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RESEARCH ARTICLE

Fasting enhances mitochondrial efficiency in duckling skeletal muscle by acting on the substrate oxidation system

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ABSTRACT

During food deprivation, animals must develop physiological responses to maximize energy conservation and survival. At the subcellular level, energy conservation is mainly achieved by a reduction in mitochondrial activity and an upregulation of oxidative phosphorylation efficiency. The aim of this study was to decipher mechanisms underlying the increased mitochondrial coupling efficiency reported in fasted birds. Mitochondrial oxidative phosphorylation activity, efficiency and membrane potential were measured in mitochondria isolated from the gastrocnemius muscle of ducklings. The content and activities of respiratory chain complexes were also determined. Results from ducklings fasted for 6 days were compared with ducklings fed *ad libitum*. Here, we report that 6 days of fasting improved coupling efficiency in muscle mitochondria of ducklings by depressing proton-motive force through the downregulation of substrate oxidation reactions. Fasting did not change the basal proton conductance of mitochondria but largely decreased the oxidative phosphorylation activity, which was associated with decreased activities of succinate–cytochrome *c* reductase (complexes II–III) and citrate synthase, and altered contents in cytochromes *b* and $c+c_1$. In contrast, fasting did not change cytochrome aa_3 content or the activity of complexes I, II and IV. Altogether, these data show that the lower capacity of the respiratory machinery to pump protons in ducklings fasted for 6 days generates a lower membrane potential, which triggers a decreased proton leak activity and thus a higher coupling efficiency. We propose that the main site of action would be located at the level of co-enzyme *Q* pool/complex III of the electron transport chain.

KEY WORDS: Bioenergetics, Proton conductance, Membrane potential, Oxidative phosphorylation, Electron transport chain, Birds

INTRODUCTION

Fasting is a major characteristic of the life cycle of many wild mammals and birds. These periods of food shortage often occur during reproduction, moult, migration or winter, when biological activities compete with feeding or when food is scarce or lacking (Mrosovsky and Sherry, 1980). To survive under these conditions, two prominent physiological mechanisms are developed: (i) a metabolic shift towards the use of internal energy storage (mainly lipids); and (ii) the reduction of energy expenditure by adjusting activity and thermoregulation (McCue, 2010; Staples, 2014). For instance, ducklings reduce their resting metabolic rate after 1 week of food deprivation through shallow hypothermia, a loss of metabolically active tissues and a hypometabolism of their mitochondria (Monternier et al., 2017). Similarly, long-term fasted king penguin chicks have lower resting metabolic rate, exhibit shallow hypothermia, reduced lean body mass and lower mitochondrial activity (Duchamp et al., 1989, 1991; Eichhorn et al., 2011; Bourguignon et al., 2017). At the subcellular level, oxygen consumption is mostly (at 90%) linked to mitochondrial activities (Rolfe and Brand, 1997). Therefore, variations in mitochondrial activity during food shortage would contribute to a change in metabolic rate and animal performance (Speakman et al., 2004; Salin et al., 2015; Bourguignon et al., 2017).

Food restriction is well known to remodel bioenergetics and dynamics in skeletal muscles of mammals and birds (Crescenzo et al., 2003; Abe et al., 2006; Ramsey and Hagopian, 2006; de Lange et al., 2007; Rey et al., 2008; Putti et al., 2015). As a whole, the reduction in mitochondrial oxidative activity appears as a general characteristic of the effect of food restriction, although duration and intensity of the restriction, as well as the age of animals, would influence the adjustment pattern of mitochondrial machinery (Bevilacqua et al., 2004; Dumas et al., 2004; Johnson et al., 2006; Lanza et al., 2012; Bourguignon et al., 2017). In long-term (5 months), calorie-restricted rats, the lower respiratory activity of muscle fibers was associated with an enhanced mitochondrial coupling efficiency (Zangarelli et al., 2006). Similarly, long-term fasted birds exhibited a higher mitochondrial efficiency in their skeletal muscles and liver which, together with mitochondrial hypometabolism, have been estimated to contribute up to 20% of the reduction of whole-body oxygen consumption (Rey et al., 2008; Monternier et al., 2017; Bourguignon et al., 2017). Mitochondrial coupling efficiency (ATP/O ratio) describes how much oxygen, and so how much substrates, are needed to produce ATP and fulfil cellular energy requirements (Brand, 2005). For this reason, a higher mitochondrial coupling efficiency triggers additional energy saving by channeling the energy of oxidized substrates to ATP synthesis and cellular energy-demanding processes. However, the underlying mechanisms remain unclear.

The aim of this work was to decipher the mitochondrial mechanisms responsible for fasting-induced changes in oxidative phosphorylation activity (downregulation) and coupling efficiency (upregulation) of skeletal muscle mitochondria in ducklings. In the mitochondrial energy transduction system, the electrochemical proton gradient builds up by the activity of respiratory chain is mainly coupled to ATP synthesis, but also consumed by futile proton cycling across the inner membrane. This means that the efficiency of mitochondrial oxidative phosphorylation will be, in part, determined by the activity of proton leakage during ATP synthesis. For instance, agents that increase inner membrane proton conductance and therefore proton leak activity, such as thyroid hormones (Hafner et al., 1988; Nogueira et al., 2002), mitochondrial uncouplers (Beavis and Lehninger, 1986; Clerc et al., 2007) or

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cachexia (Dumas et al., 2011), will tend to decrease mitochondrial coupling efficiency. Alternatively, a small inhibition of the respiratory chain has been shown to increase mitochondrial oxidative phosphorylation efficiency, with no change in membrane proton conductance (Clerc et al., 2007). Altogether, these results suggest that skeletal muscle mitochondria from fasted birds may display a higher coupling ATP/O ratio either because their inner membranes are less leaky to protons or because the proton-motive force is lower due to the depressed capacity of the respiratory machinery to pump protons. Here, we show that fasting-induced upregulation of mitochondrial coupling efficiency in birds was not accompanied by changes in the basal proton conductance of mitochondria isolated from skeletal muscle but resulted from a downregulation of their oxidative capacity.

