

RESEARCH ARTICLE

Increased mitochondrial energy efficiency in skeletal muscle after long-term fasting: its relevance to animal performance

Aurore Bourguignon, Anaïs Rameau, Gaëlle Toullec, Caroline Romestaing* and Damien Roussel*[‡]

ABSTRACT

In the final stage of fasting, skeletal muscle mass and protein content drastically decrease when the maintenance of efficient locomotor activity becomes crucial for animals to reactivate feeding behaviour and survive a very long period of starvation. As mitochondrial metabolism represents the main physiological link between the endogenous energy store and animal performance, the aim of this study was to determine how a very long, natural period of fasting affected skeletal muscle mitochondrial bioenergetics in king penguin (*Aptenodytes patagonicus*) chicks. Rates of mitochondrial oxidative phosphorylation were measured in pectoralis permeabilized fibres and isolated mitochondria. Mitochondrial ATP synthesis efficiency and the activities of respiratory chain complexes were measured in mitochondria isolated from pectoralis muscle. Results from long-term (4–5 months) naturally fasted chicks were compared with those from short-term (10 day) fasted birds. The respiratory activities of muscle fibres and isolated mitochondria were reduced by 60% and 45%, respectively, on average in long-term fasted chicks compared with short-term fasted birds. Oxidative capacity and mitochondrial content of pectoralis muscle were lowered by long-term fasting. Bioenergetic analysis of pectoralis muscle also revealed that mitochondria were, on average, 25% more energy efficient in the final stage of fasting (4–5 months) than after 10 days of fasting (short-term fasted birds). These results suggest that the strong reduction in respiratory capacity of pectoralis muscle was partly alleviated by increased mitochondrial ATP synthesis efficiency. Such oxidative phosphorylation optimization can impact animal performance, e.g. the metabolic cost of locomotion or the foraging efficiency.

KEY WORDS: Mitochondria, Oxidative phosphorylation, Bioenergetics, Starvation, Birds, Skeletal muscle

INTRODUCTION

During food deprivation, the conservation of limited endogenous energy resources becomes a key priority, and animals must exhibit adaptive physiological and biochemical responses to reduce energy expenditure and/or the cost of current biological activities (Wang et al., 2006). Approximately 90% of oxygen consumption is associated with mitochondrial function (Rolfe and Brand, 1997), which implies that variations in mitochondrial activity within tissues and organs during food shortages should contribute to

changes in metabolic rate. This is well exemplified by hibernating mammals, in which an early active suppression of mitochondrial metabolism is associated with metabolic suppression and subsequent falls in body temperature (Staples, 2014). In birds, it has been shown that 2 weeks of fasting decreased avian uncoupling-protein-mediated mitochondrial proton leak activity in adult king penguins, which has been calculated to represent nearly 20% of the fasting-induced reduction in metabolic rate (Rey et al., 2008). An upregulation of mitochondrial efficiency has been recently reported in hypo-metabolic muscle mitochondria of fasted ducklings (Monternier et al., 2015). Mitochondrial efficiency (i.e. the coupling ATP/O value) describes how much oxygen is needed to produce ATP, and so to run cellular activities (Brand, 2005). Thus, skeletal muscles from fasted birds should improve their ATP generation capacity by consuming less oxygen and nutrients to fuel their energy needs. These data clearly illustrate that substantial energy savings can be achieved at the whole-animal level by downregulating the activities and/or upregulating the efficiency of mitochondrial oxidative phosphorylation at the cell level.

King penguin chicks have evolved to survive up to 5 months of starvation (Cherel and Le Maho, 1985). This exceptional starvation resistance in young endotherms reflects their high capacity to store, mobilize and oxidize fat as fuel and their ability to minimize heat dissipation and energy expenditure (Duchamp et al., 1989; Cherel et al., 1993; Eichhorn et al., 2011; Teulier et al., 2013). At the mitochondrial level, we have recently shown that short-term fasting (10 days) increased the efficiency of oxidative phosphorylation processes in skeletal muscle mitochondria (Monternier et al., 2014). However, contrary to fasted ducklings (Monternier et al., 2015), the elevated ATP generation per oxygen uptake was not associated with a loss of oxidative phosphorylation activity (Duchamp et al., 1991; Monternier et al., 2014). In ducklings, fasting-induced mitochondrial hypo-activity and highly efficient oxidative phosphorylation were associated with decreased mitochondrial protein levels in pectoralis muscle (Monternier et al., 2015). In contrast, in king penguins, the volume and content of pectoralis muscle mitochondria and their oxidative capacity were not affected by short-term fasting, at least after up to 3 weeks of food deprivation (Duchamp et al., 1991; Rey et al., 2008). Thus, the sparing of mitochondrial protein in the pectoralis muscle of king penguin chicks during short-term fasting might contribute to the preservation of high oxygen consumption and ATP generation in their mitochondria. In line with this hypothesis is the lower succinate-supported respiration reported in long-term (4–5 months) fasted king penguin chicks, associated with a decrease in mitochondrial oxidative capacity and the loss of mitochondrial protein in pectoralis muscle (Duchamp et al., 1991). These observations raise the question of whether the loss of mitochondrial protein during long-term fasting may in turn improve mitochondrial oxidative phosphorylation efficiency in skeletal muscle. Therefore, in this study, we investigated the effects of long-term fasting on the

