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RESEARCH ARTICLE

Skeletal muscle phenotype affects fasting-induced mitochondrial oxidative phosphorylation flexibility in cold-acclimated ducklings

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ABSTRACT

Starvation is particularly challenging for endotherms that remain active in cold environments or during winter. The aim of this study was to determine whether fasting-induced mitochondrial coupling flexibility depends upon the phenotype of skeletal muscles. The rates of oxidative phosphorylation and mitochondrial efficiency were measured in pectoralis (glycolytic) and gastrocnemius (oxidative) muscles from cold-acclimated ducklings (*Cairina moschata*). Pyruvate and palmitoyl-L-carnitine were used in the presence of malate as respiratory substrates. Plasma metabolites, skeletal muscle concentrations of triglycerides, glycogen and total protein and mitochondrial levels of oxidative phosphorylation complexes were also quantified. Results from *ad libitum* fed ducklings were compared with those from ducklings that were fasted for 4 days. During the 4 days of nutritional treatment, birds remained in the cold, at 4°C. The 4 days of starvation preferentially affected the pectoralis muscles, inducing an up-regulation of mitochondrial efficiency, which was associated with a reduction of both total muscle and mitochondrial oxidative phosphorylation protein, and with an increase of intramuscular lipid concentration. By contrast, fasting decreased the activity of oxidative phosphorylation but did not alter the coupling efficiency and protein expression of mitochondria isolated from the gastrocnemius muscles. Hence, the adjustment of mitochondrial efficiency to fasting depends upon the muscle phenotype of cold-acclimated birds. Furthermore, these results suggest that the reduced cost of mitochondrial ATP production in pectoralis muscles may trigger lipid storage within this tissue and help to sustain an important metabolic homeostatic function of skeletal muscles, which is to maintain levels of amino acids in the circulation during the fast.

KEY WORDS: Mitochondria, Bioenergetics, Energy efficiency, Starvation, Birds, Oxidative metabolism

INTRODUCTION

Fasting is a major characteristic of the life cycle of many wild mammals and birds. These periods of fasting occur during reproduction, moult or migration, when these important activities for the survival of individuals or species compete with feeding, or during winter, when food is scarce or lacking (Mrosovsky and Sherry, 1980). During food deprivation, animals cannot maximize all of their physiological traits because the energy required for basal metabolism, physical activity, thermoregulation, growth and survival must come from limited body energy stores. Accordingly, limited energy resources can cause a negative functional interaction, i.e. a trade-off, between several organismal functions that compete for the same resource pool, e.g. growth and thermoregulation (Zera and Harshman, 2001). It has been clearly shown that species tolerating long fasts have evolved a whole range of energy conservation mechanisms and a great ability to reduce proteolysis and conserve total body protein, which has been shown to occur concomitant with their initial adiposity and capacity for mobilizing and oxidizing lipids (Goodman et al., 1981; Le Maho et al., 1981; Cherel et al., 1992; Lindgård et al., 1992; Boismenu et al., 1992).

Starvation remains particularly challenging for endotherms that remain active in cold environments or during winter. This is well exemplified by king penguin chicks, which have evolved to survive up to 5 months of starvation during the austral winter (Cherel and Le Maho, 1985; McCue, 2010). Although winter-acclimatized king penguin chicks retain a high capacity for thermogenesis (Duchamp et al., 1989), such starvation resistance reflects their ability to store energy as fat (34% adiposity in the pre-winter period) and control its allocation to minimize energy expenditure (growth arrest, lower basal metabolic rate, shallow hypothermia, reduced thermogenic effect of lipids) in order to maximize energy conservation (Duchamp et al., 1989; Cherel et al., 1993, 2004; Eichhorn et al., 2011; Teulier et al., 2013). In a recently published paper, it was shown that skeletal muscle mitochondria from fasted winter-acclimatized chicks minimized the cost of ATP synthesis by increasing the efficiency of oxidative phosphorylation processes, which would ultimately alleviate the need for energy substrates (Monternier et al., 2014). This finding is of particular interest for at least two reasons. Firstly, mitochondrial activity remains the main physiological link between endogenous energy stores and energy allocated to cell activities and therefore to animal performance under food deprivation. Secondly, skeletal muscles are the main site of thermogenesis in birds, with muscle mitochondria playing a pivotal role in heat production processes (Skulachev and Maslov, 1960; Duchamp et al., 1992; Duchamp and Barré, 1993; Toyomizu et al., 2002; Bicudo et al., 2002). However, the reported regulation of mitochondrial efficiency was shown in pectoral muscles (Monternier et al., 2014), which are preferentially degraded in fasted birds to provide the blood with amino acids, whereas muscles involved in terrestrial locomotion are preserved (Robin et al., 1988; Duchamp et al., 1991; Swain, 1992). These observations raise the question of whether mitochondrial efficiency is similarly affected by starvation in these relatively preserved skeletal muscles.

The aim of this work was to determine to what extent fasting-induced mitochondrial coupling flexibility depends upon the phenotype of skeletal muscles. This was done by measuring mitochondrial activity and by quantifying the levels of oxidative phosphorylation proteins in the pectoral muscle (a glycolytic muscle) and in the red part of the gastrocnemius muscle, a

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more oxidative muscle that is involved in terrestrial locomotion (Duchamp et al., 1992; Baéza et al., 1999). *Cairina moschata* (Linnaeus 1758) ducklings were cold acclimated and results from ducklings fed *ad libitum* were compared with those from birds that were fasted for 4 days. Plasma metabolites and the skeletal muscle concentrations of energy metabolites (triglycerides and glycogen) and total protein were also quantified. From the results of the present study we conclude that fasting preferentially affected the bioenergetics parameters of pectoralis muscles, inducing an up-regulation of mitochondrial efficiency, which was associated with a loss of mitochondrial oxidative phosphorylation protein. By contrast, fasting decreased the activity of mitochondrial oxidative phosphorylation in the gastrocnemius muscles without any change in either mitochondrial coupling or mitochondrial protein expression.

