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RESEARCH ARTICLE

Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks

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ABSTRACT

Energy conservation is a key priority for organisms that live in environments with seasonal shortages in resource supplies or that spontaneously fast during their annual cycle. The aim of this study was to determine whether the high fasting endurance of winter-acclimatized king penguin chicks (*Aptenodytes patagonicus*) is associated with an adjustment of mitochondrial bioenergetics in pectoralis muscle, the largest skeletal muscle in penguins. The rates of mitochondrial oxygen consumption, and ATP synthesis and mitochondrial efficiency (ATP/O ratio) were measured in winter-acclimatized chicks. We used pyruvate/malate and palmitoyl-L-carnitine/malate as respiratory substrates and results from naturally fasted chicks were compared to experimentally re-fed chicks. Bioenergetics analysis of pectoralis muscle revealed that mitochondria are on average 15% more energy efficient in naturally fasted than in experimentally fed chicks, indicating that fasted birds consume less nutrients to sustain their energy-demanding processes. We also found that moderate reductions in temperature from 38°C to 30°C further increase by 23% the energy coupling efficiency at the level of mitochondria, suggesting that king penguin chicks realize additional energy savings while becoming hypothermic during winter. It has been calculated that this adjustment of mitochondrial efficiency in skeletal muscle may contribute to nearly 25% of fasting-induced reduction in mass-specific metabolic rate measured *in vivo*. The present study shows that the regulation of mitochondrial efficiency triggers the development of an economical management of resources, which would maximize the conservation of endogenous fuel stores by decreasing the cost of living in fasted winter-acclimatized king penguin chicks.

KEY WORDS: Bird, Skeletal muscle, Mitochondria, Energy efficiency, Starvation

INTRODUCTION

Spontaneous fast is a major characteristic of the annual cycle of sea birds when breeding or moulting on land, whereas they feed exclusively at sea. In situations where food is lacking or not used, the allocation of limited endogenous resources causes trade-offs between competing traits, such as reproduction, somatic growth and maintenance. During food deprivation, animals cannot maximize all of their life-history traits and must exhibit adaptive behavioural, physiological and biochemical responses to reduce metabolism and/or the cost of current activities in order to maintain biological value (Wang et al., 2006). Among sea birds, penguins, especially the *Aptenodytes* genus, which inhabits cold environments, have evolved to tolerate some of the greatest relative body mass losses and to survive several months of starvation, which can be up to 5 months in winter-acclimatized king penguin chicks (Cherel and Le Maho, 1985; Groscolas, 1990; McCue, 2010).

King penguin chicks (*Aptenodytes patagonicus*, Miller J. F. 1778) experience a severe nutritional shortage when left alone for a long period of time, and they may be fed infrequently or not at all by their parents during the austral winter (Cherel et al., 1987). Yet, the energetic demands remain elevated because of somatic growth, tissue maturation, thermogenesis and activity against predators. Although young king penguins can tolerate the loss of 70% of their body mass, a high percentage of the growing chicks fails to support the energy cost associated with thermoregulation and predation pressure and will die by the end of winter because of a shortage of fuel (Cherel et al., 1987). Nevertheless, king penguin chicks show an exceptional fasting endurance for up to 5 months (Cherel and Le Maho, 1985), indicating that they have evolved adaptive responses to the lack of food, which primarily favours the development of a whole range of energy conservation mechanisms. Firstly, chicks are anatomically (large size, round shape, high thermal insulation) and behaviourally (huddles) well adapted to minimize heat dissipation in harsh environmental conditions (Barré, 1984; Le Bohec et al., 2005). Secondly, the winter energy expenditure is physiologically reduced by lowering the plasma level of thyroid hormones and the amount of metabolically active tissues, thereby minimizing the basal metabolic rate and the metabolic cost of growth (Barré, 1984; Cherel and Le Maho, 1985; Cherel et al., 1987; Cherel et al., 1993a; Cherel et al., 2004; Duchamp et al., 1989). Thirdly, the development of seasonal heterothermy (Eichhorn et al., 2011), together with a low thermogenic effect of circulating lipids (Teulier et al., 2013), further indicate a reduced energy investment into heat production and thermoregulation. All these energy-saving mechanisms maximize the survival of chicks in winter during this long period of food shortage.

The underlying physiological mechanisms responsible for winter fast-induced metabolic depression in chicks have not been thoroughly investigated so far. Because mitochondria provide most of the useable energy in the form of ATP, which chicks can ultimately allocate into several vital energy consuming functions (maintenance, growth, tissue maturation, locomotion, thermoregulation), mitochondrial metabolism may represent an important proximate factor that is responsible for the remarkable resistance of king penguin chicks to starvation. Therefore, the aim of this study was to determine whether the high fasting endurance of king penguin chicks was associated with an adjustment of mitochondrial ATP efficiency in skeletal muscle, which is directly involved in cold-induced thermogenesis and predator avoidance. Indeed, mitochondrial ATP synthesis is well known to have a variable degree of coupling to oxygen consumption, which mainly

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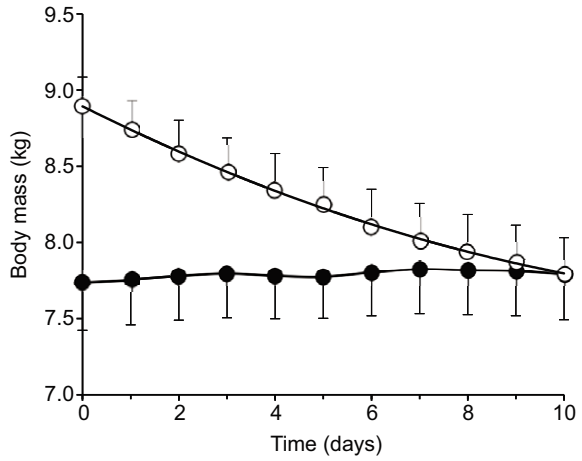

