure involved Potter homogenization, protease digestion and differential centrifugation, with all steps performed at 4°C. Finally, all skeletal muscle mitochondria were pelleted at 8700 g (10 min) and the protein concentration of the mitochondrial suspension was determined using a Biuret method with bovine serum albumin as standard.

### Mitochondrial oxidative phosphorylation activity

Mitochondrial oxidative phosphorylation efficiency was assessed at 40°C by measuring the rates of oxygen consumption and ATP synthesis in a respiratory buffer (120 mmol l<sup>-1</sup> KCl, 5 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1 mmol l<sup>-1</sup> EGTA, 2 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 0.3% fatty acid-free bovine serum albumin, 1.6 U ml<sup>-1</sup> hexokinase, 20 mmol l<sup>-1</sup>

glucose and 3 mmol l<sup>-1</sup> Hepes, pH 7.4). Basal respiration was initiated by adding a mixture of respiratory substrates consisting of pyruvate (5 mmol l<sup>-1</sup>), malate (2.5 mmol l<sup>-1</sup>) and succinate (5 mmol l<sup>-1</sup>). This combination of substrates was chosen to generate a convergent electron flow at the coenzyme-Q junction of the respiratory chain, which would reconstitute the physiological citric acid cycle function in isolated mitochondria by simultaneously generating NADH and succinate in the mitochondrial matrix. Thereafter, mitochondrial ATP synthesis was initiated by the addition of 500, 100, 20, 10 or 4 μmol l<sup>-1</sup> ADP. After recording the phosphorylating respiration (state 3) rate for 2 min in a closed glass cell fitted with a Clark oxygen electrode (Rank Brothers Ltd, Cambridge, UK), four 100 μl samples of mitochondrial suspension were withdrawn from the suspension every 30 s and were quenched in 100 μl perchloric acid solution consisting of 10% HClO<sub>4</sub> and 25 mmol l<sup>-1</sup> EDTA. ATP production was determined from the glucose 6-phosphate content of samples as described previously (Teulier et al., 2010). To make sure that the rates measured were specific to the mitochondrial ATP synthase activity, we determined oxygen consumption and ATP synthesis rates in the presence of oligomycin (2 μg ml<sup>-1</sup>). These values were taken into account to calculate the rate of mitochondrial ATP synthesis.

### Mitochondrial ROS production and associated ratios

The rate of H<sub>2</sub>O<sub>2</sub> released by isolated mitochondria was measured at 40°C using a fluorometer (SFM-25, Kontron Instrument, Augsburg, Germany) at excitation and emission wavelengths of 560 and 584 nm, respectively. The respiratory buffer (1 ml) was supplemented with 5 U ml<sup>-1</sup> horseradish peroxidase, 1 μmol l<sup>-1</sup> Amplex Red fluorescent dye, and mitochondria (15–25 μg ml<sup>-1</sup>). The respiratory substrates pyruvate/malate (5/2.5 mmol l<sup>-1</sup>) and succinate (5 mmol l<sup>-1</sup>) were added, and the rate of H<sub>2</sub>O<sub>2</sub> titration with the sequential addition of ADP at final concentrations of 4, 10, 20, 100 and 500 μmol l<sup>-1</sup> was measured. The fluorescent signal was calibrated using a standard curve obtained after successive additions of H<sub>2</sub>O<sub>2</sub> (up to 35 pmol). We calculated the free electron leak as described previously (Rey et al., 2013), after measuring the corresponding respiratory rate of isolated mitochondria (% ROS/O). Similarly, the oxidative cost of mitochondrial ATP production was based on the ratio of ROS generated (i.e. H<sub>2</sub>O<sub>2</sub> released) in isolated mitochondria divided by the corresponding rate of ATP synthesis (% ROS/ATP).