RESULTS

Body mass loss, plasma and skeletal muscle metabolites

At the beginning of nutritional manipulation, fasted ducklings were slightly heavier than control fed birds (1.58±0.02 versus 1.40±0.03 kg, $P<0.05$). After 4 days of treatment, the final body mass of fasted ducklings was significantly lower than that of control fed birds (1.12±0.02 kg in the fasted group versus 1.66±0.04 kg in the fed group, $P<0.05$). Ducklings lost 464±10 g (29±1% of initial body mass) over the 4 days of fasting (Fig. 1A). The specific daily loss in mass was rapid during the first 48 h of fasting (121±7 g kg^{-1} day^{-1}, phase I, see Materials and methods), then slowed abruptly and was subsequently maintained at a constant level of 70±4 g kg^{-1} day^{-1} up to the end of the fasting period (Fig. 1B), indicating that cold-acclimated ducklings were in phase II of fasting.

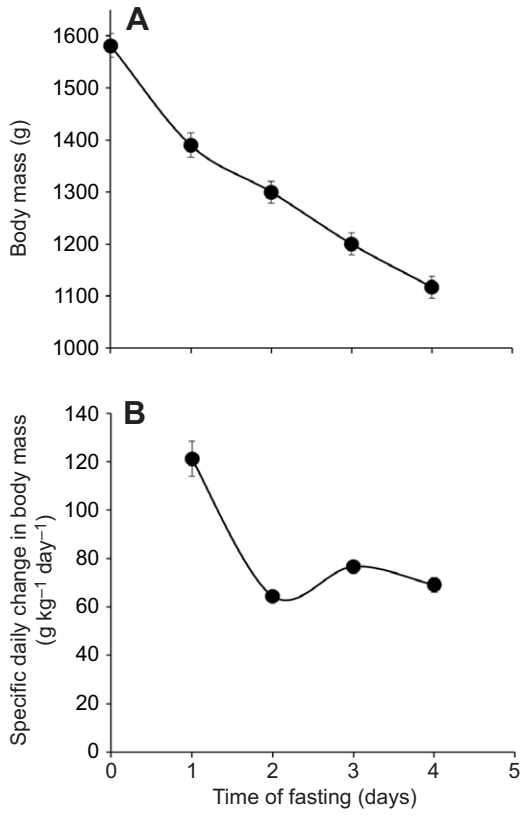

Fig. 1. Body mass changes during fasting in cold-acclimated ducklings. (A) Body mass and (B) specific daily body mass loss in ducklings during a 4 day fast. Values are means±s.e.m. for $N=5$ ducklings.

Table 1. Effect of fasting on plasma metabolites and uric acid

	Fed	Fasted
Glucose (mmol l^{-1})	13.5±1.0	13.2±0.5
Triglycerides (mmol l^{-1})	2.86±0.62	0.40±0.03*
Glycerol (μmol l^{-1})	3.6±2.4	34.2±6.9*
Non-esterified fatty acids (μmol l^{-1})	120±41	561±41*
β-Hydroxybutyrate (μmol l^{-1})	156±14	1858±183*
Uric acid (μmol l^{-1})	550±115	290±19

Cold-acclimated ducklings were either fed *ad libitum* or fasted for 4 days as described in Materials and methods.
Values are means±s.e.m. ($N=5$). *$P<0.05$, significantly different from fed group.

The effects of fasting on plasma metabolites and uric acid are shown in Table 1. Glycaemia was not significantly different between groups ($P=0.78$). After 4 days of fasting, plasma triglyceride concentration was significantly decreased ($P<0.01$), while plasma glycerol ($P<0.01$), non-esterified fatty acid ($P<0.0001$) and β-hydroxybutyrate concentration ($P<0.0001$) were significantly increased. Plasma uric acid concentration was slightly but not significantly lower in fasted ducklings than in fed birds (47%, $P=0.056$).

Fasting significantly decreased skeletal muscle mass (Table 2; $P<0.001$). However, when expressed relative to body mass, the wet mass of skeletal muscles was either significantly decreased by 38% (pectoralis muscles) or unaffected (gastrocnemius muscles). Protein (decreased by 18%, $P<0.05$) and triglyceride (increased by 79%, $P<0.01$) concentrations were significantly altered by fasting in pectoralis muscles only. Glycogen content was significantly decreased by 55% in gastrocnemius muscles ($P<0.01$) and almost completely depleted in pectoralis muscles ($P<0.001$) after 4 days of fasting (Table 2).

Muscle oxidative capacity and mitochondrial respiration

The specific (per organ mass) and total cytochrome *c* oxidase activity of pectoralis muscles was significantly lower in fasting than in fed ducklings ($P<0.01$ and $P<0.001$, respectively). These activities were not significantly altered in gastrocnemius muscles (Table 2). Regardless of the respiratory substrate used, Table 3 shows that the rate of mitochondrial oxygen consumption associated with ATP production (state 3) was significantly decreased by approximately 67% ($P<0.0001$) and 46% ($P<0.0001$) in pectoralis and gastrocnemius muscles of fasted ducklings as compared with fed birds, respectively. The basal non-phosphorylating respiration rate (state 4_{oligo}) was significantly decreased in pectoralis muscles of fasted ducklings ($P<0.0001$), but not significantly altered in gastrocnemius muscles. On the whole, gastrocnemius mitochondria had significantly higher oxygen consumption rates than pectoralis mitochondria in both active (state 3; $P<0.001$) and basal (state 4_{oligo}; $P<0.01$) steady states, irrespective of the respiratory substrate used. Respiratory control ratios were not significantly affected either by the nutritional status of ducklings or by the skeletal muscle phenotype (Table 3).