Fig. 1. Changes in body mass through the course of nutritional protocols in fasted or re-fed chicks. Values are means±s.e.m. for *N*=14 fasted chicks (open symbols) and *N*=12 fed chicks (filled symbols).

depends on mitochondrial maintenance cost, i.e. the oxygen consumed to compensate for energy wastage pathways, such as membrane permeability and/or slip reactions of the proton-pumping machinery (Brand, 2005). From the low level of circulating thyroid hormones reported in winter acclimatized chicks (Le Ninan et al., 1988; Cherel et al., 2004), one should expect a lower mitochondrial maintenance cost and thus an optimal production of ATP (Harper and Brand, 1993; Nogueira et al., 2002). To test this hypothesis, we measured muscle mitochondrial oxygen consumption and ATP synthesis at maximal and submaximal oxidative phosphorylation rates *in vitro*. Winter-acclimatized chicks were used and results from naturally fasted chicks were compared to experimentally re-fed chicks. The resting metabolic rate of birds was also measured *in vivo*. Furthermore, we also investigated the impact of heterothermy on the mitochondrial energy transduction system, by measuring *in vitro* muscle mitochondrial bioenergetics at 30°C (hypothermia) and 38°C (normothermia) in naturally fasted winter-acclimatized chicks.

RESULTS

Resting metabolic rate and body mass loss

The final body masses of chicks were not different between groups. The mass-specific resting metabolic rate of re-fed king penguin chicks (2.7±0.1 W kg^{-1}) was 17% higher than that of fasted chicks (2.3±0.2 W kg^{-1}). Chicks lost an average of 1.10±0.08 kg (13±1% of initial body mass) over the 10 days of fasting (Fig. 1). In the last 3 days of fasting, the daily loss in mass expressed per chick or per unit of body mass was 72±6 g and 9.1±0.6 g kg^{-1}, respectively. From these data, we can calculate that the mean energy equivalent of body mass loss was 21.2 kJ g^{-1}. All these values indicate that winter-acclimatized fasted chicks were in phase II of fasting at the time of experiment, a long phase of energy economy that is characterized by low energy expenditure and high lipid catabolism (Barré, 1984; Cherel and Le Maho, 1985; Cherel et al., 1993b; Robin et al., 1988; Groscolas, 1990).

Effect of nutritional status on mitochondrial activity and efficiency

Fig. 2 shows the effect of nutritional status on the activity of oxidative phosphorylation in pectoral muscle mitochondria respiring

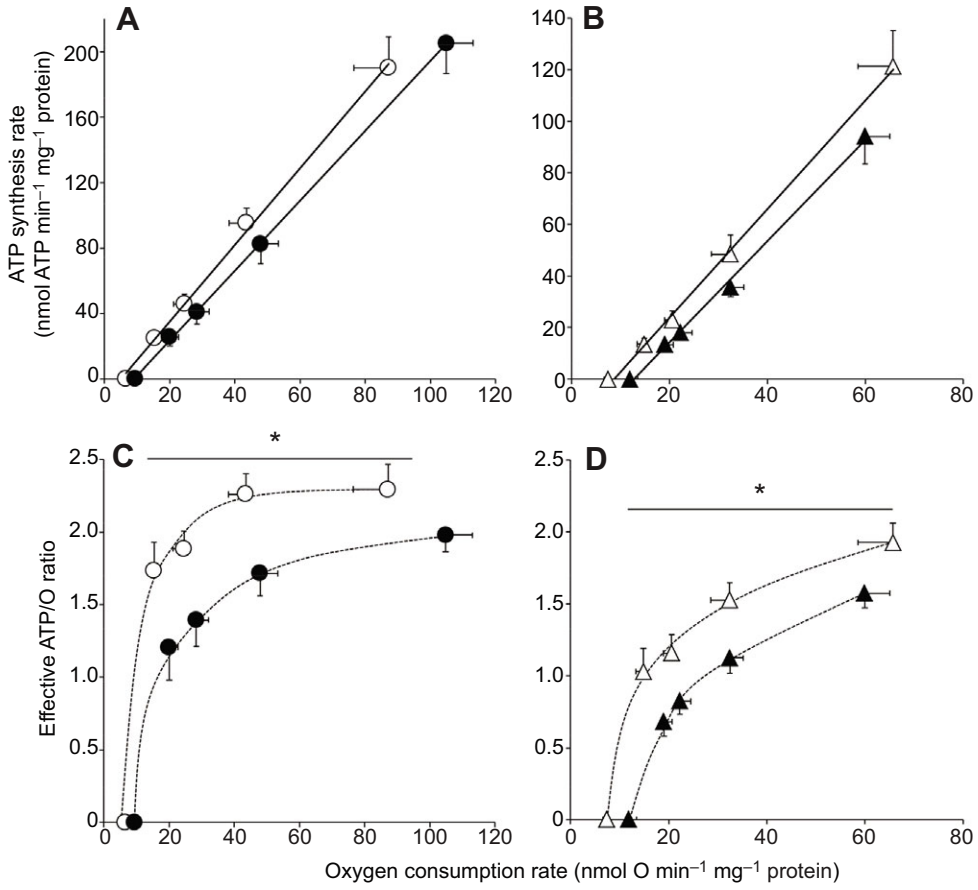

Fig. 2. Oxidative phosphorylation efficiency of mitochondria isolated from pectoralis muscle of fasted or re-fed chicks. Oxygen uptake and ATP synthesis rate were titrated by addition of increasing concentrations of ADP in the presence of glucose (20 mmol l^{-1}), hexokinase (1.5 U ml^{-1}) and either pyruvate/malate (A and C) or palmitoyl-L-carnitine/malate (B and D) at 38°C. Values are means±s.e.m. for *N*=11 (fasted, open symbols) and *N*=9 (fed, filled symbols) independent mitochondrial preparations.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.104505