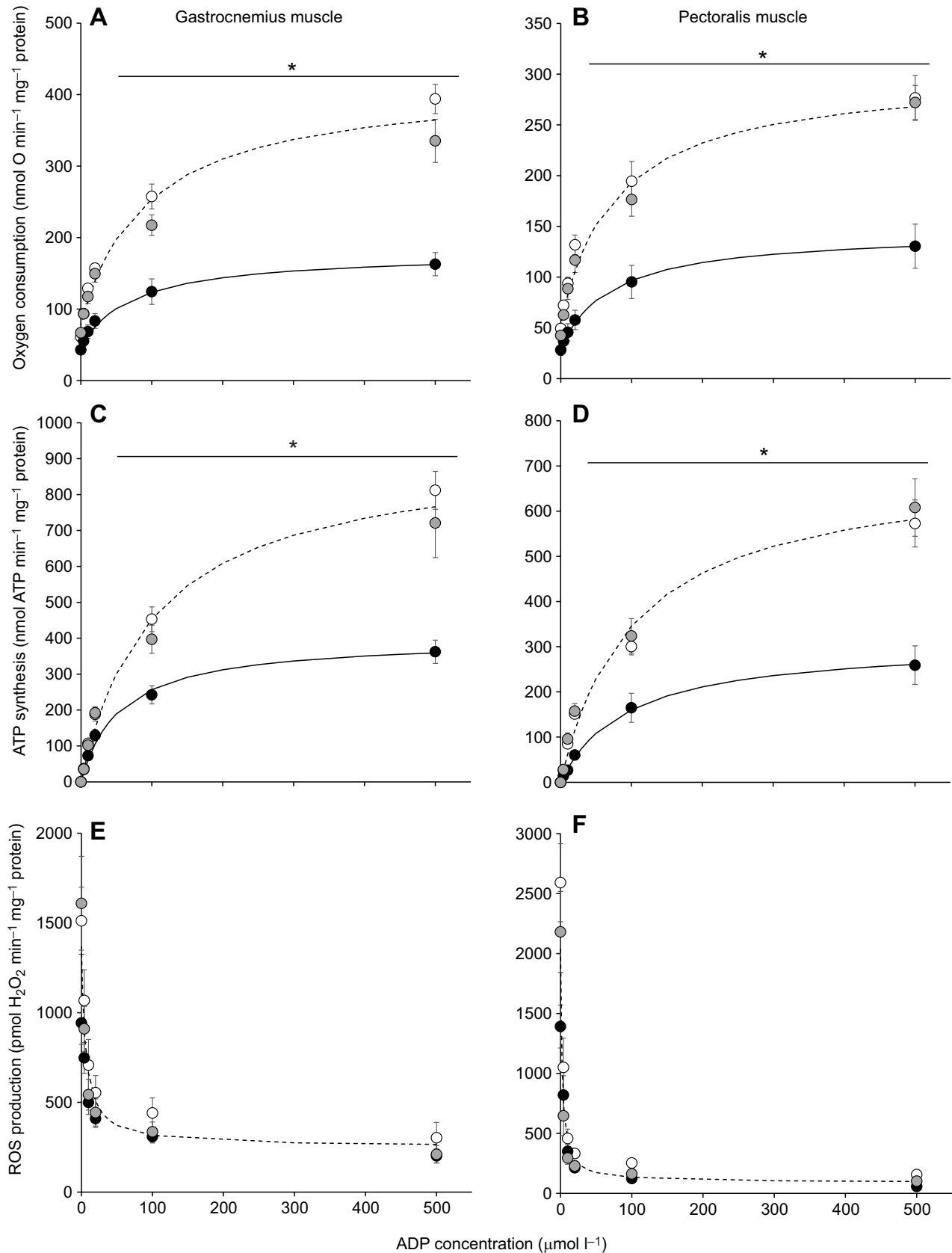
### Statistical analysis

Two-way repeated-measures ANOVA (RM ANOVA) followed by a Holm–Sidak *post hoc* test was performed to estimate the effects of group and ADP addition on mitochondrial oxygen consumption, ATP synthesis and H<sub>2</sub>O<sub>2</sub> production fluxes (SigmaPlot 12.0, Systat Software, San Jose, CA, USA). Mitochondrial and kinetic parameters were tested with ANOVA for independent values, followed by protected least significant difference tests (Statview v4.5 software, Abacus Concepts, Inc., Berkeley, CA, USA). Data are presented as means±s.e.m. with significance considered at  $P<0.05$ .