Mitochondrial oxidative phosphorylation efficiency

Fig. 2 shows the effect of starvation on oxidative phosphorylation in pectoralis (Fig. 2A,B) and gastrocnemius (Fig. 2C,D) muscle mitochondria with pyruvate (Fig. 2A,C) or palmitoyl-L-carnitine (Fig. 2B,D) as the respiratory substrate. Regardless of the respiratory substrate used, the maximal rates of oxygen consumption and ATP synthesis, the highest points on the right of the linear relationships, were significantly decreased by approximately 66% ($P<0.0001$)

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Table 2. Effect of fasting on muscle mass, nutrient content and oxidative capacity of skeletal muscles

	Pectoralis muscles		Gastrocnemius muscles	
Parameters	Fed	Fasted	Fed	Fasted
Muscle mass				
Total (g)	42.8±3.5	17.9±1.5*	27.8±0.9‡	19.2±0.9*
Relative (g 100 g^{-1} body mass)	2.6±0.2	1.6±0.1*	1.7±0.1‡	1.7±0.1
Nutrient content (mg g^{-1} dry muscle mass)				
Protein	491±19	403±30*	475±29	495±27
Triglyceride	1.9±0.4	3.4±0.3*	2.3±0.2	2.6±0.2‡
Glycogen	5.9±0.8	0.42±0.04*	5.1±0.6	2.3±0.2*,‡
Cytochrome *c* oxidase activity				
Specific (μmol O min^{-1} g^{-1} muscle)	21±3	10±1*	89±16‡	98±7‡
Total (μmol O min^{-1})	916±141	178±25*	2503±498‡	1865±119‡

Cold-acclimated ducklings were either fed *ad libitum* or fasted for 4 days as described in Materials and methods.
Values are means±s.e.m. (N=5). *P<0.05, significantly different from corresponding fed group. ‡P<0.05, significantly different from pectoralis muscle within the same nutritional group.

and 45% (P<0.001) in pectoralis and gastrocnemius muscles, respectively (Fig. 2). However, it must be noted that the maximal rates of oxygen consumption failed to reach statistical significance in gastrocnemius mitochondria respiring on pyruvate/malate (P=0.06; Fig. 2C). The basal non-phosphorylating respiration rates measured in the presence of oligomycin (the intercepts with the *x*-axis) were significantly decreased by 66% in pectoralis muscles on average (P<0.0001; Fig. 2A,B), but were not significantly altered in gastrocnemius muscles (P=0.53; Fig. 2C,D). Hence, regardless of the respiratory substrate used, the linear relationship between the rate of ATP synthesis and oxygen consumption was significantly shifted to the left in pectoralis muscles from fasted ducklings compared with that from fed birds (Fig. 2A,B). This indicates a higher mitochondrial oxidative phosphorylation efficiency (effective ATP/O ratio) because, to produce a given amount of ATP, mitochondria from pectoralis muscles consumed less oxygen in fasted than in fed ducklings. By calculating oxygen consumption at the highest common ATP synthesis rate for both substrates, it was found that the effective oxidative phosphorylation efficiency of pectoralis mitochondria was increased by an average of 50% in fasted ducklings (44±5% for pyruvate, 58±9% for palmitoyl-L-carnitine). By contrast, 4 days of fasting did not alter the mitochondrial efficiency, e.g. the amount of ATP synthesized from ADP per amount of oxygen consumed, in gastrocnemius mitochondria respiring on pyruvate (Fig. 2C) or palmitoyl-L-carnitine (Fig. 2D). Finally, mitochondrial oxidation and phosphorylation rates of fasted ducklings were significantly higher in gastrocnemius mitochondria than in pectoralis mitochondria respiring on pyruvate (P<0.05) or palmitoyl-L-carnitine (P<0.01). In fed ducklings, the oxidative and phosphorylation rates of gastrocnemius mitochondria, when palmitoyl-L-carnitine was used as the respiratory substrate, were significantly higher compared with those of pectoralis mitochondria (P<0.001).

Mitochondrial oxidation affinity for ADP

By using the respiratory data reported in Table 3 and Fig. 2, the dependence of mitochondrial oxygen consumption rate on ADP concentration ranging from 10 to 500 μmol l^{-1} can be illustrated. Kinetics parameters of mitochondrial oxidation can be investigated by determining the apparent affinity (K_m) for ADP and the maximal oxidation rate (V_{max}) of mitochondria for each substrate and each muscle type in both fasted and fed cold-acclimated ducklings (Fig. 3). Regardless of the respiratory substrates used, fasting did not significantly affect the affinity of mitochondria for ADP in both pectoralis (Fig. 3A,B) and gastrocnemius muscles (Fig. 3C,D). Considering all results and regardless of the nutritional status, the apparent affinity for ADP was significantly higher in gastrocnemius mitochondria respiring on pyruvate compared with pectoralis mitochondria (P<0.05), with no differences when mitochondria were respiring on palmitoyl-L-carnitine (P=0.14). Fasting elicited significant decreases in maximal oxidation rate in mitochondria of both skeletal muscles (P<0.001), with gastrocnemius muscle mitochondria having significantly higher maximal oxidative rates than pectoralis muscle mitochondria (P<0.001), irrespective of the respiratory substrates used (Fig. 3).

Expression of mitochondrial oxidative phosphorylation proteins

Fig. 4 shows that the protein expression of respiratory chain complexes and ATP synthase was significantly decreased by fasting in pectoralis muscles but not in gastrocnemius muscles. In pectoral muscle, the expression of the alpha subunit of ATP synthase was significantly different between groups, whereas the relative abundance of other complexes tended to be lower but failed to reach statistical significance (complex II, P=0.07; core protein 2 of complex III, P=0.06; subunit 1 of complex IV, P=0.08).