MATERIALS AND METHODS

Animals

The present investigation was conducted in accordance with the guiding principles in the care and use of animals and was approved by the Ethics Committee of Lyon (project no. DR2013-54v2). Newly hatched male Muscovy ducklings, *Cairina moschata* (Linnaeus 1758), were obtained from a commercial stockbreeder (Eclosion Grimaud Frères, Roussay, France). Ducklings were kept at thermoneutrality for 4 weeks in a constant photoperiod (light:dark 8 h:16 h) with food and water *ad libitum*. Thereafter, birds were kept at thermoneutrality (25°C) and randomly divided into two groups: one-half continued to be fed *ad libitum* and the other half was fasted for 6 days.

Mitochondrial isolation and maximal oxidative activity

Animals were stunned by cranial percussion and killed by decapitation. The red part of the gastrocnemius muscle was rapidly withdrawn, weighed and freshly used for mitochondrial isolation. Mixed skeletal muscle mitochondrial populations were isolated in an ice-cold isolation buffer (100 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 50 mmol l^{-1} Tris-base, pH 7.4 at 4°C), following a standard extraction protocol, using a Potter homogenizer, partial protease digestion and differential centrifugation, as previously described (Monternier et al., 2014). Maximal oxidative activity of muscle mitochondria (0.3 mg ml^{-1}) was determined at 40°C in respiratory buffer containing 120 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} EGTA, 2 mmol l^{-1} $MgCl_2$, 0.3% bovine serum albumin (w/v) and 3 mmol l^{-1} Hepes (pH 7.4). The basal non-phosphorylating respiration rate (state 4_{oligo}) was measured in the presence of 1 µg ml^{-1} oligomycin. The maximal fully uncoupled respiration rate (state FCCP) was initiated by the addition of 2 µmol l^{-1} carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The respiratory control ratio (RCR_{unc}) refers to the ratio of state FCCP to state 4_{oligo}. Respiration was measured using different combinations of respiratory substrates: pyruvate/malate (5/2.5 mmol l^{-1}), succinate (5 mmol l^{-1} in the presence of 5 µmol l^{-1} rotenone), and pyruvate/malate/succinate (5/2.5/5 mmol l^{-1}).

Mitochondrial oxidative phosphorylation efficiency

Mitochondrial oxidative phosphorylation efficiency was determined at 40°C by measuring ATP synthesis concurrently with the oxygen consumption in respiratory buffer supplemented with 20 mmol l^{-1} glucose and 1.6 U ml^{-1} hexokinase. Muscle mitochondria (0.3 mg ml^{-1}) were respiring on succinate (5 mmol l^{-1} in the presence of 5 µmol l^{-1} rotenone) or pyruvate/malate/succinate (5/2.5/5 mmol l^{-1}). In order to investigate the effect of the mitochondrial oxidation system on mitochondrial coupling, we also measured oxidative phosphorylation efficiency in the presence of 55 µmol l^{-1} potassium cyanide (KCN), a specific inhibitor of cytochrome *c* oxidase, in fed mitochondria respiring on pyruvate/malate/succinate. Note that this dose of KCN has been determined on each mitochondrial preparation and represents the average quantity needed to bring the non-phosphorylating respiration rate of fed mitochondria up to the fasted level. Thereafter, different steady states of phosphorylation were obtained by adding ADP at 500 µmol l^{-1}, 100 µmol l^{-1}, 20 µmol l^{-1} or 10 µmol l^{-1}. After recording the phosphorylating respiration rate for 2–3 min in a glass cell fitted with a Clark electrode (Rank Brothers Ltd, Bottisham, UK), four 100 µl samples of mitochondrial suspension were withdrawn every 30 s and immediately quenched in ice-cold 100 µl perchloric acid solution (10% $HClO_4$/25 mmol l^{-1} EDTA). Samples were centrifuged at 20,000 ***g*** for 5 min at 4°C, thereafter 180 µl of the resulting supernatants were neutralized with KOH (2 mol l^{-1} KOH/0.3 mol l^{-1} MOPS). After centrifugation of the neutralized samples (20,000 ***g*** for 5 min at 4°C), the mitochondrial ATP production was determined from the glucose-6-phosphate content of the resulting supernatants, which was measured enzymatically by spectrophotometry at 340 nm in an assay medium (7.5 mmol l^{-1} $MgCl_2$, 3.75 mmol l^{-1} EDTA, 50 mmol l^{-1} triethanolamine–HCl, pH 7.4 at room temperature) supplemented with 0.5 mmol l^{-1} NAD and 0.5 U glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Lang and Michal, 1974).