on pyruvate (Fig. 2A) or palmitoyl-L-carnitine (Fig. 2B). The maximal rates of oxygen consumption and ATP production, the highest points to the right of the linear relations (Fig. 2A,B), and the apparent affinity constants of mitochondrial fluxes (oxygen consumption or ATP synthesis) in response to added ADP (supplementary material Fig. S1) were not significantly different between groups.

Regardless of respiratory substrates, the relationship between the rates of ATP synthesis and oxygen consumption in skeletal muscle mitochondria working at different steady-state rates of ATP production were linear and parallel. Indeed, the slopes were not significantly different between re-fed and fasted mitochondria (ATP/O values for pyruvate were 2.03±0.13 and 2.52±0.22 in re-fed and fasted chicks, respectively; ATP/O values for palmitoyl-L-carnitine were 1.97±0.13 and 2.12±0.16 in re-fed and fasted chicks, respectively). It is important to understand here that the slope values describe the amount of extra oxygen that mitochondria have to consume to sustain an additional ATP production imposed by a change in the activity of the phosphorylating system. As such, the slope values would be close to the maximum overall stoichiometry (mechanistic ATP/O ratio) of mitochondrial oxidative phosphorylation and would mainly depend on the activity and coupling stoichiometry of oxidative phosphorylation complexes and the nature of the oxidized substrate. The above results therefore suggest that the mechanistic ATP/O did not differ between fasted and re-fed chicks.

The basal non-phosphorylating respiration rates measured in the presence of oligomycin (the intercepts with the *x*-axis) were significantly higher in re-fed than in fasted mitochondria respiring on pyruvate (+44%) or palmitoyl-L-carnitine (+60%). Hence, regardless of the substrate used, the linear relations concerning the re-fed chicks were significantly shifted to the right compared with fasted birds, indicating lower oxidative phosphorylation efficiency (effective ATP/O) because, to produce a given amount of ATP, mitochondria consumed more oxygen in re-fed than in fasted chicks. This is shown more clearly when effective ATP/O ratios are plotted against the oxygen consumption rate (Fig. 2C for pyruvate/malate; Fig. 2D for palmitoyl-L-carnitine/malate). In the range of oxygen consumption measured, the effective ATP/O was higher in fasted than in re-fed chicks, irrespective of the substrate used. Contrary to the mechanistic ATP/O (slope values as described above), the effective ATP/O would be affected by the activities of mitochondrial reactions that consume the proton gradient built up by the respiratory chain, mainly proton leak reactions and the phosphorylation system (ATP synthase, phosphate carrier and adenine nucleotide translocase), which compete for the same driving force (Brand et al., 1993). Altogether, these results therefore suggest that mitochondrial efficiency had been altered by extrinsic properties of oxidative phosphorylation machinery. On the whole, the effective oxidative phosphorylation efficiency of skeletal muscle mitochondria was lowered by an average of 15% in re-fed animals (Fig. 4).

Effect of temperature on mitochondrial activity and efficiency

Regardless of the respiratory substrate used, ADP was less effective at stimulating oxidative activities at 30°C, whereas no significant differences were found in the rates of ATP synthesis (supplementary material Fig. S2). The apparent affinity constants of mitochondrial fluxes (oxygen consumption or ATP synthesis) to added ADP were not significantly different between the two thermal conditions (supplementary material Fig. S2, insets). Fig. 3 shows the effect of temperature on the efficiency of oxidative phosphorylation in