Table 3. Effect of fasting on respiratory parameters of isolated muscle mitochondria

		Pectoralis muscle		Gastrocnemius muscle	
Respiratory substrate	Parameter	Fed	Fasted	Fed	Fasted
Pyruvate	State 3	239±36	80±15*	360±34‡	192±15*,‡
	State 4_{oligo}	18±2	7±1*	32±4‡	20±4‡
	RCR	14±2	12±2	12±2	11±2
Palmitoyl-L-carnitine	State 3	295±19	96±9*	459±23‡	258±47*,‡
	State 4_{oligo}	16±2	8±1*	27±6	15±2‡
	RCR	19±2	12±1	20±3	19±3

Respiration rate (state 3 and state 4_{oligo}, expressed in nmol O min^{-1} mg^{-1} of protein) was determined at 40°C in the presence of malate (2.5 mmol l^{-1}). Values are means±s.e.m. for N=5 independent mitochondrial preparations. RCR, respiratory control ratio.
*P<0.05, significantly different from corresponding fed group. ‡P<0.05, significantly different from pectoralis muscle mitochondria within the same nutritional group.

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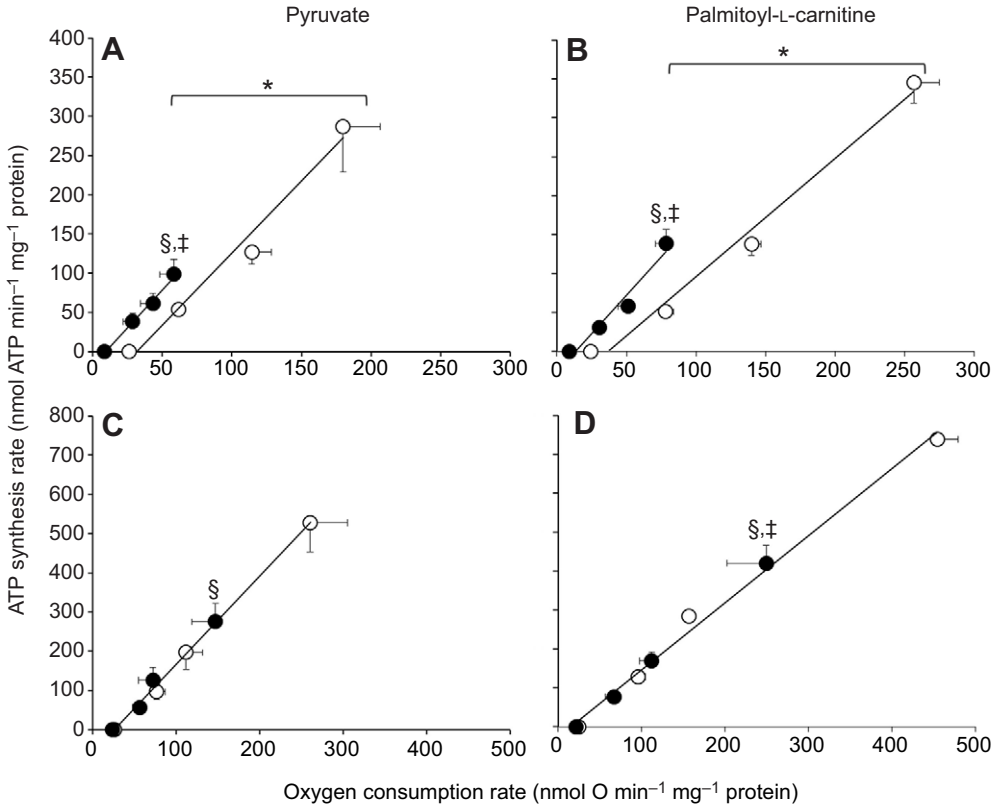

Fig. 2. Effects of fasting on mitochondrial oxidative phosphorylation efficiency. The rates of oxygen uptake and ATP synthesis were measured in mitochondria isolated from pectoralis (A,B) or gastrocnemius (C,D) muscles respiring on pyruvate or palmitoyl-L-carnitine. Values are means±s.e.m. for *N*=5 independent mitochondrial preparations for fed (open symbols) or fasted (filled symbols) ducklings. *The linear relationship between ATP synthesis and oxygen consumption is significantly shifted to the left in fasted ducklings. §Significant effect of fasting on maximal ATP synthesis rate; ‡significant effect of fasting on oxygen consumption rate (the highest points on the right of the linear relationship in both cases).

DISCUSSION

From the results obtained during the present study, it is evident that fasting-induced mitochondrial hypometabolism was associated with an improvement of ATP synthesis efficiency and a decreased amount of oxidative phosphorylation proteins in the pectoralis muscle. By contrast, both mitochondrial efficiency and protein expression were preserved in the gastrocnemius muscle throughout the fast. Hence, the adjustment of muscle mitochondrial oxidative phosphorylation efficiency in response to fasting seems to depend on the activity and/or phenotype of the skeletal muscle investigated,