Mitochondrial membrane potential

Respiration rate and membrane potential were measured simultaneously at 40°C using electrodes sensitive to oxygen and to the potential-dependent probe triphenylmethylphosphonium ($TPMP^+$), respectively. Muscle mitochondria (0.25 mg ml^{-1}) were incubated in 2 ml of respiratory buffer supplemented with 5 µmol l^{-1} rotenone, 60 ng ml^{-1} nigericin and 1 µg ml^{-1} oligomycin. The $TPMP^+$ electrode was calibrated by sequential 0.5 µmol l^{-1} additions up to 2 µmol l^{-1} $TPMP^+$, then 5 mmol l^{-1} succinate was added to start the reaction. The kinetic response of the proton conductance to its driving force (proton-motive force) was measured as the relationship between oxygen consumption and membrane potential when the potential is varied by the sequential addition of malonate up to 4 mmol l^{-1} (Brand and Nicholls, 2011). The kinetic response of substrate oxidation rate to proton-motive force was determined either in the presence of oligomycin and nigericin by manipulating the mitochondrial proton leak with increasing doses of FCCP (a mitochondrial uncoupler) or in the absence of nigericin by manipulating the ATP synthesis capacity from a fully active respiration (oligomycin absent; 500 µmol l^{-1} ADP; state 3) to non-phosphorylating resting respiration (1 µg ml^{-1} oligomycin present; state 4) (Brand and Nicholls, 2011; Roussel et al., 2004). After each run, 2 µmol l^{-1} FCCP was added to dissipate the membrane potential and release all $TPMP^+$ back into the medium for baseline correction. Membrane potentials were calculated as previously described by Brand (1995), assuming a $TPMP^+$-binding correction of 0.35 µl mg^{-1} of protein for skeletal muscle mitochondria (Rolfe et al., 1994).

Mitochondrial cytochrome content

The cytochromes of the mitochondrial respiratory chain were measured by dual-wavelength spectrophotometry by comparing the spectra of fully oxidized (with 8 mmol l^{-1} ferricyanide) versus fully reduced (with 5 mmol l^{-1} ascorbate plus few grains of sodium hydrosulfite) cytochromes (Williams, 1964). Mitochondria (0.8 mg ml^{-1}) were incubated in 600 µl of 100 mmol l^{-1}

potassium phosphate buffer (pH 7.4) supplemented with 1% Triton X-100, and absorbance spectra were recorded between 500 and 650 nm. Wavelength pairs and absorption coefficient used were: *c*-type cytochromes $(c+c_1)_{(550–535\ nm)}$ ε=18.5 l mmol^{-1} cm^{-1}; cytochrome $b_{(563–575\ nm)}$ ε=18.5 l mmol^{-1} cm^{-1}; and cytochrome $aa_{3(605–630\ nm)}$ ε=24 l mmol^{-1} cm^{-1}. Note that mitochondrial cytochromes are mainly associated with complexes of the electron transport chain: the *a*-type cytochromes belong to complex IV (cytochrome *c* oxidase), the *b*-type cytochromes are associated with complex III (two b-type haems) and complex II (one b-type haem), and the *c*-type cytochromes are either found in complex III (cytochrome c_1) or in the intermembrane space (cytochrome *c*) of mitochondria (Nicholls and Ferguson, 2001).

Mitochondrial enzyme activity

Cytochrome *c* oxidase activity was measured in freshly isolated mitochondria respiring on 4 mmol l^{-1} ascorbate plus 50 µmol l^{-1} *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene-diamine (TMPD) in respiratory buffer (see above) supplemented with 10 µmol l^{-1} antimycin, 1 µg ml^{-1} oligomycin and 2 µmol l^{-1} FCCP. Note that ascorbate and TMPD were successively added in the respiratory chamber. Then, the maximal respiration rate associated with isolated cytochrome *c* oxidase activity was calculated as the rate of oxygen consumption measured in the presence of ascorbate/TMPD minus the oxygen consumed in the presence of ascorbate alone.

Citrate synthase, 3-hydroxyacyl-CoA dehydrogenase, NADH– and succinate–ubiquinone reductases were measured spectrophotometrically at 40°C (Medja et al., 2009; Monternier et al., 2017; Bourguignon et al., 2017). Frozen mitochondria underwent three freeze/thaw cycles before being diluted in 100 mmol l^{-1} phosphate buffer supplemented with 2 mmol l^{-1} EDTA. Citrate synthase activity was assayed at 412 nm in a reaction medium containing 100 mmol l^{-1} Tris buffer (pH 8), 100 µmol l^{-1} 5,5′-dithiobis(2-nitrobezoic acid), 300 µmol l^{-1} acetyl-CoA and 15 µg mitochondrial protein ml^{-1}. After 3 min of incubation, the reaction was started by adding 500 µmol l^{-1} oxaloacetate, and enzyme activity was quantified using an extinction coefficient of 13.6 l mmol^{-1} cm^{-1}. Rotenone-sensitive activity of NADH–ubiquinone reductase and 3-hydroxyacyl-CoA dehydrogenase activity were measured at 340 nm in a reaction medium containing 50 mmol l^{-1} phosphate buffer (pH 7.5), 100 µmol l^{-1} decylubiquinone, 3.75 mg ml^{-1} bovine serum albumin and 15 µg mitochondrial protein ml^{-1} (in the presence or absence of 12.5 µmol l^{-1} rotenone) or 40 mmol l^{-1} imidazole (pH 7), 1 mmol l^{-1} EDTA, 200 µmol l^{-1} NADH and 30 µg mitochondrial protein ml^{-1}, respectively. After 3 min of incubation, reactions were started by adding either 100 µmol l^{-1} NADH (NADH–ubiquinone reductase) or 200 µmol l^{-1} aceto-acetyl-CoA (3-hydroxyacyl-CoA dehydrogenase), and enzyme activities were quantified using an extinction coefficient of 6.22 l mmol^{-1} cm^{-1}. Succinate–ubiquinone reductase was assayed at 600 nm in a reaction medium containing 25 mmol l^{-1} phosphate buffer (pH 7.5), 20 mmol l^{-1} succinate, 50 µmol l^{-1} 2,6-dichlorophenolindophenol (DCPIP), 1 mmol l^{-1} KCN, 100 µmol l^{-1} ATP, 2 mg ml^{-1} bovine serum albumin and 5 µg mitochondrial protein ml^{-1}. After 3 min of incubation, the reaction was started by adding 100 µmol l^{-1} decylubiquinone, and enzyme activity was quantified using an extinction coefficient of 19.2 l mmol^{-1} cm^{-1}. Succinate–cytochrome *c* reductase was assayed at 550 nm in a reaction medium containing 50 mmol l^{-1} phosphate buffer (pH 7.5), 2 mg ml^{-1} bovine serum albumin, 1 mmol l^{-1} KCN, 12.5 µmol l^{-1} rotenone, 100 µmol l^{-1} cytochrome *c* and 5 µg mitochondrial protein ml^{-1}. After 3 min of incubation, the reaction was started by adding 10 mmol l^{-1} succinate, and enzyme activity was quantified using an extinction coefficient of 18.5 l mmol^{-1} cm^{-1}