Université de Lyon, Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, UMR 5023 CNRS, Université Claude Bernard Lyon 1, ENTPE, 69622 Villeurbanne cedex, France.

*These authors contributed equally to this work.

[‡]Author for correspondence (damien.roussel@univ-lyon1.fr)

 D.R., 0000-0002-8865-5428

oxidative phosphorylation and cytochrome *c* oxidase activities of permeabilized fibres and isolated mitochondria from pectoralis muscle in king penguin chicks. We also measured oxygen consumption and ATP synthesis at maximal and submaximal oxidative phosphorylation rates in pectoralis muscle mitochondria. Results from long-term (4–5 months) fasted king penguin chicks were compared with those from short-term (10 days) fasted chicks.

MATERIALS AND METHODS

Animal studies

The present work was carried out at the French Alfred Faure station on Possession Island (Crozet Archipelago, 46°25'S, 51°45'N) from July to September during three austral winter campaigns (2014, 2015 and 2016). According to the Agreed Measures for the Conservation of Antarctic and Sub-Antarctic Fauna, the project and all of the present experimental procedures received the ethical approval of the French Polar Research Institute (IPEV, program no. 131). Two groups of king penguin (*Aptenodytes patagonicus* Miller 1778) chicks of either sex, 7–9 months old, captured at the breeding colony of Baie du Marin and kept outside in an open-top enclosure, were established. The first group of 30 chicks was captured during the winter (July–August) and left to fast for 8.2±0.4 days. By the end of the fasting period, the specific daily change in body mass was 10±1 g kg⁻¹ day⁻¹, indicating that birds were in early phase II of fasting (Cherel and Le Maho, 1985). These chicks constituted the short-term (phase II) fasting group. The second group of 26 chicks was captured in September after several months of natural winter fast. These chicks constituted the long-term fasting group and were deemed to be in phase III of fasting (Cherel and Le Maho, 1985). Superficial pectoralis muscle was surgically biopsied under isoflurane-induced general anaesthesia, as previously performed on king penguin chicks (Monternier et al., 2014). Muscle samples were immediately used for fibre preparation or mitochondrial extraction. On completion of the study, all penguin chicks were fed and kept until full recovery, and then released at the site of their capture.

Permeabilized muscle fibre preparation, respiration and cytochrome *c* oxidase activity

Muscle biopsies (50 mg) were immersed in ice-cold isolation solution [BIOPS; containing (in mmol l⁻¹): 2.77 Ca-EGTA, 7.23 EGTA, 20 imidazole, 20 taurine, 50 K-MES, 0.5 DTT, 6.56 MgCl₂, 5.77 ATP, 15 phosphocreatine, pH 7.2], and muscle strips were dissected to separate muscle fibres (Pesta and Gnaiger, 2012). Fibre bundles were transferred to BIOPS solution supplemented with saponin (50 µg ml⁻¹) and mixed gently at 4°C for 30 min. The permeabilized fibres were then washed once by gentle mixing for 10 min at 4°C in the BIOPS solution. Muscle fibres were weighed, and their respiration was monitored with an Oroboros oxymeter at 38°C in a hyperoxygenated respiratory buffer [Mir05: 110 mmol l⁻¹ sucrose, 0.5 mmol l⁻¹ EGTA, 3 mmol l⁻¹ MgCl₂, 60 mmol l⁻¹ K-lactobionate, 20 mmol l⁻¹ taurine, 10 mmol l⁻¹ KH₂PO₄, 1 g l⁻¹ fatty acid-free bovine serum albumin (w/v) and 20 mmol l⁻¹ Hepes, pH 7.1 at 38°C] using a mixture of respiratory substrates (5 mmol l⁻¹ pyruvate/2.5 mmol l⁻¹ malate/5 mmol l⁻¹ succinate). Hyperoxygenated conditions were obtained by pre-bubbling Mir05 with pure oxygen and were used with permeabilized muscle fibres to avoid experimental oxygen limitation of respiration (Pesta and Gnaiger, 2012). The phosphorylating state of respiration was determined in the presence of 1 mmol l⁻¹ ADP. Uncoupled respiration was initiated by the addition of 2 µmol l⁻¹ carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Thereafter, antimycin (10 µmol l⁻¹) was added to fully inhibit pyruvate/malate/

succinate-supported respiration. Then, ascorbate (5 mmol l⁻¹) and *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine (1.5 mmol l⁻¹) were added and the maximal respiration rate associated with isolated cytochrome *c* oxidase activity was recorded.