## RESULTS

### Mitochondrial activity and ROS production

Fig. 1 shows the dependence of oxygen consumption, ATP synthesis and H<sub>2</sub>O<sub>2</sub> release rates on ADP concentration in both gastrocnemius and pectoralis muscles from ducklings in the fed, fasted and refed groups. Regardless of the skeletal muscle type, the rates of oxidation and phosphorylation were significantly lower in fasted than in fed and refed ducklings (Fig. 1A–D). The rates of



**Fig. 1. Effect of fasting and refeeding on kinetic responses of oxygen consumption, ATP synthesis and  $\text{H}_2\text{O}_2$  release to added ADP.** Oxygen consumption (A,B), ATP synthesis (C,D) and  $\text{H}_2\text{O}_2$  release (E,F) rates were determined at  $40^\circ\text{C}$  in mitochondria respiring on pyruvate/malate/succinate and isolated from gastrocnemius (A,C,E) or pectoralis muscles (B,D,F) of fed (open circles), fasted (black circles) or re-fed (grey circles) ducklings. Values are means  $\pm$  s.e.m. for 6 animals. \*Overall kinetic response to added ADP in fasted ducklings is significantly different from that of fed and re-fed ducklings ( $P < 0.05$ ).

basal non-phosphorylating (state 4) oxygen consumption, maximal (state 3) phosphorylating oxygen consumption and associated ATP synthesis were significantly decreased by fasting in both gastrocnemius and pectoralis muscles (Table 1). The maximum (state 3) oxygen consumption and ATP synthesis fluxes were not significantly different between fed and refed groups (Table 1). The overall kinetics of ADP-inhibited mitochondrial H<sub>2</sub>O<sub>2</sub> release from fasted ducklings superimposed on those of the other two groups (Fig. 1E,F). However, the maximum rates of H<sub>2</sub>O<sub>2</sub> release measured under basal non-phosphorylating respiration were significantly lowered by fasting, compared with values in fed and refed ducklings, in both gastrocnemius and pectoralis muscles (Table 1). The lower rates of H<sub>2</sub>O<sub>2</sub> release measured under phosphorylation state 3 were not affected by nutritional status in gastrocnemius muscle, but were significantly lowered by fasting in pectoralis muscle (Table 1). Neither % ROS/O nor % ROS/ATP was significantly affected by the nutritional status of ducklings, irrespective of the muscle type (Table 1).

However, the lower respiration rates of fasted mitochondria also indicate that for a given amount of ADP, fasted mitochondria did not transfer electrons at the same rate as fed and refed mitochondria. As mitochondrial ROS are produced by electrons leaking from the respiratory chain, we correlated the rate of H<sub>2</sub>O<sub>2</sub> release and associated ratios with oxidative activity (Fig. 2). Regardless of nutritional status, the relationships between the rates of H<sub>2</sub>O<sub>2</sub> release and oxygen consumption in both skeletal muscles were not linear and decreased as phosphorylation respiration rate increased (Fig. 2A,B). A sharp decrease occurred at low oxygen consumption (i.e. at low concentrations of ADP), up to a phosphorylating steady-state rate above which H<sub>2</sub>O<sub>2</sub> release decreased more slowly as oxidative phosphorylation capacity increased close to maximum steady state (e.g. above 100 μmol l<sup>-1</sup> ADP). Fig. 2 also shows similar patterns for the electron leak (%ROS/O; Fig. 2C,D) and the oxidative cost of ATP production (%ROS/ATP; Fig. 2E,F). On the whole, fasted curves were significantly shifted to the left compared with fed and refed groups.

### Thresholds in mitochondrial ROS production and associated ratios

Altogether, these results indicate that for a given oxygen consumption, fasted mitochondria displayed lower mitochondrial