Statistical analyses

Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by protected least-significant difference tests (Statview v4.5 software, Berkeley, CA, USA). Data are presented as means±s.e.m. with significance considered at $P<0.05$.

RESULTS

Muscle mitochondrial oxidative phosphorylation efficiency

Fig. 1A shows the linear relationship between the rates of ATP synthesis and oxygen consumption in skeletal muscle mitochondria respiring on succinate at different steady-state rates of ATP production. After 6 days of fasting, the maximal rates of ATP synthesis and corresponding oxygen consumption (the highest points to the right of the linear relationships) was significantly decreased by an average of 45% in fasted ducklings compared with fed birds. The slope values of the linear relationships were significantly increased by fasting (ATP/O_{fasted}=1.90±0.02 versus ATP/O_{fed}=1.73±0.05; $P<0.01$), indicating that the relationships between the rates of ATP synthesis and oxygen consumption were divergent. The basal non-phosphorylating respiration rates measured in the presence of oligomycin (the intercepts with the *x*-axis) were also significantly decreased by 30% in fasted animals, indicating that the whole linear relationship in fasted ducklings was significantly shifted to the left compared with fed birds (Fig. 1A). Thereby, at any steady-state rates of ATP production, less oxygen was consumed by skeletal muscle mitochondria of fasted ducklings. In other words, the mitochondrial oxidative phosphorylation of fasted ducklings was more efficient, consuming less oxygen and thus oxidizing less succinate to produce a given amount of ATP. This is clearly illustrated in Fig. 1B showing that at any given rate of oxidative flux, the effective ATP/O ratio was higher in fasted than in fed ducklings. This result extends our previous findings on fasted birds that reported lower oxidative phosphorylation activity and higher efficiency of mitochondria respiring on pyruvate/malate or palmitoyl-L-carnitine/malate (Monternier et al., 2014).

Kinetic responses of proton leak and substrate oxidation system to changes in membrane potential

In the non-phosphorylating state (the intercepts with the *x*-axis in Fig. 1), all the proton current generated by the activity of the respiratory chain return back into the matrix via the leak pathway, the ATP/O ratio is zero and the proton leak-dependent respiration is maximal. In order to understand whether the significant reduction in maximal leak-dependent respiration in skeletal muscle mitochondria of fasted ducklings was associated with a change in inner membrane proton conductance, we measured the kinetic response of the proton leak to the membrane potential, its driving force. Fig. 2A shows that membrane proton conductance was the same in fasted and fed mitochondria, because at any given membrane potential, the rates of oxygen consumed to counteract the proton leak were not significantly different. It is worth noting here that the steep increase in the rate of proton leaks during the transition from the phosphorylating to the non-phosphorylating respiratory state accounts for the non-linear relationship between the effective ATP/O and mitochondrial oxidative phosphorylation activity shown in Fig. 1B (Brand et al., 1993; Brand and Nicholls, 2011).

In contrast, 6 days of fasting resulted in a decrease in substrate oxidation capacity of mitochondria to pump protons. Indeed, at any

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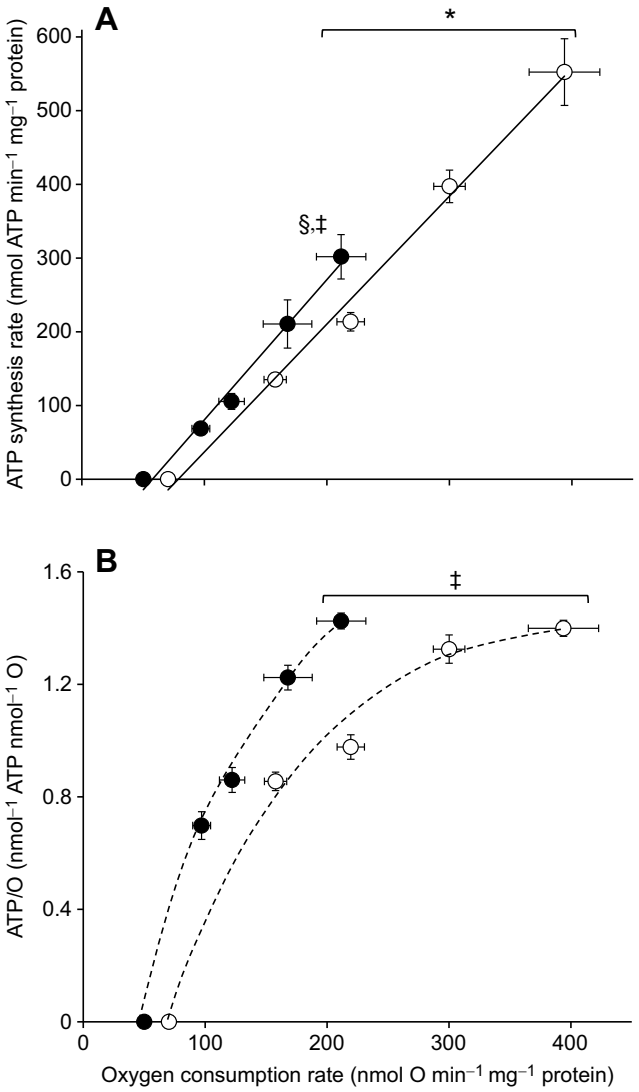