Mitochondrial isolation, respiration and cytochrome *c* oxidase activity

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), using a standard extraction protocol, involving Potter homogenization, partial protease digestion and differential centrifugations, as previously described (Monternier et al., 2014). Protein content of the mitochondrial preparation was assayed at 540 nm using the biuret method, with bovine serum albumin as a standard. Note that the absorbance of the same volume of mitochondria was assayed at 540 nm in a solution containing 0.6% Na-K-tartrate and 3% NaOH and subtracted in order to take into account any contamination with haemoglobin. Oxygen consumption was measured in a glass cell fitted with a Clark oxygen electrode (Rank Brothers Ltd, Bottisham, UK), at 38°C, and calibrated with air-saturated respiratory buffer (120 mmol l⁻¹ KCl, 5 mmol l⁻¹ KH₂PO₄, 1 mmol l⁻¹ EGTA, 2 mmol l⁻¹ MgCl₂, 0.3% fatty acid-free bovine serum albumin (w/v), and 3 mmol l⁻¹ Hepes, pH 7.4). Muscle mitochondria (1 mg ml⁻¹) were incubated in the respiratory buffer and 5 mmol l⁻¹ pyruvate/2.5 mmol l⁻¹ malate/5 mmol l⁻¹ succinate was added to start the oxygen consumption. Phosphorylating respiration was initiated by the addition of 1 mmol l⁻¹ ADP. The uncoupled state of respiration was initiated by the addition of 2 µmol l⁻¹ FCCP (in the presence of 2 µg ml⁻¹ oligomycin). Thereafter, antimycin (10 µmol l⁻¹) was added to fully inhibit pyruvate /malate/succinate-supported respiration. Then, ascorbate (5 mmol l⁻¹) and TMPD (1 mmol l⁻¹) were added and the maximal respiration rate associated with isolated cytochrome *c* oxidase activity was recorded.

Mitochondrial oxidative phosphorylation efficiency

Oxygen consumption and ATP synthesis rates were measured at 38°C in 500 µl respiratory buffer supplemented with glucose (20 mmol l⁻¹) and hexokinase (1.5 U ml⁻¹), as previously described (Teulier et al., 2010; Monternier et al., 2014). Muscle mitochondria were respiring on a mixture of substrates consisting of pyruvate (5 mmol l⁻¹), malate (2.5 mmol l⁻¹) and succinate (5 mmol l⁻¹). Mitochondrial ATP synthesis was initiated by the addition of 1 mmol l⁻¹, 100 µmol l⁻¹, 20 µmol l⁻¹, 10 µmol l⁻¹ or 5 µmol l⁻¹ ADP. After recording the phosphorylating respiration rate for 2–3 min in a glass cell fitted with a Clark oxygen electrode (Rank Brothers), four 100 µl samples of mitochondrial suspension were withdrawn every minute and quenched in a perchloric acid solution (10% HClO₄, 25 mmol l⁻¹ EDTA). After centrifugation of the denatured protein (15,000 g for 5 min) and neutralization of the resulting supernatant with a KOH solution (2 mol l⁻¹ KOH, 0.3 mol l⁻¹ MOPS), ATP production was determined from the glucose-6-phosphate content of samples, which was measured enzymatically by spectrophotometry according to Lang and Michal (1974). Basal non-phosphorylating respiration rates were determined in the presence of 2 µg ml⁻¹ oligomycin. Kinetic parameters of mitochondrial oxidation, i.e. the apparent affinity (K_m) for ADP and the maximal oxidation rate (V_{max}) of mitochondria, were determined from the dependence of mitochondrial oxygen consumption rate on ADP concentration ranging from 5 to 1000 µmol l⁻¹.

Mitochondrial enzyme activity

Citrate synthase, NADH ubiquinone reductase (complex I) and succinate ubiquinone reductase (complex II) activities were measured spectrophotometrically following the protocols of Medja et al. (2009). Frozen mitochondria were thawed and diluted 1:20 in 100 mmol l⁻¹ phosphate buffer supplemented with 2 mmol l⁻¹ EDTA. All assays were performed in duplicate at 38°C using a final volume of 260 µl.