H<sub>2</sub>O<sub>2</sub> release rates and associated ratios (% ROS/O, % ROS/ATP). However, the maximal phosphorylation and respiration rates of these mitochondria were also significantly lower, implying that for a given oxygen consumption rate, fasted mitochondria were not working at the same coupling activity as fed and refed mitochondria. For this reason, we determined the percentage maximal oxidative phosphorylation rate at which H<sub>2</sub>O<sub>2</sub> production started to increase sharply, which defines a threshold in mitochondrial H<sub>2</sub>O<sub>2</sub> release. Indeed, the curves in Fig. 2 can be divided into two ascending phases from right to left, showing that mitochondrial H<sub>2</sub>O<sub>2</sub> production increased slowly with decreasing oxidative phosphorylation activity (right part of curves), up to an inflexion point at which H<sub>2</sub>O<sub>2</sub> generation greatly increased in response to a marked decrease in the rate of oxidative phosphorylation activity (left part of curves). First, we determined the rate of oxygen consumption at the inflexion point from the abscissa of the intersection point between the two regression lines for the two ascending phases (Fig. 3A). The inflexion points were significantly lower in the pectoralis than in the gastrocnemius muscle, and in both skeletal muscles, they were significantly lower in fasted than in fed and refed ducklings. Then, we calculated the threshold values by expressing the oxygen consumption at the inflexion point (Fig. 3A) as a percentage of maximal oxidative phosphorylation activity (Fig. 3B). In both skeletal muscles, fasted mitochondria exhibited higher threshold values than those in the refed group ( $P < 0.05$ ), but failed to reach statistical significance compared with the fed group ( $P = 0.06$  in gastrocnemius and pectoralis muscles; Fig. 3B). On the whole, pectoralis muscle mitochondria had lower threshold values than gastrocnemius muscle mitochondria. The data suggest that in fed and refed muscle mitochondria, the oxidative phosphorylation activity needs to be decreased by approximately 65% and 70%, respectively, before a major increase in H<sub>2</sub>O<sub>2</sub> release occurs, whereas in fasted mitochondria, oxidative phosphorylation activity needs to be decreased by only 50% and 60% in gastrocnemius and pectoralis muscle, respectively. Hence, H<sub>2</sub>O<sub>2</sub> release was initiated at a higher coupling state in fasted muscle than in the other two groups. Similar values and results applied for both % ROS/O and % ROS/ATP (data not shown).