Fig. 1. Effect of fasting on mitochondrial oxidative phosphorylation efficiency. The rates of oxygen uptake and ATP synthesis were measured in mitochondria isolated from the gastrocnemius muscle of ducklings fasted for 6 days (filled symbols; *N*=8) and ducklings fed *ad libitum* (open symbols; *N*=7). Succinate was used as the respiratory substrate. Values are means±s.e.m. from 7–8 independent mitochondrial preparations. (A) Relationship between ATP synthesis and oxygen consumption. *The linear relationship between ATP synthesis and oxygen consumption is significantly shifted to the left in fasted ducklings (see Results for more details). §,‡Significant effect of fasting (P<0.05) on maximal ATP synthesis rate (§) and oxygen consumption (‡) (the highest points to the right of the linear relationships). (B) Relationship between mitochondrial efficiency (ATP/O ratio) and mitochondrial respiratory activity. ‡Significant effect of fasting (P<0.05) on oxygen consumption at any specified steady-state rate (e.g. for each single point in the relationship).

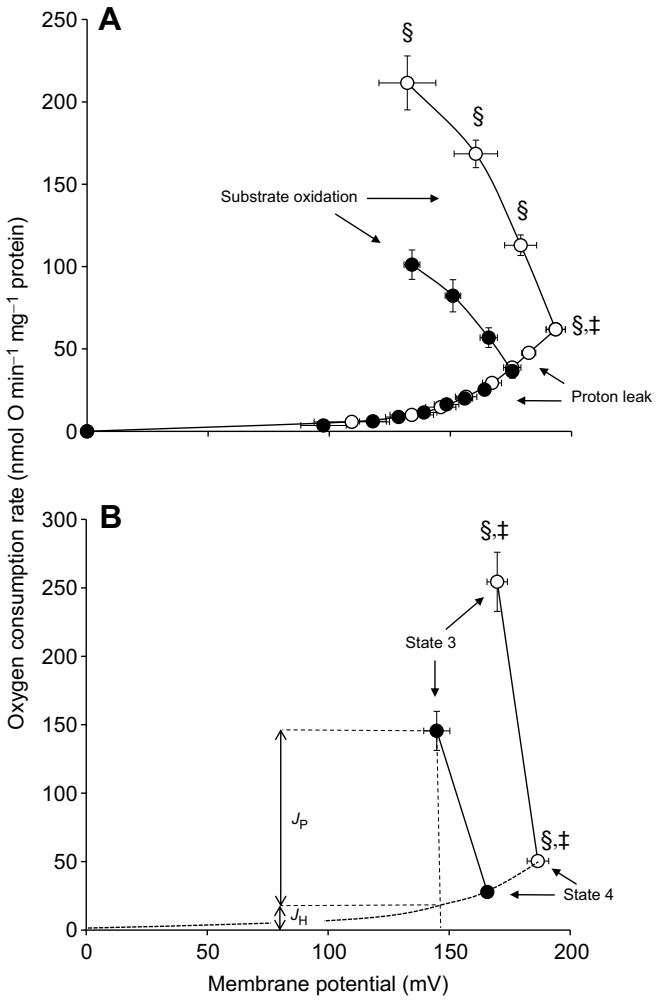

Fig. 2. Kinetic responses of the mitochondrial proton leak and substrate oxidation reactions in fasted and fed ducklings. The rates of oxygen uptake and ATP synthesis were measured in mitochondria isolated from the gastrocnemius muscle of ducklings fasted for 6 days (filled symbols; *N*=6) and ducklings fed *ad libitum* (open symbols; *N*=5). Values are means±s.e.m. from 5–6 independent mitochondrial preparations. (A) The response of substrate oxidation rate to membrane potential was determined by titration of maximal leak-dependent respiration (state 4 oligomycin) with FCCP. The response of proton leak to membrane potential was determined by titration of non-phosphorylating respiration with malonate. (B) The response of substrate oxidation rate to membrane potential was completed in the absence of nigericin with ADP (state 3) and titrated with oligomycin (state 4). See Materials and methods for more details. As illustrated for fasted mitochondria, J_P and J_H are fractions of state 3 respiration that are linked to ATP synthesis or proton leak activity, respectively. §,‡Significant effect of fasting (P<0.05) on oxygen consumption rate (§) and membrane potential values (‡).

given value of membrane potential, the oxygen used to support the activity of the substrate oxidation reactions was significantly lower in fasted than in fed mitochondria (Fig. 2). Conversely, at any given rate of oxygen consumption, the corresponding membrane potential was lower in fasted mitochondria, which would drive a lower proton leak activity and thus a higher effective ATP/O than in fed mitochondria. For instance, the rate of oxygen consumption and corresponding membrane potential at maximum rate of ATP synthesis (state 3) were significantly decreased by fasting, resulting in a lower proton leak activity (J_H) in muscle mitochondria of fasted ducklings compared with fed birds ($J_{H,fasted}$=18±3.2 nmol O min^{-1} mg versus $J_{H,fed}$=37±8 nmol O min^{-1} mg; P<0.05; Fig. 2B). As expected, these data clearly illustrate how changes in the capacity of substrate oxidation reactions to generate proton-motive force alter proton leak activity (Fig. 2) and so the yield of mitochondrial ATP production (Fig. 1).