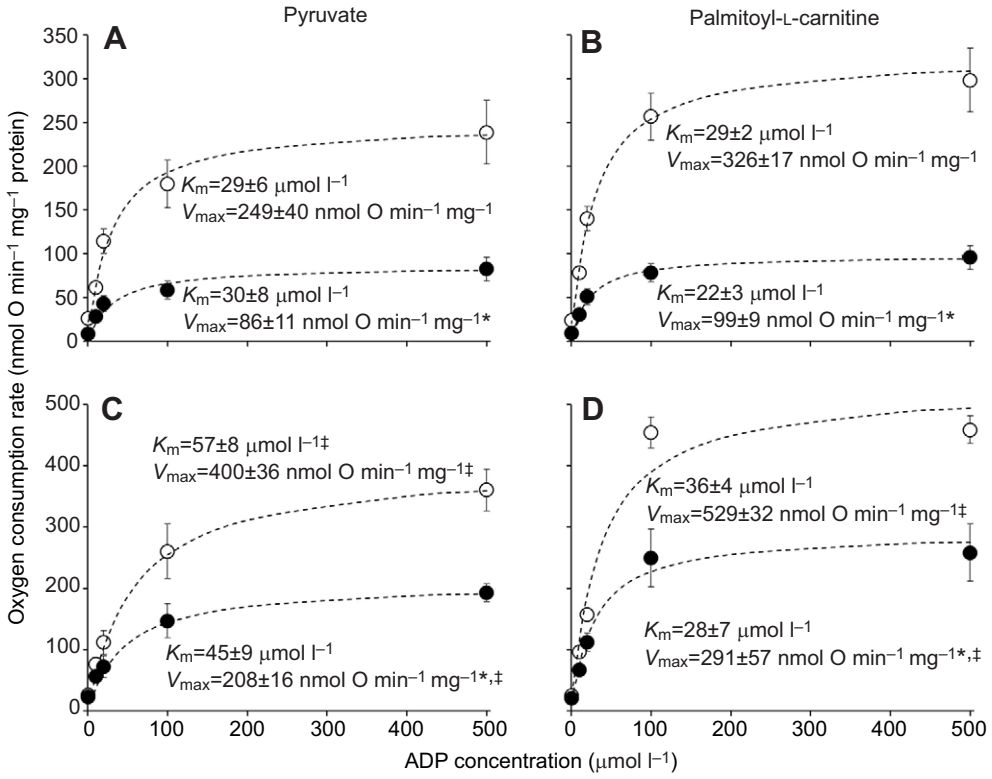

Fig. 3. Effects of fasting on the dependence of oxidation rate on ADP concentration. The rate of oxygen uptake was measured in mitochondria isolated from pectoralis (A,B) or gastrocnemius (C,D) muscles. Data are presented as means±s.e.m. for *N*=5 independent mitochondrial preparations for fed (open symbols) or fasted (filled symbols) ducklings. *$P<0.05$, significantly different from corresponding fed group. ‡$P<0.05$, significantly different from pectoralis muscle mitochondria within the same nutritional group.

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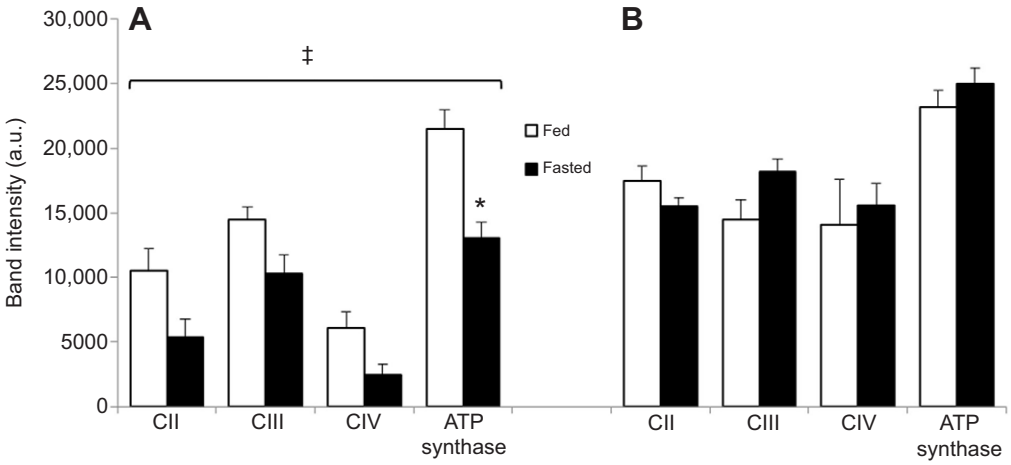

Fig. 4. Effects of fasting on the expression of mitochondrial oxidative phosphorylation proteins. Western blot analyses were performed on mitochondria isolated from pectoralis (A) and gastrocnemius (B) muscles of fed or fasted ducklings, using antibodies against respiratory chain complexes (complex II–IV) and ATP synthase. Results are means+ s.e.m. for *N*=3–4 independent mitochondrial preparations. ‡Significant effect of fasting on the global expression of mitochondrial proteins. *Significantly different from fed groups. See Results for more details.

supporting recently reported observations on muscle mitochondria from cold-acclimatized king penguin chicks (Monternier et al., 2014).

In the present study, the constant daily change in body mass (Fig. 1B; supplementary data Fig. S1) indicates that cold-acclimated ducklings were in phase II of fasting (Le Maho et al., 1981; Cherel et al., 1988; Robin et al., 1988). This metabolic stage was further confirmed by (1) the increase in circulating non-esterified fatty acids, indicating body fat mobilization, (2) the high plasma levels of β-hydroxybutyrate, a by-product of fatty acid breakdown, and (3) the relatively low plasma levels of uric acid, the end-product of proteolysis in birds (Le Maho et al., 1981; Cherel and Le Maho, 1985; Robin et al., 1988; Cherel et al., 1988; Bourgeon et al., 2010). Indeed, phase II of fasting is widely regarded as a period of economy, characterized by high fat mobilization and oxidation and by a decrease in protein breakdown (Le Maho et al., 1981; Cherel et al., 1988; Robin et al., 1988; Boismenu et al., 1992; McCue, 2010). Yet, the specific daily body mass change of 70 g kg^{-1} day^{-1} found in cold-acclimated ducklings was 2–4 times higher than in adult birds fasting in the cold (Thouzeau et al., 1999; Mata et al., 2001; Bourgeon et al., 2010), and 10–11 times higher than the specific daily body mass loss of emperor penguins and winter-acclimatized king penguin chicks, two avian species that tolerate a very long fast during winter (Cherel and Le Maho, 1985; Robin et al., 1988). These data suggest that cold-acclimated ducklings were less efficient in sparing their protein reserves during phase II. The initial adiposity and the energy cost imposed by cold exposure are among the main factors that may explain the limited fasting endurance of ducklings compared with winter-acclimatized king penguin chicks and other birds (Goodman et al., 1981; Cherel et al., 1992; Lindgård et al., 1992; Boismenu et al., 1992; Thouzeau et al., 1999). In accordance with this, it can be estimated from previously published data (Bénistant et al., 1998) that cold-acclimated ducklings had a very low adiposity, 2% of the initial body mass, compared with the pre-winter adiposity of 34% found in king penguin chicks (Cherel et al., 1993). Furthermore, cold-acclimated ducklings fasting at 4°C were well below their lower critical body temperature (20°C; Teulier et al., 2010), whereas king penguin chicks fasting ashore during the austral winter remain most of the time above their lower critical temperature (−7°C; Duchamp et al., 1989). Hence, the biological and physiological traits of cold-acclimated ducklings (low adiposity and high energy cost for thermoregulation) mainly explain the poor fasting endurance of ducklings in the cold, and suggest that the intensity of fasting may not be strictly comparable between the present study and the recently published study in king penguin chicks (Monternier et al., 2014).