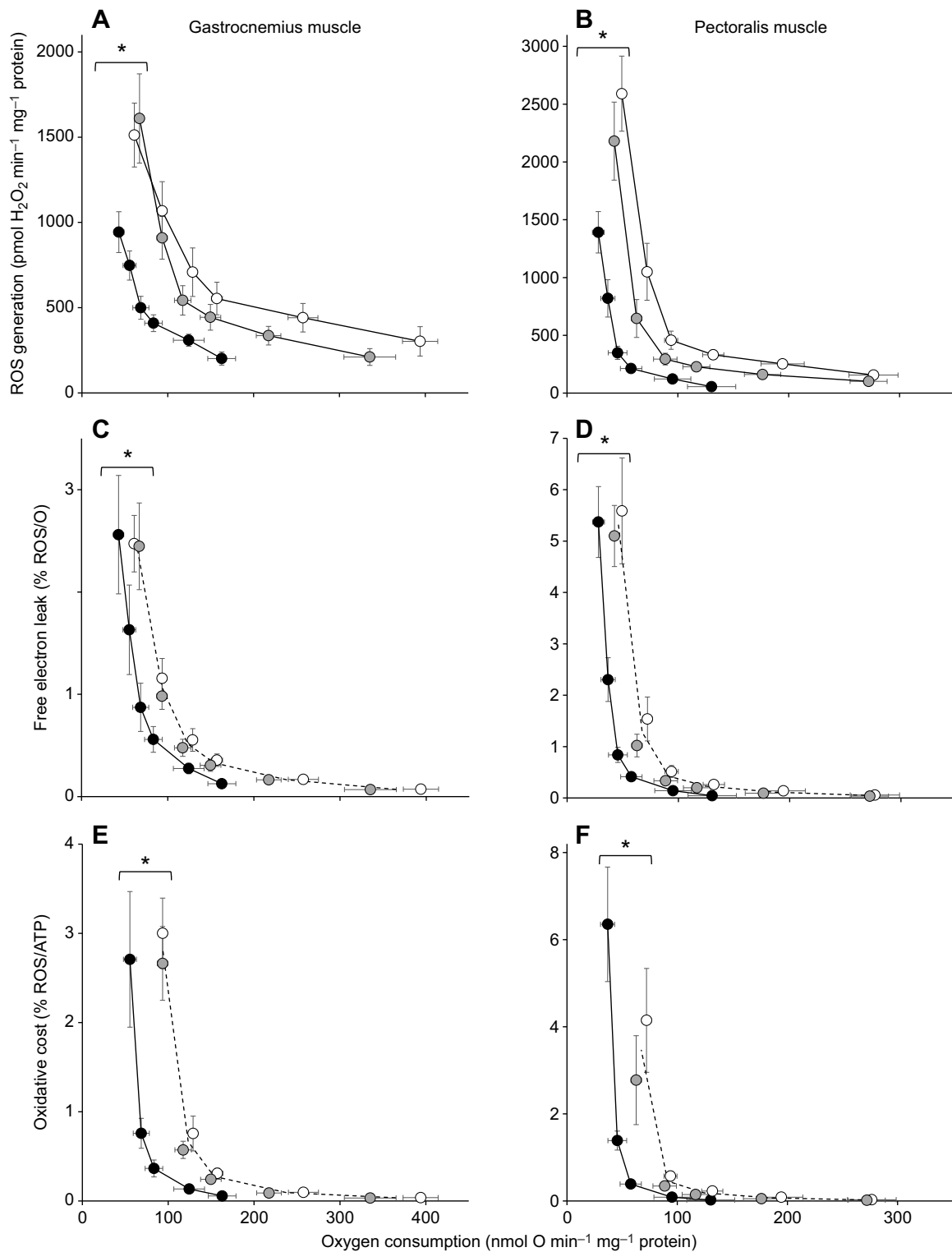
### DISCUSSION

The fundamental conclusion of the present study is that H<sub>2</sub>O<sub>2</sub> release, electron leak (% ROS/O) and the oxidative cost of ATP

**Table 1. Mitochondrial parameters**

Parameters	Gastrocnemius muscle			Pectoralis muscle			Statistical analysis		
	Fed	Fasted	Refed	Fed	Fasted	Refed	Diet	Muscle	Diet×Muscle
ROS production (pmol H <sub>2</sub> O <sub>2</sub> min <sup>-1</sup> mg <sup>-1</sup> protein)									
State 4	1512±187 <sup>a,b</sup>	943±120 <sup>b</sup>	1609±262 <sup>a</sup>	2591±325 <sup>a,*</sup>	1391±179 <sup>b</sup>	2180±338 <sup>a</sup>	<0.01	<0.01	n.s.
State 3	303±86	202±38	211±49	156±23 <sup>a</sup>	56±20 <sup>b,*</sup>	101±20 <sup>a,b</sup>	0.09	<0.01	n.s.
Oxygen consumption rate (nmol O min <sup>-1</sup> mg <sup>-1</sup> protein)									
State 4	61±4 <sup>a</sup>	43±6 <sup>b</sup>	67±4 <sup>a</sup>	49±4 <sup>a</sup>	28±5 <sup>b</sup>	43±3 <sup>a,*</sup>	<0.001	<0.001	n.s.
State 3	394±20 <sup>a</sup>	163±16 <sup>b</sup>	335±30 <sup>a</sup>	277±22 <sup>a,*</sup>	130±22 <sup>b</sup>	272±17 <sup>a</sup>	<0.0001	<0.001	n.s.
ATP synthesis rate (nmol ATP min <sup>-1</sup> mg <sup>-1</sup> protein)									
State 3	812±53 <sup>a</sup>	362±32 <sup>b</sup>	721±96 <sup>a</sup>	573±52 <sup>a,*</sup>	259±43 <sup>b</sup>	608±64 <sup>a</sup>	<0.0001	<0.01	n.s.
Ratios									
ATP <sub>State3</sub> /O <sub>State3</sub>	2.07±0.12	2.29±0.26	2.12±0.15	2.06±0.02	2.01±0.11	2.22±0.16	n.s.	n.s.	n.s.
ROS <sub>State4</sub> /O <sub>State4</sub> (%)	2.47±0.28	2.56±0.58	2.45±0.42	5.59±1.03 <sup>*</sup>	5.37±0.69 <sup>*</sup>	5.10±0.59 <sup>*</sup>	n.s.	<0.0001	n.s.
ROS <sub>State3</sub> /ATP <sub>State3</sub> (%)	0.37±0.10	0.55±0.09	0.33±0.09	0.28±0.04	0.21±0.05 <sup>*</sup>	0.16±0.03	n.s.	<0.01	n.s.