Involvement of the substrate oxidation system

To further investigate whether the observed increase in oxidative phosphorylation efficiency was a consequence of substrate oxidation inhibition *per se*, we used a submaximal concentration of KCN and measured coupling efficiency in skeletal muscle mitochondria respiring on pyruvate/malate/succinate. As with succinate (Fig. 1)

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and in accordance with our recently published data (Monternier et al., 2017), fasting significantly shifted to the left the linear relationship between ATP synthesis and oxygen consumption (Fig. 3A). In the presence of KCN, fed mitochondria exhibited the same mitochondrial efficiency as fasted mitochondria, because the linear relationship concerning KCN-treated fed mitochondria was superimposed to that of fasted mitochondria (Fig. 3A). Hence, KCN decreased the oxygen consumed by fed mitochondria to produce a given amount of ATP, fully mimicking the effect of fasting in skeletal muscle mitochondria. This is clearly shown in Fig. 3B where, at any given rate of oxygen consumption, ATP/O was the same in the KCN-treated and fasted mitochondria.

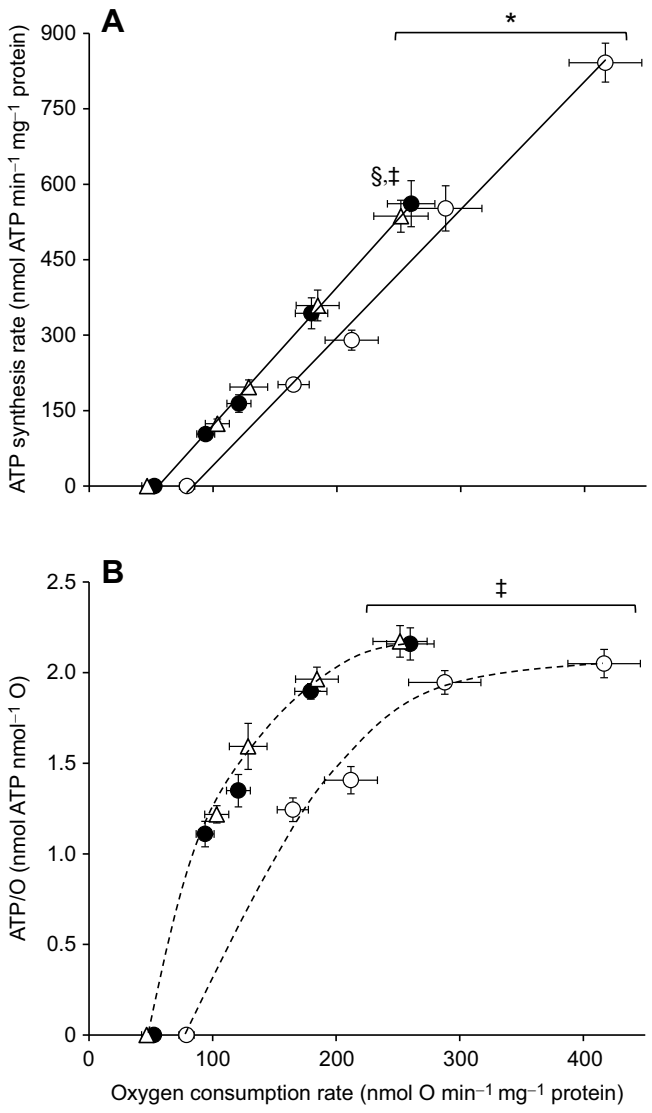

Fig. 3. Effect of KCN on mitochondrial oxidative phosphorylation efficiency. The rates of oxygen uptake and ATP synthesis were measured in mitochondria isolated from the gastrocnemius muscle of ducklings fasted for 6 days (filled symbols; *N*=8) and ducklings fed *ad libitum* (open symbols; *N*=8). Pyruvate/malate/succinate were used as respiratory substrates in the absence (circles) or in the presence of 55 µmol l^{-1} KCN on average (triangles). Values are means±s.e.m. from eight independent mitochondrial preparations. (A) Relationship between ATP synthesis and oxygen consumption. *The linear relationship between ATP synthesis and oxygen consumption is significantly shifted to the left in fasted ducklings (see Results for more details). §,‡Significant effect of fasting (P<0.05) on maximal ATP synthesis rate (§) and oxygen consumption (‡) (the highest points to the right of the linear relationships). (B) Relationship between mitochondrial efficiency (ATP/O ratio) and mitochondrial respiratory activity. ‡Significant effect of fasting (P<0.05) on oxygen consumption at any specified steady-state rate (e.g. for each single point in the relationship).

The substrate oxidation system consists of all reactions involved in the generation of the proton-motive force, and thus includes substrate uptake, dehydrogenases and electron transport chain complexes. Table 1 shows that 6 days of fasting significantly lowered the maximal activity of the respiratory chain with all substrates tested. Basal non-phosphorylating respiration was also significantly affected by fasting with all substrates tested, even though it failed to reach statistical significance with palmitoyl-L-carnitine (P=0.09). The respiratory control ratio (RCR_{unc}) was not affected by the nutritional status, except with succinate (Table 1). Altogether, these data indicate that fasting significantly impacted the oxidation of NADH- and $FADH_2$-linked substrates. In addition, Table 2 shows that the activities of NADH–ubiquinone reductase, succinate–ubiquinone reductase and cytochrome *c* oxidase (complex I, II and IV of the electron transport chain, respectively) were not significantly different between experimental groups. In contrast, the activity of succinate–cytochrome *c* reductase, which involves complex III and co-enzyme *Q* pool, was significantly decreased by 25% in fasted compared with fed mitochondria (Table 2). Table 2 also shows that the activities of citrate synthase (an enzyme of the citric acid cycle) and 3-hydroxyacyl-CoA dehydrogenase (an enzyme of the β-oxidation pathway) were significantly decreased by fasting. After 6 days of fasting, the contents of cytochrome *b* (−17%, P<0.05) and c+c_1 (−26%, P<0.05) of the electron transport chain were significantly decreased compared with fed mitochondria (Table 3). In contrast, the content of cytochrome aa_3, which belongs to complex IV of the electron transport chain, was not significantly affected (Table 3).