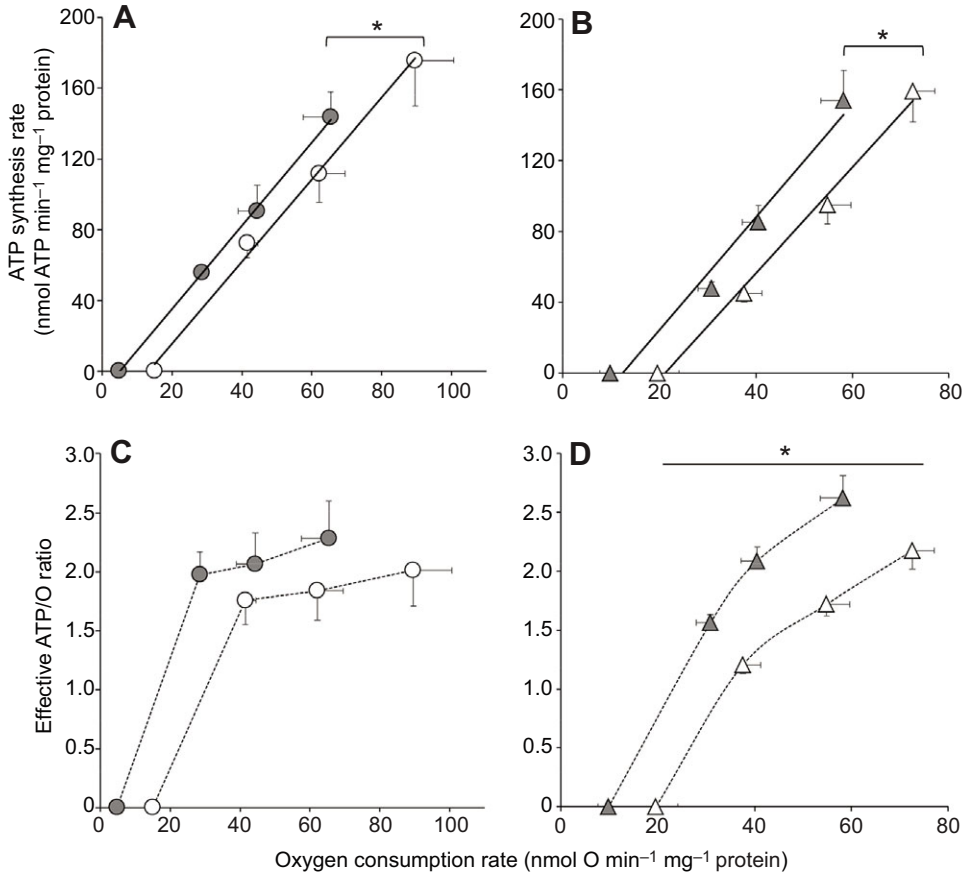

Fig. 3. Oxidative phosphorylation efficiency of mitochondria isolated from pectoralis muscle of fasted chicks. Oxygen uptake and ATP synthesis were determined at 38°C (open symbols) and at 30°C (grey symbols) with either pyruvate/malate (A) or palmitoyl-L-carnitine/malate (B). Mitochondrial fluxes were titrated by addition of increasing concentrations of ADP in the presence of glucose (20 mmol l^{-1}) and hexokinase (1.5 U ml^{-1}). Values are means±s.e.m. for *N*=5 independent mitochondrial preparations.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.104505

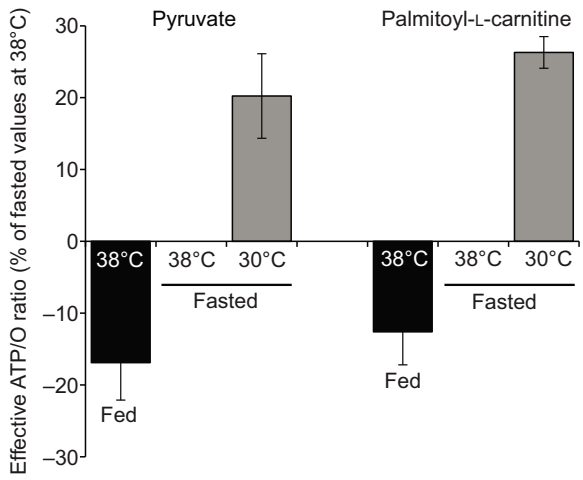

Fig. 4. Effect of feeding or low temperature on mitochondrial efficiency. To compare data from the two experimental protocols, oxygen consumption rates were calculated at the highest common ATP synthesis rate for both substrates (94.2 nmol ATP min^{-1} mg^{-1} protein) by using individual linear relation curves. Then, the resulting effective ATP/O ratios for each respiratory substrate were expressed as a percentage of the corresponding fasted ATP/O ratio measured at 38°C. Black bars, feeding; grey bars, low temperature.

pectoral muscle mitochondria respiring on pyruvate (Fig. 3A) or palmitoyl-L-carnitine (Fig. 3B). Regardless of respiratory substrate used, the slopes were not different between the two thermal conditions, whereas the non-phosphorylating respiration rates (the intercepts with the *x*-axis) were significantly lowered by an average of 58% in mitochondria functioning at 30°C in comparison with those at 38°C. Hence, the linear relations between the rates of ATP synthesis and oxygen consumption were parallel and significantly shifted to the left at 30°C. This implies that at any given rate of oxygen consumption, the rate of ATP synthesis was higher at 30°C, indicating that the effective efficiency of oxidative phosphorylation (effective ATP/O) increases in skeletal muscle mitochondria when temperature decreases (Fig. 3C,D). It follows that, to sustain energy demanding processes, the mitochondrial energy transduction system becomes intrinsically more 'economic', consuming less oxygen (−23% on average, Fig. 4) and thus oxidizing less fuel at 30°C (hypothermia) than at 38°C (normothermia).

DISCUSSION

The results show that the effective efficiency of mitochondrial oxidative phosphorylation adjusted to the nutritional status of winter king penguin chicks. This may be summarized as follows: (i) mitochondria from fasted chicks minimized the cost of ATP synthesis by decreasing the needs for energy substrates. (ii) This plasticity would be more pronounced when birds become hypothermic during winter. (iii) Mitochondria from re-fed chicks were in turn more 'thermogenic', oxidizing more nutrients and thus producing more heat to synthesize the same amount of ATP as fasted chicks. This phenotypic flexibility of mitochondrial energy transduction processes allows king penguin chicks to adjust the cost of their metabolic performance to cope with stochastic parental food provision during the austral winter.