Ducklings were fed *ad libitum* (Fed) or fasted for 6 days (Fasted) or fasted for 6 days and then refed for 3 days (Refed). ROS, reactive oxygen species; State 3, maximal ADP-stimulated oxidative phosphorylation activity; State 4, basal non-phosphorylating oxygen consumption rate measured in the presence of oligomycin; ATP<sub>State3</sub>/O<sub>State3</sub>, oxidative phosphorylation efficiency calculated under state 3; ROS<sub>State4</sub>/O<sub>State4</sub>, free electron leakage calculated under state 4; ROS<sub>State3</sub>/ATP<sub>State3</sub>, oxidative cost of ATP production calculated under state 3. Values are means±s.e.m. from N=6 ducklings. Data with different superscript letters are significantly different at  $P < 0.05$  within the same skeletal muscle. \* $P < 0.05$ , significantly different from corresponding gastrocnemius muscle value within the same nutritional group; n.s., not significant.

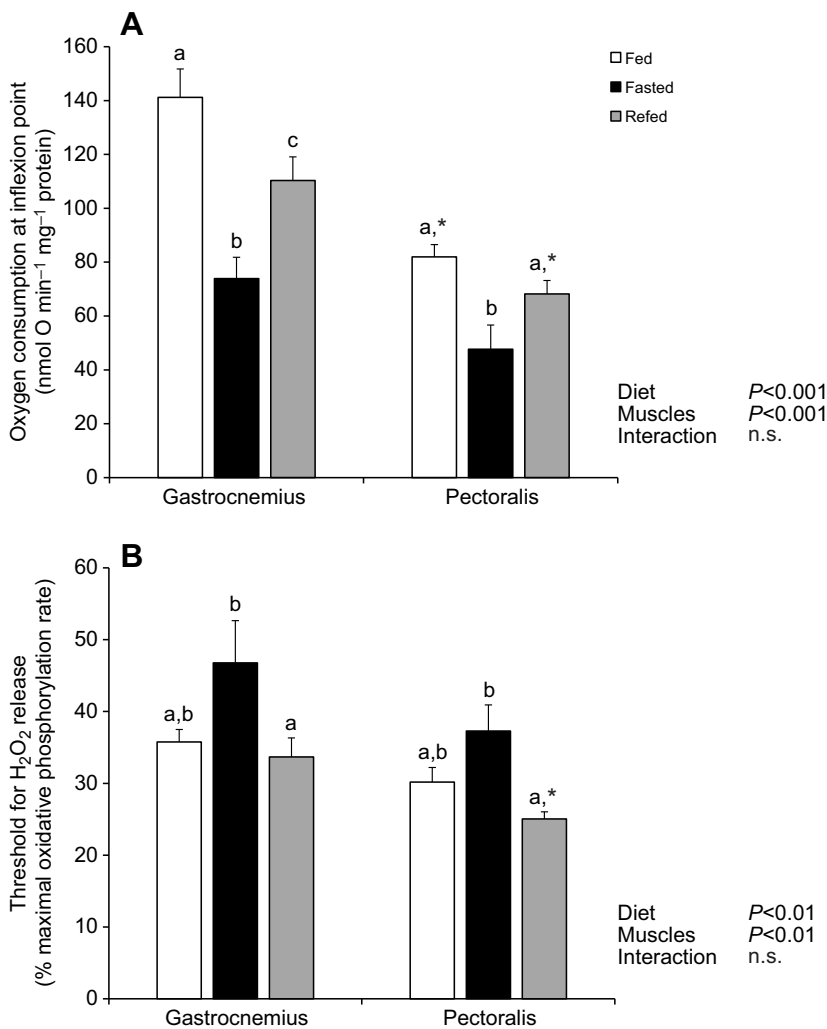


**Fig. 2. Effect of fasting and refeeding on the relationship between oxidative phosphorylation activity and H<sub>2</sub>O<sub>2</sub> release, free radical electron leak and the oxidative cost of ATP production.** Reactive oxygen species (ROS) generation measured as H<sub>2</sub>O<sub>2</sub> release (A,B), free radical electron leak (% ROS/O; C,D) and the oxidative cost of ATP production (% ROS/ATP; E,F) were determined in mitochondria isolated from gastrocnemius (A,C,E) or pectoralis muscles (B,D,F) of fed (open circles), fasted (black circles) or re-fed (grey circles) ducklings. Values are means  $\pm$  s.e.m. for 6 animals. \*The relationship between H<sub>2</sub>O<sub>2</sub> release and associated ratios and oxygen consumption has significantly shifted to the left in fasted ducklings ( $P < 0.05$ ).

production (% ROS/ATP) did not linearly evolve with the level of oxidative phosphorylation activity. Instead, these parameters remained low until a threshold of oxidative phosphorylation activity was reached, at which point they abruptly increased.