Citrate synthase activity was assayed in a reaction medium containing 100 mmol l⁻¹ Tris buffer (pH 8), 100 µmol l⁻¹ 5,5'-dithiobis(2-nitrobenzoic acid) and 300 µmol l⁻¹ acetyl-CoA, and supplemented with 10 to 30 µg ml⁻¹ of mitochondrial suspension. After 4 min of incubation, the reaction was started by adding 500 µmol l⁻¹ oxaloacetate, and the reduction of DNTB by CoASH was followed at 412 nm. Enzyme activity was quantified using an extinction coefficient of 13.6 mmol l⁻¹ cm⁻¹.

The rotenone-sensitive activity of mitochondrial complex I (NADH ubiquinone reductase) was assayed by following NADH oxidation in the presence or absence of 12.5 µmol l⁻¹ rotenone. Mitochondria (10 to 30 µg ml⁻¹) were incubated in reaction medium containing 50 mmol l⁻¹ phosphate buffer (pH 7.5), 100 µmol l⁻¹ decylubiquinone and 3.75 mg ml⁻¹ bovine serum albumin. After 4 min of incubation, the reaction was started by adding 100 µmol l⁻¹ NADH and the change in optical density at 340 nm was recorded for 4 min. Enzyme activity was quantified by using an extinction coefficient of 6.22 mmol l⁻¹ cm⁻¹.

The activity of succinate dehydrogenase (complex II or succinate ubiquinone reductase) was assayed 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 and 2 mg ml⁻¹ bovine serum albumin, and supplemented with 10 to 30 µg ml⁻¹ of mitochondrial suspension. After 4 min of incubation, the reaction was started by adding 100 µmol l⁻¹ decylubiquinone, and the reduction of DCPIP was followed at 600 nm. Enzyme activity was quantified by using an extinction coefficient of 19.2 mmol l⁻¹ cm⁻¹.

Statistical analysis

Mitochondrial apparent K_m values for ADP were determined for oxygen consumption by fitting the Michaelis–Menten equation, $V = (V_{max} \times [ADP]) / (K_m + [ADP])$, to the experimental data using SigmaPlot 12.0 software (Systat Software, San Jose, CA, USA). Two-way repeated-measures ANOVA (RM ANOVA) followed by protected least significant difference (LSD) tests were performed to estimate the effects of groups and ADP addition on mitochondrial respiration rates (SigmaPlot 12.0). 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

Morphometric measurements

Long-term (phase III) fasted chicks weighed significantly less than short-term (phase II) fasted chicks (Table 1). Phase III fasted chicks also exhibited lower beak and flipper lengths than phase II fasted birds, indicating a smaller body size (Table 1).

Muscle oxidative activity, mitochondrial respiration and enzyme activity

Fig. 1 shows the oxygen consumption of permeabilized muscle fibres (Fig. 1A) or isolated muscle mitochondria (Fig. 1C) energized

Table 1. Summary data of morphometric measurements on fasted king penguin chicks during three successive austral winter campaigns on Possession Island in the Crozet Archipelago

Morphometric parameter	Phase II	Phase III
Body mass (kg)	8.4 ± 0.1	3.0 ± 0.1*
Bill length (mm)	91 ± 1	76 ± 1*
Flipper length (mm)	307 ± 2	264 ± 4*

Phase II, short-term (8 days) fasting; phase III, long-term (4–5 months) fasting. Values are means ± s.e.m. from 30 short-term fasted and 26 long-term fasted king penguin chicks. * $P < 0.05$, significantly different between groups.

with pyruvate–malate–succinate (PM+S), a standard mixture of respiratory substrates that provides electrons to complexes I and II of the respiratory chain. At the fibre level, rates of oxygen consumption during basal (PM+S), ADP-induced maximal phosphorylating (ADP) and FCCP-induced maximal oxidative (FCCP) activities decreased significantly by 60% on average in long-term (phase III) fasted chicks compared with short-term (phase II) fasted chicks (Fig. 1A). At the level of isolated mitochondria, rates of oxygen consumptions also dropped significantly by 45% on average in long-term fasted chicks compared with short-term fasted ones (Fig. 1C). Specific cytochrome *c* oxidase activities (per muscle mass or per