DISCUSSION

After 6 days of fasting, skeletal muscle mitochondria exhibited a decreased oxidative phosphorylation activity with all of the respiratory substrates used but did not affect inner membrane proton conductance. Hence, the present study shows that fasting induced a reduction of mitochondrial oxidative activity, which

Table 1. Effect of fasting on respiratory parameters in skeletal muscle mitochondria

Respiratory substrates	Parameters	Fed	Fasted
Palmitoyl-L-carnitine/malate	State 4_{oligo}	24±4	17±2
	State FCCP	329±35	201±21*
	RCR_{unc}	14.2±1.0	12.5±1.2
Pyruvate/malate	State 4_{oligo}	31±2	17±2*
	State FCCP	437±64	228±28*
	RCR_{unc}	14.6±2.1	14.2±1.6
Succinate	State 4_{oligo}	71±5	50±3*
	State FCCP	467±50	248±18*
	RCR_{unc}	6.6±0.5	5.0±0.3*
Pyruvate/malate/succinate	State 4_{oligo}	73±7	52±4*
	State FCCP	588±43	369±35*
	RCR_{unc}	8.2±0.3	7.1±0.3*

Respiration rates are expressed in nmol O min^{-1} mg^{-1} mitochondrial protein. Values are means±s.e.m. from eight independent mitochondrial preparations. *P<0.05 significantly different from fed birds. Mitochondria were isolated from the gastrocnemius muscle as described in Materials and methods. Respiration rates were determined at 40°C. State 4_{oligo}, basal non-phosphorylating respiration measured in the presence of 1 µg ml^{-1} oligomycin. State FCCP, maximal fully uncoupled respiration measured after the addition of 2 µmol l^{-1} FCCP; RCR_{unc}, respiratory control ratio calculated as the uncoupling state FCCP-to-state 4_{oligo} ratio.

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Table 2. Effect of fasting on mitochondrial enzyme activities

Enzymes	Fed	Fasted
3-Hydroxyacyl-CoA dehydrogenase	84±5	57±6*
Citrate synthase	944±62	732±37*
NADH–ubiquinone reductase	312±20	303±30
Succinate–ubiquinone reductase	559±48	506±52
Succinate–cytochrome *c* reductase	249±16	183±13*
Cytochrome *c* oxidase	1573±90	1345±152

Enzymatic activities were determined at 40°C in skeletal muscle mitochondria as described in Materials and methods. They are expressed in mU mg^{-1} mitochondrial protein. Values are means±s.e.m. from 7–8 independent mitochondrial preparations. *P<0.05 significantly different from fed birds.

Table 3. Effect of fasting on mitochondrial content of cytochromes

	Cytochrome (pmol mg^{-1} protein)		
	b	$c+c_1$	aa_3
Fed (7)	301±12	449±35	289±12
Fasted (6)	251±19*	331±41*	276±23

Values are means±s.e.m. from (N) independent mitochondrial preparations. *P<0.05 significantly different from fed birds.

triggered an improvement of ATP synthesis efficiency. Moreover, a small concentration of KCN, an inhibitor of cytochrome *c* oxidase, fully mimicked fasting-induced change in mitochondrial oxidative phosphorylation activity and efficiency. Given that such a submaximal concentration of KCN does not alter mitochondrial membrane conductance (Clerc et al., 2007; Romestaing et al., 2008), these results provide evidence that a slight constraint on substrate oxidation *per se* is sufficient to increase the yield of oxidative phosphorylation in skeletal muscle mitochondria of ducklings, in accordance with previously reported data on rat liver mitochondria treated with nitric oxide, KCN or sodium azide (Clerc et al., 2007; Romestaing et al., 2008; Piquet et al., 2000). In addition, we report that within the electron transport chain, only the activity of succinate–cytochrome *c* reductase, which involves complex III and co-enzyme *Q* pool, was significantly decreased by fasting. In addition, this result is consistent with the fact that fasting specifically decreased the contents of *b*-type and *c*-type cytochromes, which are cytochromes mainly associated with complex III of the electron transport chain. Noteworthy, there is a close correlation between the activity of succinate–cytochrome *c* activity and the maximal oxidative phosphorylating activity in mitochondria respiring on succinate (r^2=0.72; P<0.001; Fig. 4) or pyruvate/malate/succinate (r^2=0.54; P<0.01; not shown). Collectively, these results suggest that fasting induces a lower electron transfer along the respiratory chain, which would be located at the level of ubiquinol–cytochrome *c* reductase (complex III) and/or the co-enzyme *Q* pool. Although this hypothesis needs further experimental testing, it is reinforced by the fact that 2 days of fasting has been shown to decrease the total amount of mitochondrial co-enzyme *Q* in rat skeletal muscle (Moreno et al., 2003).