These threshold values depended on muscle type and nutritional status.

Published studies show that mitochondrial ROS production and free electron leak are steeply and non-linearly sensitive to proton



**Fig. 3. Effect of fasting and refeeding on threshold ROS production values.** Data are expressed as the oxygen consumption rate at the inflexion point of H<sub>2</sub>O<sub>2</sub> release (A) or as a percentage of maximal oxidative phosphorylation activity (B) (see Materials and Methods for more details) in mitochondria isolated from gastrocnemius or pectoralis muscles from fed, fasted and refed ducklings. Values are means±s.e.m. for 6 animals. Data with different superscript letters are significantly different within the same skeletal muscle. \*Significantly different from corresponding gastrocnemius muscle value within the same nutritional group ( $P<0.05$ ).

motive force and the redox state of the respiratory chain (Korshunov et al., 1997; Starkov and Fiskum, 2003; Quinlan et al., 2012; Kikusato and Toyomizu, 2013; Treberg et al., 2018). Hence, ROS generation is negatively correlated to the intensity of phosphorylating oxygen consumption and ATP production (Barja, 2007). These kinetics are clearly explained by the fact that the amount of electron leak from the respiratory chain, which semi-reduced oxygen to generate ROS (% ROS/O), strongly decreases during the transition from basal to maximal ATP synthesis activity (Fig. 2), as a result of the enhanced oxidized state of electron transport chain intermediates (Starkov and Fiskum, 2003; Quinlan et al., 2012). However, quantitative and qualitative questions still remain regarding % ROS/ATP and its dependence on the rate of electron flow. The method used here clearly illustrates the steep and non-linear relationship between mitochondrial oxidative phosphorylation activity and H<sub>2</sub>O<sub>2</sub> production and associated ratios. As for free electron leak (% ROS/O), % ROS/ATP is highly sensitive to oxidative phosphorylation activity, with values ranging from 0.02% in a fully active and strongly coupled state to 4% in the least active and highly uncoupled state measured in both skeletal muscles of fed animals (Fig. 2). This is in line with the much lower rates of mitochondrial superoxide production measured in skeletal muscle mitochondria *ex vivo* under mild and intense aerobic metabolism than at rest (Goncalves et al., 2015). Interestingly, the steep and non-linear shape of the relationships further indicates the

existence of a critical oxidative activity below which H<sub>2</sub>O<sub>2</sub> production and associated ratios increased sharply. In fed and refed birds, we calculated that the oxidative phosphorylation capacities of muscle mitochondria must be decreased by approximately 70% before ROS production rapidly increases (Fig. 3).

In fasted ducklings, a significant decrease in mitochondrial H<sub>2</sub>O<sub>2</sub> production was detected during basal non-phosphorylating respiration in both gastrocnemius and pectoralis muscles. In addition, when the whole relationship between H<sub>2</sub>O<sub>2</sub> production and mitochondrial oxidative phosphorylation activity is considered (Fig. 2), muscle mitochondria from birds fasted for 6 days exhibited a lower H<sub>2</sub>O<sub>2</sub> production and lower oxidative costs (% ROS/O and % ROS/ATP) at any given rate of mitochondrial substrate oxidation compared with mitochondria from nourished birds. In the present study, ROS production was measured as the net H<sub>2</sub>O<sub>2</sub> release from mitochondria using Amplex Red. Thus, the observed changes in H<sub>2</sub>O<sub>2</sub> efflux could be due to lower ROS production and/or a higher endogenous ROS scavenging capacity of mitochondria (Quinlan et al., 2012; Munro et al., 2016). In birds, activities of antioxidant enzymes (glutathione peroxidase, Mn- and Cu,Zn-superoxide dismutase) were not altered by long-term fasting in skeletal muscles (Rey et al., 2008). Alternatively, mitochondrial ROS production can also be sensitive to endogenous mitochondrial proton conductance and subsequent changes in the proton motive