To cope with the energetic limitations imposed by starvation, most organisms reduce their energy expenditure, which may be achieved by a reduction of unnecessary physical activity, thermogenesis, basal metabolic rate, body temperature and/or protein turnover (Cherel et al., 1988; Wiersma et al., 2005; Ben-Hamo et al., 2010; McCue, 2010). In the present study, a general mitochondrial hypometabolism was observed in both pectoralis and gastrocnemius muscles of fasted ducklings. Given that skeletal muscle contributes up to 30% of the resting metabolic rate in ducklings (Duchamp and Barré, 1993), such an overall reduction in mitochondrial oxidative phosphorylation activity, together with decreased skeletal muscle mass, would reduce energy expenditure in cold-acclimated fasted ducklings, similarly to other birds undergoing food deprivation in winter (Duchamp et al., 1989; Mata et al., 2001; Monternier et al., 2014). However, starvation-induced mitochondrial hypometabolism has not been found in king penguin chicks during the winter fast (Duchamp et al., 1991; Monternier et al., 2014), suggesting that a reduction in muscle mitochondrial activity may not be a general feature of metabolic adjustment to food shortage in birds. It is well known that skeletal muscle mitochondria can rapidly adjust their functional characteristics in response to manipulations of the muscle used and environmental conditions (Krieger et al., 1980; Hood, 2001; Flück and Hoppeler, 2003; Staples and Brown, 2008). In this context, it must be kept in mind that king penguin chicks have to maintain muscular activity against predators while fasting under their natural environment. By contrast, experimental ducklings were reared in laboratory conditions with cold and starvation as the only energetic constraints. Therefore, such species differences can be explained by more active and mature pectoralis muscles in winter-fasted king penguin chicks than in cold-acclimated fasted ducklings.

Although the maximal rates of oxidative phosphorylation are differently regulated in ducklings and king penguin chicks, the mitochondrial efficiency of pectoralis muscles increased during fasting in both species (present study; Monternier et al., 2014). An increase in the yield of mitochondrial ATP synthesis may involve an increase in coupling H^+/O stoichiometry at the level of the respiratory complexes (slip reactions) or a decrease in proton leak reactions or both (Groen et al., 1990; Brand, 2005). In the present study, the improvement of mitochondrial efficiency in pectoralis muscles was clearly associated with a reduction of mitochondrial oxidative phosphorylation proteins, including cytochrome *c* oxidase subunit. By contrast, the mitochondrial efficiency was preserved in

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the oxidative gastrocnemius muscle, owing to the fact that the mitochondrial content of oxidative phosphorylation proteins was unaffected by the fast. Interestingly, it has been previously reported that a decrease in cytochrome *c* oxidase content and/or activity increased the overall ATP synthesis yield of mitochondria, resulting in a significant shift of the linear relationship between oxygen consumption and ATP synthesis to the left, with a significant decrease in basal non-phosphorylating respiration (Piquet et al., 2000; Nogueira et al., 2002; Clerc et al., 2007), similar to what was found in the present study. These data thus suggest that the fasting-induced increase in the yield of mitochondrial ATP synthesis might involve slip reactions (changes in the coupling H^+/O stoichiometry) at the level of the respiratory complexes (Groen et al., 1990).

However, the large decrease in basal non-phosphorylating respiration found in fasted ducklings cannot be fully ascribed to such an intrinsic coupling mechanism, i.e. H^+/O stoichiometry of respiratory complexes. As 75% of muscle mitochondrial oxygen consumption is controlled by proton leak reactions under non-phosphorylating conditions in birds (Kikusato et al., 2010), the present 60% decrease in the content of cytochrome *c* oxidase subunit should have decreased non-phosphorylating respiration by 26% at the most. Given the decrease of 65% shown in Fig. 2, it is therefore reasonable to suggest that proton leak reactions also significantly contributed to the fasting-induced change in mitochondrial efficiency. In support of this hypothesis, one previous study has reported a significant decrease in avian UCP-mediated inducible mitochondrial proton leak activity in pectoralis muscles of long-term fasted king penguins (Rey et al., 2008). In addition, it is clear from the literature that a decreased inner membrane proton conductance is expected to shift the linear relationship between oxygen consumption and ATP synthesis to the left (Beavis and Lehninger, 1986; Clerc et al., 2007; Salin et al., 2010), similar to what was found in the present study. Notwithstanding the underlying mechanism (electron slip and/or proton leak), the present data, together with those reported in king penguin chicks (Monternier et al., 2014), strongly suggest that pectoralis muscles would specifically help to maximize the conservation of endogenous fuel stores in birds fasting in the cold, by decreasing the cost of ATP production and so the need for energy substrates. The reported increase in mitochondrial ATP synthesis yield may further facilitate the accumulation of triglycerides found in pectoralis muscles of fasted ducklings. Indeed, a fasting-induced up-regulation of mitochondrial efficiency would allow the pectoralis muscle to oxidize less fuel to sustain its energy-demanding processes, and consequently to divert a greater portion of fatty acid influx towards lipogenesis pathways, increasing lipid accumulation within the tissue in the form of triglycerides. This process would be reinforced in agreement with an earlier observation that fasting induced a coordinated increase in the transport of lipids in skeletal muscles, which preferentially affected fast-twitch (glycolytic) muscles (Swain, 1992; Hildebrandt and Neufer, 2000; Abe et al., 2006). Whether fasting triggers similar mitochondrial flexibility in adult birds with fully mature and functional pectoralis muscles remains unknown, but clearly warrants further investigation.