In aerobic living systems, mitochondrial activity is the main physiological link between environmental resources or endogenous energy stores and energy allocated to animal performances. Mitochondrial oxidative phosphorylation is an energy transduction process coupling fuel oxidation and oxygen reduction (i.e. respiration) with the production of useable cellular energy in the form of ATP. The coupling ATP/O value (i.e. mitochondrial efficiency) is an important parameter of oxidative phosphorylation as it affects how much oxygen, and so how much nutrients, are needed to run such energy productive processes (Brand, 2005). In the present study, muscle mitochondrial efficiency was higher in fasted than in re-fed king penguin chicks, indicating that fasted chicks would consume fewer nutrients and oxygen to fuel their energy budgets. Such differences in the yield of mitochondrial ATP synthesis may be explained by intrinsic or extrinsic coupling mechanisms. The intrinsic mechanisms involve modifications in the properties of oxidative phosphorylation complexes, i.e. coupling stoichiometries H^+ and O at the level of the respiratory chain or H^+ and ATP at the level of ATP synthase (Brand, 2005). It has been observed that when such intrinsic mechanisms affect mitochondrial efficiency, then mitochondrial fluxes (oxygen consumption or ATP synthesis) are altered in association with modifications in the slope of the linear relation between oxygen consumption and ATP production (Rigoulet et al., 1990; Nogueira et al., 2002; Clerc et al., 2007; Salin et al., 2012). In the present study, oxygen consumption, ATP synthesis and slope values of linear relation between these two fluxes were not significantly different between fasted and re-fed chicks. These data would rule out a change in intrinsic coupling properties of the respiratory chain (H^+/O) or the ATP synthase (H^+/ATP) as a proximate mechanism of mitochondrial efficiency flexibility in winter-acclimatized chicks. The extrinsic mechanisms mainly involve proton leak reactions, which are processes that consume the proton motive force at the expense of ATP synthesis, and would greatly explain the non linear relationship between the effective ATP/O ratio and the oxygen consumption shown in Fig. 2C,D and Fig. 3C,D. Indeed, the non-ohmic nature of proton leak reactions implies that when mitochondrial phosphorylating activity increases, the flux through proton leak pathways greatly decreases, and the effective ATP/O ratio increases (Brand et al., 1993). At the maximal oxidative phosphorylation rate, leaks still occur, and the effective ATP/O ratio remains lower than the maximum possible value (Brand, 2005). If proton leak reactions are involved in the present increased ATP/O, we can predict from the literature a shift of the linear relationship between oxygen consumption and ATP synthesis to the left, with little effect on the slope and a significant decrease in basal non-phosphorylating respiration (Beavis and Lehninger, 1986; Clerc et al., 2007; Salin et al., 2010), which is clearly the case in the present study. In support of these data, one previous study has shown fatty acid and superoxide inducible mitochondrial proton leak activity to be decreased by 45% after a period of long-term caloric restriction in adult king penguins, an effect that was partly reversed by 3 days of re-feeding (Rey et al., 2008). Altogether, these data would suggest that skeletal muscle mitochondria are more energy efficient in long-term fasting chicks because a greater part of oxygen consumption and energy derived from nutrient oxidation is invested directly into ATP synthesis rather than dissipated as heat through proton leak reactions. By contrast, the fact that the apparent affinity of mitochondrial oxidation for ADP, which appears to be affected by proton leak rates (Gouspillou et al., 2011), did not differ between experimental groups would not support a difference in proton leak over the range of oxidative phosphorylation activity measured in the present study. However, we must keep in mind that the present study was not designed to accurately determine kinetic parameters of the oxidative phosphorylation (supplementary material Figs S1, S2). Hence, whether the higher mitochondrial oxidative phosphorylation efficiency of fasted winter chicks involves changes in proton leak

The Journal of Experimental Biology (2014) doi:10.1242/jeb.104505

reactions (e.g. membrane fatty acyl and phospholipids composition, and membrane protein activities, such as those of uncoupling protein and adenine nucleotide translocase) and/or in processes giving rise to a difference in membrane potential (e.g. citric acid cycle, electron transport chain) are currently not known but clearly warrant further investigations.

The significant energy savings found in fasted king penguin chicks at the mitochondrial level was reflected at the whole-animal level with the reduction of resting metabolic rate. The reduction of mass-specific resting metabolic rate observed in fasted chicks is in line with the low level of resting metabolic rate previously reported in chick (Barré, 1984; Duchamp et al., 1989) and adult of king penguins (Fahlman et al., 2004; Rey et al., 2008) after several weeks of fasting. In the present study, the reduction in whole-animal metabolic rate was not ascribed to a difference in body temperature between fasted and re-fed birds. Indeed, all of the birds were resting at thermoneutrality during the indirect calorimetry experiments, an experimental condition for which the body temperature of winter-acclimatized chicks remains high and constant, independently of the nutritional status (Duchamp et al., 1989). In turn, the fact that resting metabolism is determined by the proportion of lean body mass raises the question of whether the observed variations could be due to differences in body composition between fasted and re-fed birds. It has been previously reported that lean body mass accounts for 46% of total body mass loss in fasted king penguin chicks during phase II of fasting, and the remaining 54% coming from lipids (Cherel et al., 1993b). From these respective contributions to body mass loss, we should expect a progressive increase in the proportion of lean body mass over the course of starvation. By using the pre-winter body composition of king penguin chicks (Cherel et al., 1993a), we can estimate the enrichment in the proportion of lean body mass to amount to 3.5% of body mass after 10 days of fasting, which might explain up to 20% of differences in mass-specific resting metabolism between fasted and re-fed chicks. However, it must be kept in mind that re-fed chicks were included in the present feeding protocol after an initial natural winter fast, once they had reached similar body weight, and therefore similar body composition, to fasted chicks. The fact that the feeding protocol was designed to maintain the initial body weight of chicks indicates that chicks were given enough food to cover daily energy expenditure but not to substantially change body mass composition. Therefore, the reported differences in mass-specific resting metabolism probably mainly reflect changes in cellular activity and mitochondrial energy efficiency, and may only moderately be influenced by variations in lean body mass proportion.