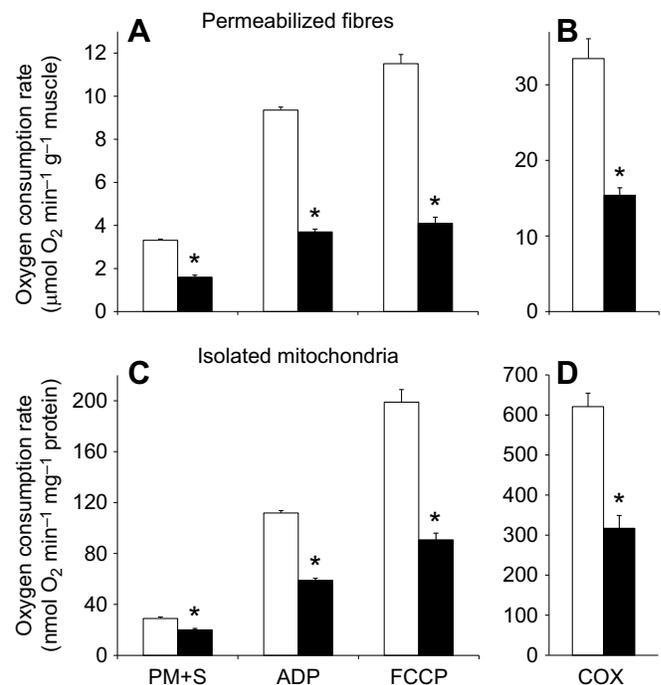


Fig. 1. Effect of fasting on muscle and mitochondrial oxidative activities. Respiration rates of permeabilized muscle fibres (A,B) and isolated mitochondria (C,D) from pectoralis muscle of short-term (phase II, white bars) and long-term (phase III, black bars) fasted king penguin chicks were measured at 38°C. (A) Fibres were energized with pyruvate–malate–succinate (PM+S). Phosphorylating respiration was initiated with 1 mmol l⁻¹ ADP (ADP), and maximal respiration was initiated with 2 µmol l⁻¹ FCCP. (B) Fibres were energized with ascorbate-TMPD (COX) in the presence of antimycin and FCCP. (C) Mitochondria were energized with PM+S. Phosphorylating respiration was initiated with 1 mmol l⁻¹ ADP (ADP), and maximal respiration was initiated with 2 µmol l⁻¹ FCCP in the presence of oligomycin. (D) Mitochondria were energized with ascorbate-TMPD (COX) in the presence of antimycin, oligomycin and FCCP (see Materials and methods for details). Values are means ± s.e.m. from $N = 8–10$ independent preparations. * $P < 0.05$, significantly different between groups.

Table 2. Effect of fasting on mitochondrial enzyme activities

Enzyme	Phase II	Phase III
Citrate synthase	(10)	(11)
nmol min ⁻¹ mg ⁻¹ mitochondrial protein	296±23	273±50
U g ⁻¹ muscle	18.8±1.4	12.5±2.3*
NADH-ubiquinone reductase (Complex I)	(9)	(8)
nmol min ⁻¹ mg ⁻¹ mitochondrial protein	165±13	166±13
U g ⁻¹ muscle	10.5±0.8	7.6±0.6*
Succinate-ubiquinone reductase (Complex II)	(10)	(11)
nmol min ⁻¹ mg ⁻¹ mitochondrial protein	102±8	131±17
U g ⁻¹ muscle	6.5±0.5	6.0±0.8

Values are means±s.e.m. The number of independent mitochondrial preparations is given in parentheses. Phase II, short-term (10 day) fasting; phase III, long-term (4–5 months) fasting. * $P<0.05$, significantly different between groups.

mitochondrial protein mass) were significantly lower after long-term fasting in both pectoralis muscle fibres (Fig. 1B) and mitochondria (Fig. 1D). Mitochondrial levels in muscle can be estimated from the ratio between the oxidative activities of muscle fibres (expressed per gram of muscle) and mitochondria (expressed per milligram of protein). Using FCCP-induced maximal oxygen consumption activities, we found significantly fewer mitochondria per muscle mass in long-term than in short-term fasted chicks (46±4 versus 63±4 mg of mitochondrial protein per gram of pectoralis muscle in phase III versus phase II chicks, respectively; $P<0.001$).

Mitochondrial oxidative phosphorylation efficiency

Fig. 2 shows the dependence of oxygen consumption rates on ADP concentrations ranging from 5 to 1000 $\mu\text{mol l}^{-1}$ (Fig. 2A) and the relationship between the rates of ATP synthesis and oxygen consumption (Fig. 2B) in a mixed population of mitochondria isolated from pectoralis muscle and respiring on PM+S. The maximal oxidation rates (V_{max}) of mitochondria were significantly lowered by long-term fasting compared with short-term fasting, and the apparent affinity constants of mitochondrial oxidation for ADP were not significantly different between groups (Fig. 2A). In accordance with the respiratory parameters reported above, maximal rate of ATP synthesis and corresponding oxygen consumption, the highest points to the right of the linear relationships, decreased significantly by 52% in long-term fasted chicks compared with short-term fasted chicks (Fig. 2B). The basal non-phosphorylating respiration rates measured in the presence of oligomycin (the intercepts with the x -axis) decreased significantly by 48% in long-term fasted mitochondria. Hence, the linear relationships concerning long-term fasted chicks was significantly shifted to the left compared with short-term fasted chicks, indicating that to produce a given amount of ATP, less oxygen was consumed by long-term fasted mitochondria (Fig. 2B). On the whole, at any specified respiration rate, the effective oxidative phosphorylation efficiency of pectoralis mitochondria was increased by approximately 25% in long-term fasted chicks compared with short-term fasted chicks.