Fasting induces other metabolic changes, among which is an increase in fat utilization and sparing of protein, with less than 10% of the energy derived from protein (Cherel et al., 1988; Mata et al., 2001). The downward trend of plasma uric acid concentration during the fast would suggest a lower protein catabolism and therefore whole-body protein sparing in fasted ducklings. Nevertheless, it must be kept in mind that skeletal muscles are a major reservoir for protein, and have an important metabolic homeostatic function in various wasting conditions by providing the plasma with amino acids. Therefore, a period of fasting always involves a certain amount of muscle protein breakdown, despite adaptive mechanisms of protein sparing (Cherel et al., 1988). Skeletal muscles are also the main tissue involved in thermogenesis in birds (Duchamp and Barré, 1993; Bicudo et al., 2002). It emerges from the literature on birds fasting in the cold that such metabolic trade-offs between the need for protein breakdown to maintain plasma amino acid homeostasis and the need for protein conservation to maintain thermogenesis and locomotor activity would be resolved by different groups of skeletal muscles dedicated to different metabolic tasks (Robin et al., 1988; Duchamp et al., 1991; Thouzeau et al., 1999). In agreement with this is the finding during the present study that pectoralis muscles were preferentially degraded during the fast. This is highlighted by a loss of both total and mitochondrial proteins in pectoralis muscles after 4 days of fasting, which led to a reduction in its mass-specific aerobic capacity. By contrast, both total and mitochondrial proteins are totally spared in gastrocnemius muscles, which contribute to preserve its aerobic capacity during the fast. The flightless growing ducklings used in the present study display well-developed and functional leg muscles, which are also the major sites of regulative thermogenesis in the cold (Duchamp and Barré, 1993), whereas pectoralis muscles are little used, developing and becoming fully functional much later. Accordingly, an explanation for the relative protected status of gastrocnemius muscles would lie in the maintenance of aerobic capacity in order to sustain efficient locomotor activity and possibly the need for more-'thermogenic' loose-coupled mitochondria to sustain thermogenesis in ducklings that are fasting in the cold (Skulachev and Maslov, 1960; Duchamp et al., 1992). In contrast, pectoralis muscles would play an important metabolic homeostatic function during the fast, by maintaining acceptable levels of amino acids in the circulation, in order to sustain hepatic gluconeogenesis and protein synthesis in other organs.

In conclusion, the muscular mitochondrial efficiency is a flexible parameter that can be differently adjusted to the nutritional status of birds, depending on the muscle phenotype. In pectoralis muscles, starvation increases the energy coupling efficiency of mitochondria, which triggers an economical management of fuels and might drive the shift from oxidation to storage of fatty acids within the tissue. In gastrocnemius muscles, mitochondria from fasted cold-acclimated ducklings remained as efficient as those from their fed counterparts, which may allow fasted ducklings to maintain a certain level of heat production for thermoregulatory purposes, despite a reduced mitochondrial activity.

MATERIALS AND METHODS

Animal, plasma and muscle sampling

All experiments were conducted in accordance with animal care guidelines and were approved by the Ethics Committee of Lyon (project no DR2013-54v2) and the Ministère de la Recherche et de l'Enseignement Supérieur. Male Muscovy ducklings (*C. moschata*) were obtained from a commercial stockbreeder (Eclosion Grimaud Frères, Roussay, France). The cold acclimation schedule described by Barré et al. (1986) was used. Briefly, from the age of 8 days, the ducklings were reared at 4°C in a constant photoperiod (8 h:16 h light:dark) for 4 weeks.

A preliminary study was first conducted on five cold-acclimated ducklings to determine the duration of nutritional manipulation used in the present study. Of the three phases described from the changes in body mass during fasting (Cherel et al., 1988), only two phases were clearly characterized in cold-acclimated ducklings (supplementary material Fig. S1): a short phase I characterized by a rapid drop in body mass,

followed by a steady decline in specific daily change in body mass during phase II of fasting, which lasted 5–6 days. This preliminary study was interrupted after 7 days of fasting, when ducklings had lost 40–47% of their initial body mass.

In the present study, birds were divided into two groups at the end of the cold acclimation schedule (see above). One half continued to be fed *ad libitum* (N=5) and the other half was fasted for 4 days and weighed daily (N=5); both groups of birds were kept at 4°C. At the end of the experimental protocol, animals were stunned by cranial percussion and killed by decapitation. Blood was collected in heparinized tubes at 4°C, centrifuged shortly after sampling and aliquots of plasma were stored at −80°C until analysis. Pectoralis muscles and the red part of gastrocnemius muscles were rapidly removed and weighed (±10 mg). One small sample of each muscle was frozen in liquid nitrogen and kept at −80°C until assayed for enzyme activity. A second small sample of each muscle was freeze-dried (Christ® ALPHA 1-4LD lyophilisator) and stored at −80°C until metabolite content analysis. The remainder of the tissue was used fresh for mitochondrial isolation.

Plasma metabolites

Glucose, β-hydroxybutyrate, uric acid and triglyceride concentrations were determined using an Automate Architect C16000 clinical chemistry analyser (Abbott©, Wiesbaden, Germany). Non-esterified fatty acids and glycerol concentrations were determined using an ABX® PENTRA 400 clinical chemistry analyser (Horiba, Montpellier, France) with commercially available kits (Wako®, Neuss, Germany and Randox®, Crumlin, Antrin, UK).