Given that skeletal muscle contributes to a large proportion of resting energy expenditure due to its relative mass and its high oxidative capacity, the specific variation of the degree of mitochondrial coupling in skeletal muscle may play a considerable metabolic role at the whole-animal level. If we assume that skeletal muscle contributes up to 30% of the resting metabolic rate in birds (Duchamp and Barré, 1993), we can estimate that the observed 15% increase in mitochondrial oxidative phosphorylation efficiency contributes to a decrease of almost 5% in whole-body oxygen consumption of fasted chicks. This could represent up to 30% of the fasting-induced reduction in mass-specific metabolic rate. In penguins fasting onshore, pectoral muscles are little used and are therefore preferentially degraded to provide the blood with amino acids, whereas skeletal muscles involved in terrestrial locomotion are spared (Robin et al., 1988; Duchamp et al., 1991). Hence, because pectoralis muscles undergo a selective and progressive degradation during fasting, losing almost 15 g of fresh mass or 4.4 g of muscle protein per day in fasted chicks (Duchamp et al., 1991), the above calculated contribution of muscle mitochondrial efficiency to changes in chicks' metabolic rate might be overestimated. However, in contrast to total muscle proteins, mitochondrial proteins are spared during phase II of fasting and so is the specific oxidative activity of pectoralis muscles (Duchamp et al., 1991). By taking into account these data and the proportion of pectoralis muscle mass in winter chicks (Duchamp et al., 1991; Cherel et al., 1993a), the above calculated contribution of mitochondrial function might be overestimated by 5%. On the whole, the present data suggest that upregulation of the muscle mitochondrial energy efficiency may contribute to a lower metabolic rate by minimizing the metabolic need for oxygen and nutrients in cellular energy production.

Energy conservation is a key priority for organisms living in environments with seasonal or unpredictable shortages in resource supplies. This is exemplified in hibernating or aestivating animals that undergo long periods of torpor (Geiser, 2004). However, previous studies have shown that energy conservation in animals undergoing dormancy is mainly achieved by an overall metabolic depression in conjunction with a reduction in mitochondrial oxidative phosphorylation activity rather than a downregulation of mitochondrial coupling efficiency (Staples and Brown, 2008). As commonly reported in food-restricted birds (Walker et al., 1983; McKechnie and Lovegrove, 2002), king penguin chicks experience shallow hypothermia, resulting in progressive body temperature decline throughout the winter fast that can fall as low as 30–33°C in the deep core of a chick's body near to the heart and the liver (Eichhorn et al., 2011). Even though those authors did not report a temperature for pectoral muscle, the fact that the thermal gradient between the deep core and the body shell can be as large as 8–15°C would indicate that pectoral muscles undergo a physiological hypothermia, at least similar to that measured in the thorax and the upper abdominal site near to the liver, i.e. down to 30–33°C (Eichhorn et al., 2011). Here, we show that such a level of heterothermy induced a significant 23% increase in the energy coupling efficiency at the level of mitochondria with an overall decrease in mitochondrial oxidative activity. Furthermore, we found coupling efficiency to be increased in conjunction with decreased basal non-phosphorylating oxygen consumption and oxidative activity. It is now well known that the effect of temperature on basal mitochondrial respiration reflects that on mitochondrial proton leak activities (Dufour et al., 1996; Chamberlin, 2004; Keller et al., 2004; Brown et al., 2007; Brown et al., 2012). In addition, in the high temperature range used in the present study, the inner membrane potential would be unaffected (Dufour et al., 1996; Chamberlin, 2004) and the occurrence of intrinsic coupling mechanisms, i.e. stoichiometry of oxidative phosphorylation complexes, negligible (Canton et al., 1995; Brand et al., 1994). In light of these studies, we interpret our findings as evidence that hypothermic mitochondria operate with higher efficiency because the lower oxidative activity (e.g. lower production of mitochondrial membrane potential) would be compensated for by a decrease in inner membrane proton leak rate (e.g. lower consumption of mitochondrial membrane potential), resulting in homeostasis of the proton motive force, and thus the flux of ATP synthesis would remain stable. Notwithstanding the underlying mechanisms, the present results clearly indicate that king penguin chicks realize additional energy savings while becoming hypothermic during winter.

It remains that skeletal muscle is also the main tissue involved in thermogenesis, an energetically demanding process that remains operative during winter (Barré, 1984; Duchamp et al., 1989). Is there a cost of having more energy efficient mitochondria when these

energetic processes are activated? Teulier et al. (Teulier et al., 2010) have shown that the thermogenic capacities of growing birds are associated with enhanced mitochondrial phosphorylation intensity but not with innate uncoupling or changes in the efficiency of ATP generation. In king penguin, the volume and content of mitochondria in skeletal muscle (Duchamp et al., 1991; Rey et al., 2008) and the rate of mitochondrial ATP synthesis (present study) are kept high during phase II of fasting in relation to the high capacity for thermogenesis that has been reported in winter fast chicks (Barré, 1984; Duchamp et al., 1989). Hence, changes in skeletal muscle mitochondrial efficiency would have no major impact on the intrinsic capacity of a chick's thermogenic mechanism during phase II of fasting. In conclusion, the regulation of the degree of mitochondrial coupling reported in fasted winter-acclimatized chicks triggers an economical management of resources at the level of mitochondria, which would, in association with short-term hypothermic stage, maximize the conservation of endogenous fuel stores and may contribute to the ability of king penguins to withstand fasting for several months (Cherel and Le Maho, 1985; Cherel et al., 1993a; Eichhorn et al., 2011).