Mitochondrial enzyme activities

The levels of citrate synthase (an enzyme of the citric acid cycle), NADH-ubiquinone reductase and succinate-ubiquinone reductase (complex I and II of the electron transport chain, respectively) per milligram of mitochondrial protein were not significantly different between experimental groups (Table 2). When taking into account the mitochondrial content of pectoralis muscle (see above), the activities of citrate synthase and NADH-ubiquinone reductase per gram of pectoralis muscle were significantly lower in phase III than

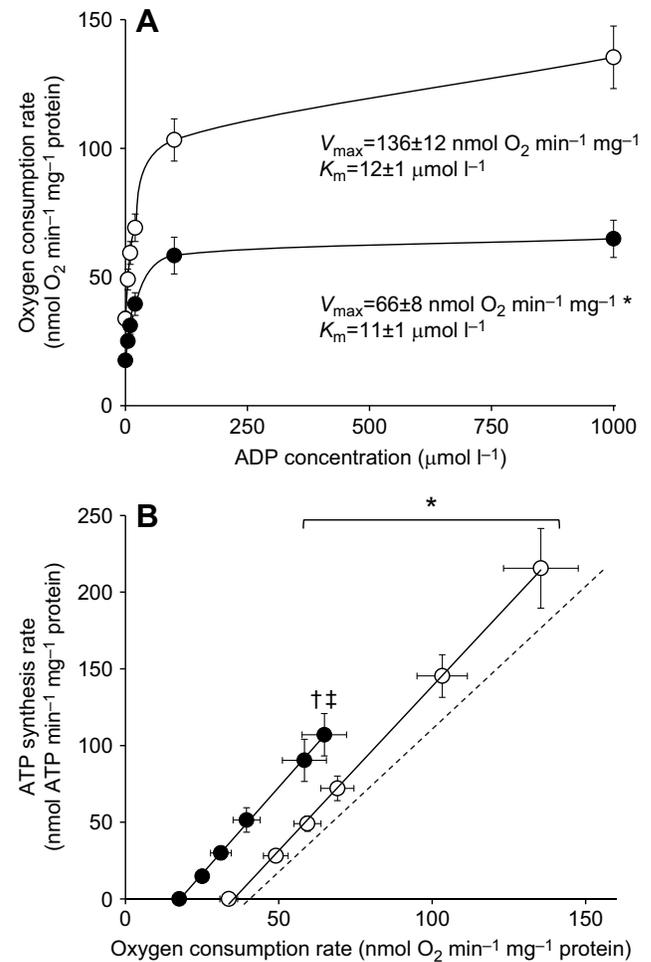


Fig. 2. Effect of fasting on mitochondrial kinetics oxidation parameters for ADP and oxidative phosphorylation efficiency. Respiration rates and oxidative phosphorylation efficiency of mitochondria isolated from pectoralis muscle of short-term (phase II, open circles) and long-term (phase III, black circles) fasted king penguin chicks. Mitochondrial activity was determined at 38°C with pyruvate/malate/succinate as respiratory substrates. (A) Dependence of oxidation rate on ADP concentrations. (B) Linear relationship between rates of ATP synthesis and oxygen consumption. Values are means±s.e.m. from $N=10$ and $N=8$ independent mitochondrial preparations for short-term and long-term fasted chicks, respectively. * $P<0.05$, significantly different between groups. † and ‡ indicate a significant effect of long-term fasting on maximal ATP synthesis rate and corresponding oxygen consumption, respectively (the highest points on the right of the linear relationship in both cases). The dashed line was calculated from data previously measured in king penguin chicks (Montermer et al., 2014) and illustrates the effect of re-feeding on the mitochondrial efficiency of pectoralis muscle.

in phase II chicks, whereas succinate-ubiquinone reductase activity per muscle mass was not significantly altered after long-term fasting (Table 2). It has been previously reported that pectoralis muscles diminished drastically after long-term (phase III) fasting in king penguin chicks (Duchamp et al., 1991). Consequently, long-term fasted chicks have much less overall muscle respiratory capacity and mitochondrial enzyme activities than short-term fasted chicks.