Muscle metabolites

Extraction and assays of glycogen, triglycerides and proteins from freeze-dried skeletal muscle samples were processed as described by Renault et al. (2002). Total lipids were extracted by homogenizing muscle samples in 20 volumes of a chloroform–methanol mixture (2:1 v:v) according to Folch et al. (1957), and triglycerides were measured using the enzymatic triglyceride test (GPO Trinder, Sigma-Aldrich, France). To measure protein and glycogen content, muscle samples were homogenized in 10 volumes of 1 mol l^{-1} NaOH. Protein and glycogen were subsequently measured using the enzymatic Pierce® Bicinchoninic Acid Protein Assay (Thermo Scientific, Rockford, IL, USA) and Glucose HK (Sigma-Aldrich, France) kits, respectively. All assays were performed using a spectrophotometer (AQUAMATE, Thermo Electron Corporation) at room temperature.

Cytochrome *c* oxidase activity

Frozen skeletal muscle samples were homogenized with a Polytron® homogenizer in medium containing 1 mmol l^{-1} ATP, 50 mmol l^{-1} Hepes, 100 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA and 5 mmol l^{-1} EGTA (pH 7.4). The muscle homogenates were incubated in the presence of 5 mg ml^{-1} lubrol for 30 min in an ice bath. Cytochrome *c* oxidase activity was determined polarographically at 40°C using a thermostatic cell glass fitted with a Clark oxygen electrode (Rank Brothers Ltd, UK).The oxygen consumption was initiated with 4 mmol l^{-1} ascorbate and 300 µmol l^{-1} *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine in reaction buffer containing 30 µmol l^{-1} cytochrome *c*, 4 µmol l^{-1} rotenone, 50 µmol l^{-1} carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone (FCCP), 10 mmol l^{-1} malonate and 75 mmol l^{-1} Hepes (pH 7.4) as described previously (Roussel et al., 2000).

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 and 50 mmol l^{-1} Tris-base, pH 7.4 at 4°C). Briefly, skeletal muscles were cut up finely with sharp scissors, homogenized with a Potter–Elvehjem homogenizer (five passages) and treated with subtilisin (1 mg g^{-1} muscle wet mass) for 5 min in an ice bath. The mixture was diluted 1:2, homogenized (three passages), and then centrifuged at 1000 ***g*** for 10 min. The supernatant containing mitochondria was centrifuged at 8700 ***g*** for 10 min, and the pellet was re-suspended in 10 ml of isolation buffer and centrifuged at 1000 ***g*** for 10 min to pellet any remaining nucleus or cell debris contamination. The resulting supernatant was filtered through cheesecloth and centrifuged at 8700 ***g*** for 10 min. The pellet containing mitochondria was washed once by suspension in the isolation buffer and re-centrifuged at 8700 ***g*** for 10 min. All steps were carried out at 4°C. The protein concentration of mitochondrial suspensions was determined at 540 nm using the Biuret method with bovine serum albumin as standard (Beyer, 1983).

Mitochondrial respiration and oxidative phosphorylation efficiency

Respiration rates were measured at 40°C as described by Teulier et al. (2010) in a thermostatic cell glass using a Clark oxygen electrode (Rank Brothers Ltd, UK). Isolated mitochondria were incubated (0.3 mg ml^{-1}) in a respiratory buffer (120 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} EGTA, 2 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} Hepes, 2.5 mmol l^{-1} malate and 0.3% BSA, pH 7.4) with pyruvate (5 mmol l^{-1}) or palmitoyl-L-carnitine (40 µmol l^{-1}) as respiratory substrates. The phosphorylation state (state 3) was obtained after addition of 500 µmol l^{-1} ADP. The basal non-phosphorylating state (state 4_{oligo}) was obtained by the addition of oligomycin (2.5 µg ml^{-1}). The respiratory control ratio (RCR) refers to the ratio of oxygen consumed after adding ADP to that consumed in the presence of oligomycin. The mitochondrial oxidative phosphorylation efficiency was assessed at 40°C by measuring the rate of oxygen consumption and ATP synthesis in the respiratory buffer supplemented with glucose (20 mmol l^{-1}), hexokinase (1.6 U ml^{-1}) and different concentrations of ADP (10, 20 or 100 µmol l^{-1}), as described previously (Teulier et al., 2010; Monternier et al., 2014).

Western blot analysis

Frozen mitochondria were used for western blot analysis; 50 µg samples of protein were loaded on 10% SDS-polyacrylamide gels and separated by electrophoresis, then transferred to PVDF membranes. A cocktail of monoclonal antibodies was used to detect the complexes of the respiratory chain: subunit NDUFB8 of complex I, complex II, Core protein 2 of complex III, subunit 1 of complex IV and the alpha subunit of ATP synthase (1:1000; ab110413, Abcam). Ponceau staining was used to ensure consistent loading and transfer. Blots were revealed using the ECL chemiluminescence method (ThermoScientific). Densitometric analysis of specific bands on immunoblots was performed by Quantity One software (Bio-Rad, USA).

Statistical analysis

Mitochondrial apparent K_m values for ADP were determined for oxygen consumption by fitting experimental data by the Michaelis–Menten equation: $V=(V_{max}\times[ADP])/(K_m+[ADP])$ using Sigma plot 12.0 software. Mitochondrial and kinetic parameters were tested with ANOVA for independent values, followed by protected least significant difference tests (Statview v4.5 software). Data are presented as means±s.e.m. with significance considered at $P<0.05$.

Competing interests

The authors declare no competing or financial interests.

Author contributions

P.-A.M. and D.R. conducted the bioenergetics studies, analysed the data, assembled the figures and wrote the manuscript. A.F. and P.-A.M. performed western blot experiments. F.H. and D.R. performed the experimental determination of skeletal muscle energy metabolite contents. J.D. and D.C.-C. performed the plasma metabolites analyses. J.L.R. and P.-A.M. performed cold and fasting acclimation protocols and revised the article. D.R. conceived and designed the study.

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Supplementary material

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