MATERIALS AND METHODS

Animals and experimental design

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 August during two austral winter campaigns (2010 and 2011). 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 chicks of either sex, 6–8 months old, captured at the breeding colony of Baie du Marin and kept outside in an open-top enclosure, were established. In the first group, fourteen chicks were left to fast for 10 days. The mean body mass was 8.9±0.2 kg at capture and 7.8±0.2 kg after 10 days of fasting (Fig. 1). In the second group, twelve naturally fasted chicks were force fed defrosted fishes (*Scomber vernalis*) up to 200 g day^{-1} for 10 days. The mean body mass was 7.7±0.3 kg at the beginning and 7.8±0.3 kg after 10 days of feeding (Fig. 1). The day before experimentation, chicks were fasted. After stabilization within the thermostatic chamber at thermoneutrality, the resting metabolic rate was recorded *in vivo* by indirect calorimetry as already described in king penguin chicks (Duchamp et al., 1989; Teulier et al., 2013). The day after the indirect calorimetry experiments, ~3 g of the superficial pectoralis muscle was surgically biopsied under isoflurane-induced general anaesthesia and immediately used for mitochondrial extraction (Rey et al., 2008). On completion of the study, all penguin chicks were fed and kept until full recovery and then released at the site of their capture.

Mitochondrial isolation

Mixed skeletal muscle mitochondrial populations were isolated by a standard extraction protocol, involving potter homogenization, partial protease digestion and differential centrifugations. Briefly, pectoral muscle was cut up finely with sharp scissors in an ice-cold isolation buffer containing 100 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 50 mmol l^{-1} Tris-base, and pH 7.4 at 4°C. The minced tissue was homogenized with a Potter-Elvehjem homogenizer (five passages) and centrifuged at 800 ***g*** for 10 min. The supernatant containing the subsarcolemmal mitochondria was centrifuged at 1000 ***g*** for 10 min and the pellet containing the intermyofibrillar mitochondria was re-suspended in 10 ml of isolation buffer and treated with subtilisin (1 mg g^{-1} muscle wet weight) 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. Both resulting supernatants were filtered through cheesecloth and centrifuged at 8700 ***g*** for 10 min to pellet subsarcolemmal and intermyofibrillar mitochondria. The pellets were suspended in 10 ml of isolation medium, mixed together and centrifuged at 8700 ***g*** for 10 min. The resulting pellet was washed once by suspension in the isolation buffer and re-centrifuged at 8700 ***g*** for 10 min. All steps were performed at 4°C. The protein concentration of mitochondrial suspensions was determined by a Biuret method with bovine serum albumin (BSA) as the standard.

Mitochondrial respiration and ATP synthesis

Respiration and ATP synthesis rates were measured in the same mitochondrial aliquots as described previously for bird muscle mitochondria (Teulier et al., 2010; Salin et al., 2010). Oxygen consumption was measured in a glass cell fitted with a Clark oxygen electrode (Rank Brothers Ltd, UK), at 38°C and calibrated with air-saturated respiratory buffer (120 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} EGTA, 2 mmol l^{-1} $MgCl_2$, 2.5 mmol l^{-1} malate, 20 mmol l^{-1} glucose, 1.5 U ml^{-1} hexokinase, 0.3% BSA (w/v), and 3 mmol l^{-1} Hepes, pH 7.4 at 38°C). Muscle mitochondria (1 mg protein ml^{-1}) were incubated in the respiratory buffer supplemented with 5 mmol l^{-1} pyruvate or 40 µmol l^{-1} palmitoyl-L-carnitine. Different steady states of phosphorylation were initiated by adding different amounts of ADP from 5 to 100 µmol l^{-1}. After recording the phosphorylating respiration rate for 2 min, four 100 µl aliquots of mitochondrial suspension were withdrawn every 30 s and quenched in a perchloric acid solution (10% $HClO_4$, 25 mmol l^{-1} EDTA). The denatured proteins were centrifuged at 15,000 ***g*** for 5 min and the resulting supernatant neutralized with a KOH solution (2 M KOH, 0.3 M Mops). Then, the ATP production was determined from the glucose-6-phosphate content of the samples, which was measured enzymatically by spectrophotometry according to Lang and Michal (Lang and Michal, 1974). Basal non-phosphorylating respiration rates were determined in the presence of 2 µg ml^{-1} oligomycin.

Statistical analysis

Statistical significance of oxygen consumption and ATP synthesis changes induced by ADP was assessed using two-way analysis of variance (ANOVA) with ADP and penguins or temperature as the main factors. Mitochondrial apparent K_m for ADP were determined for both oxygen consumption and ATP production 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 one-way ANOVA for independent (effect of nutritional status) or repeated (effect of temperature) values followed by protected least significant difference tests or paired Student's *t*-tests (Statview v.4.5 software). Data are presented as means±s.e.m. with significance considered at $P<0.05$.

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Competing interests

The authors declare no competing financial interests.