DISCUSSION

In king penguin chicks, the long natural winter fast can be divided into three phases (Cherel and Le Maho, 1985). After a short period of metabolic adaptation (8 days), marked by a rapid drop in body

mass and an increased mobilization of fat stores (phase I), the daily fall in body mass stabilizes and remains at a minimum for an average of 4 months (phase II). Phase II is a period of economy, characterized by high fat mobilization and oxidation while body proteins are efficiently spared (reviewed in Cherel et al., 1988; Groscolas and Robin, 2001). Thereafter follows the shorter phase III (21 days), which is characterized again by a rapid decrease in body mass and increased protein catabolism, characterized by myofibrillar degeneration and protein loss in pectoralis muscle (Cherel and Le Maho, 1985; Duchamp et al., 1991; Cherel et al., 1994). In this ultimate period of fasting, the enhanced proteolysis in skeletal muscle leads to a drastic muscle atrophy in birds (Le Maho et al., 1981; Robin et al., 1988; Duchamp et al., 1991; Cherel et al., 1994; Thouzeau et al., 1999). In the present study, long-term fasting induced a loss of approximately 30% of mitochondrial protein per pectoralis muscle mass and a general decrease in the specific activity of mitochondrial enzymes (citrate synthase, NADH-ubiquinone reductase, cytochrome *c* oxidase) expressed per gram of pectoralis muscle. Of note is the relative preservation of succinate-ubiquinone reductase activity in naturally long-term fasted chicks, which was not significantly different when expressed per gram of pectoralis muscle than that in short-term fasted chicks. Of course, on account of the drastic reduction in pectoralis muscle mass (Duchamp et al., 1991), the mitochondrial enzyme capability was almost totally depleted (>90% on average) in pectoralis muscle of long-term (phase III) fasted king penguin chicks compared with short-term (phase II) fasted chicks. These results are in line with and extend previously published data on king penguin chicks, showing losses in mitochondrial protein content and cytochrome *c* oxidase capacity in both pectoralis and gastrocnemius muscles after natural long-term fasting (Duchamp et al., 1991). Skeletal muscles are the primary site of heat production in birds, contributing to a large proportion of energy expenditure owing to their relative mass and high oxidative capacity (Duchamp and Barré, 1993; Duchamp et al., 1999; Bicudo et al., 2002). In particular, breast muscle is an important determinant of metabolic rate (Weber and Piersma, 1996; Chappell et al., 1999) and changes in its mass have been shown to drive the seasonal phenotypic flexibility of metabolic rates in birds (Swanson and Vézina, 2015). Thus, the drastic diminution of the total oxidative capacity of skeletal muscles is consistent with the lower peak metabolic rate measured in king penguin chicks after a long-term fast (Duchamp et al., 1989).

The loss of oxidative capacity reported in long-term fasted king penguin chicks at the pectoralis muscle and whole-animal levels was reflected at the mitochondrial level by a reduced maximal rate of oxidative phosphorylation, as shown using the substrate combination PM+S. Nevertheless, when succinate was used as respiratory substrate, the phosphorylating state of respiration measured in mitochondria from pectoralis muscle of long-term fasted king penguin chicks was not significantly different from that in short-term fasted chicks (Duchamp et al., 1991). Among the mitochondrial enzymes involved in substrates or reduced coenzyme oxidation, succinate-ubiquinone reductase (complex II) was slightly increased by 28% in late fasting, and citrate synthase and complex I of the electron transport chain were not significantly altered by long-term fasting (Table 2). Only cytochrome *c* oxidase activity was reduced by approximately 50% in long-term fasted chicks compared with short-term fasted chicks (Fig. 1; see also Duchamp et al., 1991). In birds, the mitochondrial active state of respiration in skeletal muscle appears to be mainly controlled by oxidation processes, e.g. substrate transport, oxidation and respiratory chain (Kikusato et al., 2010). Therefore, the reason for the selective effect

of fasting on substrate oxidation in penguins is unclear, but may be due to the glucose–fatty acid cycle, based on the concept that fatty acid oxidation would inhibit the uptake and catabolism of glucose in muscle, with, for example, the inhibition of the pyruvate dehydrogenase complex at the mitochondrial level (Randle, 1998; Roden, 2004). Another non-exclusive hypothesis is based on the critical dependence on the substrate supply for the control exerted by cytochrome *c* oxidase over oxygen consumption (Kunz et al., 2000; Quarato et al., 2011). In saponin-permeabilized muscle fibres, the flux control coefficient of cytochrome *c* oxidase appears higher in the presence of both complex I and II substrates (glutamate/malate/succinate) than with glutamate/malate or succinate alone (Wiedemann and Kunz, 1998; Kunz et al., 2000). Similarly, the flux control coefficient of cytochrome *c* oxidase depends on the combination of respiratory substrates used in digitonin-permeabilized HepG2 cells, these being higher in the presence of complex I and II substrates, compared with pyruvate/malate or succinate alone, or pyruvate/malate/succinate>pyruvate/malate>succinate (Quarato et al., 2011). The pyruvate carrier also exerts a rather important control on the oxygen consumption flux in muscle mitochondria (Rossignol et al., 2000). Even though large respiratory substrate concentrations were used in the present study, we cannot rule out that part of the effect of late fasting on the mitochondrial oxidative phosphorylation rate could be ascribed to the selective impairment of pyruvate transport and/or oxidation in mitochondria. These speculations clearly deserve further investigation.