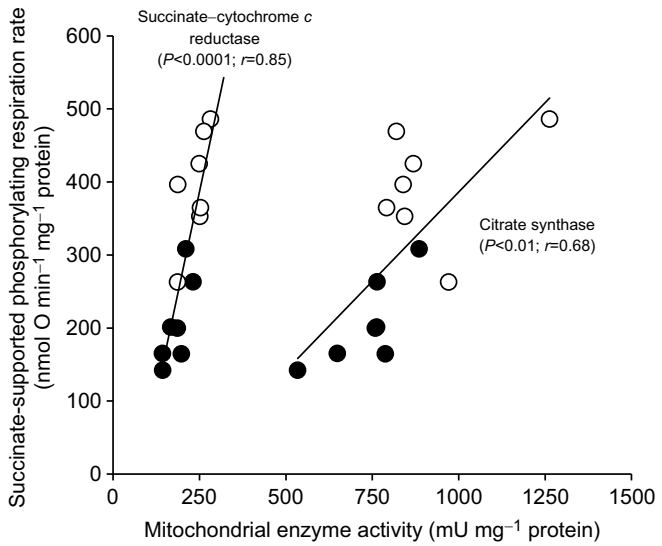

Fig. 4. Relationships between mitochondrial enzyme activities and phosphorylating respiration rate. Correlation of mitochondrial succinate–cytochrome *c* and citrate synthase activities (means values are reported in Table 2) with the rate of phosphorylating respiration using succinate (plus rotenone) in the presence of 500 µmol l^{-1} ADP as the respiratory substrate (mean values are reported in Fig. 1). Individual values show seven independent mitochondrial preparations from the gastrocnemius muscle of fed (open symbols) or fasted ducklings (closed symbols).

The activity of citrate synthase, a key enzyme of the citric acid cycle which converts oxaloacetate to citrate, was also significantly reduced by 22% in fasted ducklings when compared with fed birds. This inhibition could lead to an accumulation of oxaloacetate within mitochondria, an inhibitor of succinate dehydrogenase (Gutman, 1978), which in turn would trigger a reversible suppression of succinate oxidation. In mammals, oxaloacetate has been found to partly contribute to reversible mitochondrial hypometabolism during torpor, by inhibiting succinate dehydrogenase activity (Fedotcheva et al., 1985; Armstrong and Staples, 2010). Interestingly, when plotting mitochondrial succinate oxidation under phosphorylating state against the activity of citrate synthase measured in the same mitochondrial preparations, we found a significant linear correlation (r^2=0.47; P<0.01; Fig. 4). Note that the r^2 value is quite low, indicating that the variation observed in the data between fasted and fed groups was only partially explained by this correlation. Although, a causal link between the inhibition of citrate synthase activity and succinate oxidation remains to be clearly demonstrated, these results suggest that a change in the activity of metabolic pathways involved in substrate oxidation might also contribute to lower the oxidative capacity of mitochondria caused by food restriction in ducklings

It remains that the effective ATP/O ratio is largely controlled by the proton leak and the phosphorylating systems, the substrate oxidation system having a small degree of control (Brand et al., 1993; Rolfe et al., 1994; Roussel et al., 2004). In the present study, we clearly demonstrate that mitochondrial inner membrane proton conductance was not different between fasted and fed ducklings. This is consistent with previously published data in mammals showing that short-term food deprivation or restriction have no, or very little, effect on mitochondrial proton conductance in skeletal muscle (Cadenas et al., 1999; Bevilacqua et al., 2004, 2010; Johnson et al., 2006; Crescenzo et al., 2006). These data raise the question as to whether intrinsic mitochondrial coupling efficiency was really impacted by fasting in ducklings. In this context, it is worth noting that at maximum rate of ATP synthesis (the higher point to the right of the relationships in Figs 1 and 3), and more generally at any given steady-state rate of ATP production (e.g. for the same amount of ADP-induced phosphorylating activity), the effective ATP/O ratios were not different between fasted and fed ducklings (Figs 1B and 3B). This is further supported by data reported in Fig. 2. The oxygen consumed by mitochondria under state 3 respiration is used to drive both ATP synthesis and proton leak; therefore, a P/O ratio can be calculated from the flow ratio of the oxygen consumption used to drive phosphorylation (J_P) divided

by the state 3 respiration rate (Fig. 2B) as described in Brand et al. (1993). The calculated P/O ratios were not significantly different between fasted and fed ducklings (P/O_{fasted}=0.87±0.02 versus P/O_{fed}=0.84±0.04), indicating that ∼85% of the oxygen consumed under state 3 respiration was used to drive ATP synthesis in both fasted and fed birds. This fraction of oxygen used to drive ATP synthesis is consistent with previous calculations using the same experimental conditions, e.g. mitochondria respiring on succinate (plus rotenone) in the presence of nigericin (Brand et al., 1993; Roussel et al., 2004; Johnson et al., 2006; Toyomizu et al., 2011; Amo et al., 2011). However, the use of nigericin could lead to an underestimation of the contribution of oxygen consumption in the production of ATP, by depressing the phosphorylating (state 3) respiration rate (Brand et al., 1993; Rolfe et al., 1994). Indeed, nigericin is used to clamp the transmembrane difference in pH and thus would have some effect on the mitochondrial processes driven by ΔpH. In this condition, a more precise measure of coupling efficiency might be given by subtracting the oxygen consumed to drive proton leak activity (J_H; Fig. 2) from the phosphorylating (state 3) respiration rate measured in the absence of nigericin (Fig. 1). By doing so, P/O values of 0.91 were calculated for both fasted and fed mitochondria, showing that the proton leak is less but still contributes to ∼10% of the total oxygen consumed under state 3. Altogether, these results indicate that, even though mitochondrial state 3 respiration was altered by fasting, mitochondrial oxidative phosphorylation efficiency was preserved because ATP synthesis (J_P) and proton leak (J_H) were affected in a similar way.

In conclusion, 6 days of fasting improves mitochondrial ATP synthesis efficiency in skeletal muscle of ducklings by depressing proton-motive force and consequently the proton leak activity, through the downregulation of substrate oxidation reactions. The lower capacity of the respiratory machinery to pump protons in 6 day fasted birds generates a lower membrane potential, which triggers a large decrease in proton leak activity and thus a higher coupling efficiency at any given rate of substrate oxidation. We propose that the site of action would be mainly located at the level of co-enzyme *Q* pool/complex III within the electron transport chain.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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