force and redox state of the Q pool (Brand, 2000). But again, fasting did not change muscle mitochondrial proton conductance in ducklings (Roussel et al., 2018). Therefore, the present downregulation of H<sub>2</sub>O<sub>2</sub> generation could be explained by an overall decrease in the substrate oxidation and electron fluxes within the respiratory chain (Roussel et al., 2018). In turn, this implies that for a given oxygen consumption rate, fasted mitochondria are not working at the same coupling intensity as fed and refed mitochondria. The threshold values allow calculation of the percentage maximal oxidative phosphorylation coupling activity at which mitochondrial ROS generation starts to increase sharply. After 6 days of fasting, the threshold values for H<sub>2</sub>O<sub>2</sub> production and associated ratios were increased compared with those of nourished birds. In other words, muscle mitochondria from fasted ducklings must be more strongly coupled than those of nourished birds to achieve low levels of H<sub>2</sub>O<sub>2</sub> production.

The two skeletal muscles used in the present study display contrasting metabolic phenotypes, with a more oxidative phenotype for gastrocnemius muscle than for pectoralis muscle (Monternier et al., 2015; Monternier et al., 2017). In line with previous studies (Anderson and Neuffer, 2006; Picard et al., 2012; Rey et al., 2013), mitochondria from the glycolytic pectoralis muscle exhibited greater H<sub>2</sub>O<sub>2</sub> release and free electron leak than that of mitochondria from the more oxidative gastrocnemius muscle. Nevertheless, muscle differences in H<sub>2</sub>O<sub>2</sub> production were only found in the basal non-phosphorylating state. Indeed, mitochondrial H<sub>2</sub>O<sub>2</sub> production in the maximal (state 3) phosphorylating state was lower in the glycolytic pectoralis muscle than in the oxidative gastrocnemius muscle (Table 1). This opposing difference in mitochondrial metabolism of H<sub>2</sub>O<sub>2</sub> in phosphorylating respiration might simply result from a higher capacity of the gastrocnemius muscle to supply electrons to the electron transport chain, as neither the free electron leakage under phosphorylating respiration (present study) nor the constitutive membrane proton leak activity (Rey et al., 2013) was significantly different between the two skeletal muscles. Notwithstanding the underlying mechanisms, it is worth noting that the skeletal muscle with the highest capacity to generate H<sub>2</sub>O<sub>2</sub> (i.e. the glycolytic pectoralis muscle) is preferentially degraded during fasting (Monternier et al., 2017). In flightless growing ducklings, pectoralis muscles are little used and poorly developed, whereas leg muscles, including gastrocnemius muscles, are highly functional and active. Inactive pectoralis muscles of fasting ducklings might therefore be at risk as they would be a site of low mitochondrial oxidative activity and high ROS generation. The selective pectoralis muscle wasting during fasting might not be surprising when considering that (1) inactivity induces skeletal muscle atrophy (McCue, 2010; Pierre et al., 2016) and (2) mitochondrial oxidative stress could contribute to muscle wasting by promoting autophagy (Calvani et al., 2013; Rahman et al., 2014).

In conclusion, the energy-saving benefits of mitochondrial hypo-metabolism observed during fasting were associated with a lower maximal rate of H<sub>2</sub>O<sub>2</sub> release. This lower absolute capacity of H<sub>2</sub>O<sub>2</sub> emission was mainly achieved by an overall decrease in citric acid cycle activity and the subsequent lower entry of electrons into the respiratory chain (Roussel et al., 2018). All these changes were fully reversed by 3 days of refeeding, highlighting the flexibility of mitochondrial processes. Yet, the fundamental finding of the present study is that H<sub>2</sub>O<sub>2</sub> release and associated ratios remain low until a threshold level of mitochondrial inactivity is exceeded. In the present study, it was shown that mitochondrial metabolism must decrease by 60–70% before oxidative stress occurs in skeletal muscle. Whether the ROS generation threshold varies between

tissues and how it differs in other species or physiological conditions remains to be determined, to better understand the oxidative stress processes.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: D.R., C.D., Y.V.; Methodology: D.R., Y.V.; Validation: D.R., Y.V.; Formal analysis: D.R., M.B., C.R., Y.V.; Investigation: D.R., M.B., M.M., C.R., Y.V.; Writing - original draft: D.R., Y.V.; Writing - review & editing: M.B., C.D.

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