In the present study, long-term (4–5 months) fasting induced a large decrease in respiratory activity but also an increase in the efficiency of mitochondrial ATP synthesis in pectoralis muscle. These findings are in keeping with those reported in fasted ducklings, whose pectoralis muscle mitochondria displayed lower oxidative phosphorylation activity and an improvement in ATP synthesis efficiency when respiring on pyruvate/malate or palmitoyl-carnitine/malate (Monternier et al., 2015). In king penguin chicks, the effective oxidative phosphorylation efficiency increased by approximately 15% at the beginning of phase II after 10 days of fasting (Monternier et al., 2014) and up to 40% in phase III after several months of fasting (present study) in comparison with fed chicks (dashed line in Fig. 2B). Mitochondrial efficiency (i.e. the ATP/O coupling value) is an important parameter in the mitochondrial energy transduction system as it describes how much oxygen, and thus the level of nutrients, is needed to produce useable cellular energy in the form of ATP (Brand, 2005). Mitochondria from fasted birds therefore optimize oxidative phosphorylation output by consuming fewer nutrients and oxygen to synthesize ATP and fuel the cell's energy needs. In other words, the skeletal muscles of fasted birds could function at a lower cost for any given metabolic activity.

Relevance for animal performance and conclusions

As mitochondrial oxidative phosphorylation processes provide most of the useable energy in the form of ATP, these represent the main physiological link between the environmental or endogenous energy store and animal performance (maintenance, locomotion, thermoregulation, growth or reproduction). This situation suggests that variations in mitochondrial efficiency would substantially affect an animal's performance. In this context, it is worth noting that fasted penguins reduce their metabolic cost of terrestrial locomotion and increase their foraging efficiency at sea (Fahlman et al., 2004; Schull et al., 2016). In the former example, the cost of terrestrial locomotion in penguins decreases over the course of fasting, being reduced by either 45% or by 22% after 28 days of fasting in king penguins, when expressed per individual or per unit

of body mass (kg), respectively (Fahlman et al., 2004). Although the cost of locomotion benefits greatly from the loss of body mass (Pinshow et al., 1977; Dewasmes et al., 1980), other aspects of metabolic adjustments to fasting would contribute to improved exercise performance, such as a mitochondrial optimization of oxidative phosphorylation efficiency (present study). In support of this hypothesis, treatments that elevate mitochondrial coupling efficiency have been shown to raise both exercise efficiency and the capacity for sustained exercise in mammals (Conley, 2016). In a more recent study, Schull et al. (2016) compared the effort of phase II and phase III fasted king penguins to recover body mass after their foraging trip at sea. After a longer period of fasting, phase III fasted penguins weighed less than phase II fasted birds at the time of their release, but when they returned from their first re-feeding trip, phase III and phase II birds did not differ in body mass. They had all recovered their initial body mass while having spent the same time foraging at sea (Schull et al., 2016). Consequently, the total mass gain was higher in phase III- than in phase II-fasted birds, suggesting a higher feeding efficiency in birds having fasted significantly longer (Schull et al., 2016). There are several physiological phenomena that can explain this catch-up growth, such as a faster recovery of digestive organs during re-feeding and/or a lower energy expenditure and lower circulating thyroid hormones in phase III-fasted birds (Robin et al., 1991; Duchamp et al., 1989; Cherel et al., 2004). But a higher mitochondrial efficiency may also favour the restoration of tissue mass and function by minimizing the metabolic need for nutrients in ATP synthesis, thereby diverting nutrients from oxidation towards anabolic pathways. Although speculative, these links between mitochondrial functioning and penguin performance clearly deserve further investigation.

In conclusion, long-term fasting decreased the respiratory activity and content of mitochondria in pectoralis muscle, which strongly reduced the oxidative capacity of this organ. However, the loss of mitochondrial power was partly alleviated by increased mitochondrial energy coupling. Such optimization of oxidative phosphorylation should favour the preservation of some skeletal muscle functions and animal performance, such as locomotion and thermoregulation, which seem to be essential at the ultimate phase of fasting (Duchamp et al., 1989; Groscolas and Robin, 2001).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.R.; Methodology: C.R., D.R.; Validation: C.R., D.R.; Formal analysis: A.B., A.R., G.T., C.R., D.R.; Investigation: A.B., A.R., G.T.; Writing - original draft: D.R.; Writing - review & editing: C.R., D.R.; Supervision: C.R., D.R.; Project administration: D.R.; Funding acquisition: D.R.

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