Author contributions

P.A.M. and V.M. performed the experiments, analyzed the data and revised the article; J.L.R. conceived the indirect calorimetry experiments, analyzed the data and revised the article; D.R. conceived and designed the experiments, analyzed and interpreted the findings being published, and drafted and wrote the final version of the manuscript.

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Supplementary material

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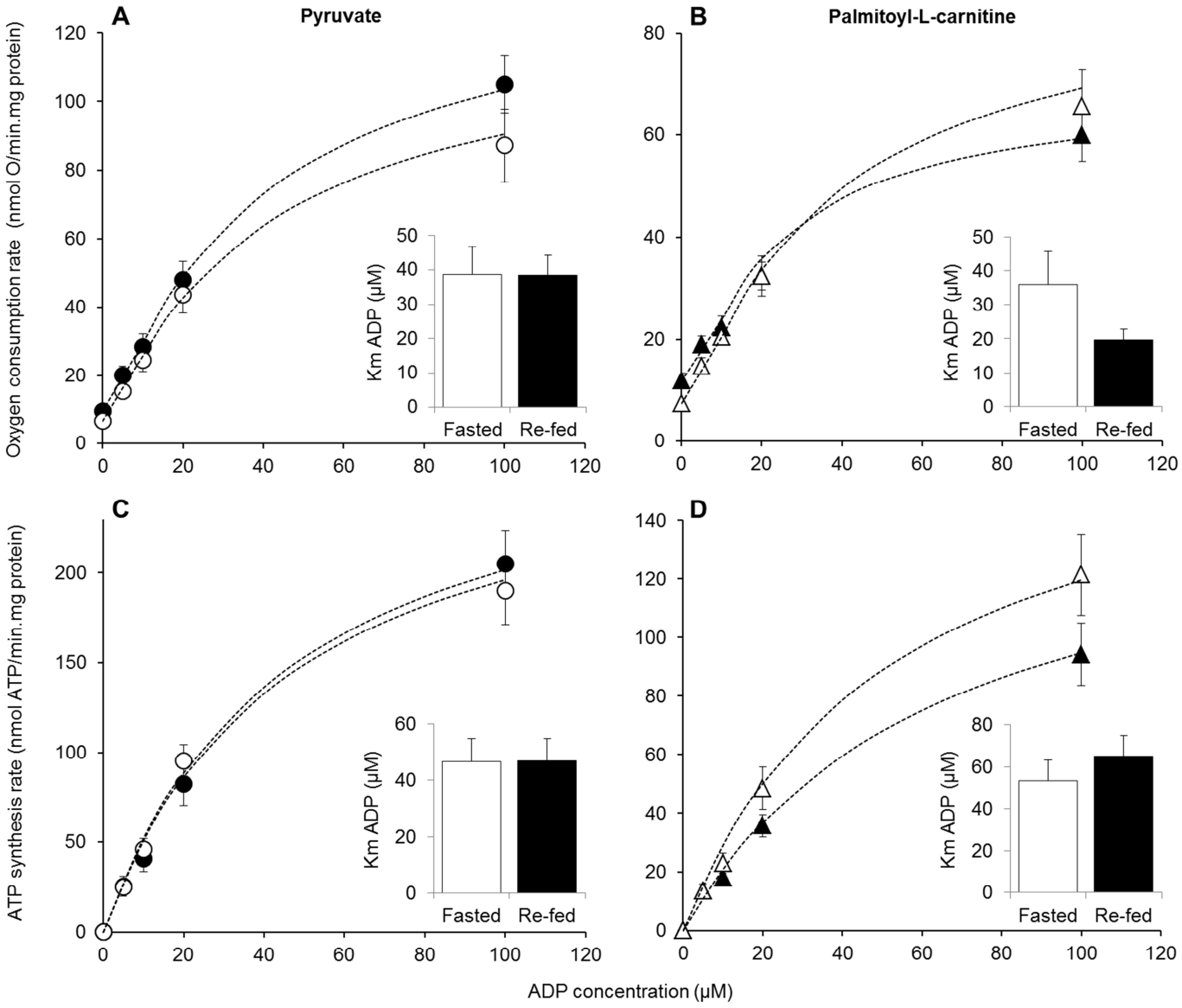
Supplemental data 1

Mitochondrial kinetics parameters of fasted versus re-fed king penguin chicks

The rates of respiration (panels A-B) and ATP synthesis (panels C-D) were measured at 38°C over a range of ADP concentrations with either pyruvate/malate (panels A and C) or palmitoyl-L-carnitine/malate (panels B and D) in the presence of glucose (20 mM) and hexokinase (1.5 U/ml). Mitochondria were isolated from pectoralis muscle of fasted (open symbols) or re-fed chicks (closed symbols). Inset: Apparent affinity constant for ADP (K_m) expressed in µM. Values are means ± S.E.M. for n=11 (fasted) and n=9 (fed) independent mitochondrial preparations.



Supplemental data 2

Effects of temperature on mitochondrial kinetics parameters

The rates of respiration (panels A-B) and ATP synthesis (panels C-D) were measured at 38°C (open symbols) and at 30°C (grey symbols) over a range of ADP concentrations with either pyruvate/malate (panels A and C) or palmitoyl-L-carnitine/malate (panels B and D) in the presence of glucose (20 mM) and hexokinase (1.5 U/ml). Mitochondria were isolated from pectoralis muscle of fasted chicks. Inset: Apparent affinity constant for ADP (K_m) expressed in µM. Values are means ± S.E.M. for n = 5 independent mitochondrial preparations. * indicates significant different between the two thermal conditions.