© 2020. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2020) 223, jeb233684. doi:10.1242/jeb.233684

RESEARCH ARTICLE

Skeletal muscle metabolism in sea-acclimatized king penguins. II. Improved efficiency of mitochondrial bioenergetics

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ABSTRACT

At fledging, juvenile king penguins (*Aptenodytes patagonicus*) must overcome the tremendous energetic constraints imposed by their marine habitat, including during sustained extensive swimming activity and deep dives in cold seawater. Both endurance swimming and skeletal muscle thermogenesis require high mitochondrial respiratory capacity while the submerged part of dive cycles repeatedly and greatly reduces oxygen availability, imposing a need for solutions to conserve oxygen. The aim of the present study was to determine *in vitro* whether skeletal muscle mitochondria become more 'thermogenic' to sustain heat production or more 'economical' to conserve oxygen in sea-acclimatized immature penguins (hereafter 'immatures') compared with terrestrial juveniles. Rates of mitochondrial oxidative phosphorylation were measured in permeabilized fibers and mitochondria from the pectoralis muscle. Mitochondrial ATP synthesis and coupling efficiency were measured in isolated muscle mitochondria. The mitochondrial activities of respiratory chain complexes and citrate synthase were also assessed. The results showed that respiration, ATP synthesis and respiratory chain complex activities in pectoralis muscles were increased by sea acclimatization. Furthermore, muscle mitochondria were on average 30–45% more energy efficient in sea-acclimatized immatures than in pre-fledging juveniles, depending on the respiratory substrate used (pyruvate, palmitoylcarnitine). Hence sea acclimatization favors the development of economical management of oxygen, decreasing the oxygen needed to produce a given amount of ATP. This mitochondrial phenotype may improve dive performance during the early marine life of king penguins, by extending their aerobic dive limit.

KEY WORDS: Bioenergetics, Marine birds, Mitochondria, Oxidative phosphorylation

INTRODUCTION

King penguins (*Aptenodytes patagonicus*) are endotherms that spend more than three-quarters of their life swimming long distances in cold seawater and diving to great depths to catch their prey (Charrassin and Bost, 2001; Pütz, 2002). At the end of their post-hatching development, which takes more than a year (Cherel et al., 2004), pre-fledging juveniles must overcome the tremendous energetic expense imposed by marine life, and swim in cold seawater for several hundred kilometers from their natal colony (Orgeret et al., 2019). Although the greatest mortality in juveniles occurs during the preceding winter on land (Saraux et al., 2011), the survival of these young king penguins during the early months at sea also depends on their ability to improve their diving performance, i.e. diving depths and durations (Orgeret et al., 2016). The development of adaptive responses to increase foraging proficiency might, therefore, represent a key pre-requisite in penguins' survival (Daunt et al., 2007; Orgeret et al., 2016). To increase foraging proficiency, birds must reduce heat losses, sustain intensive and prolonged energetic demands imposed by endurance swimming and thermoregulation in cold water, and develop flexible metabolic phenotypes to fulfil requirements for both aerobic (endurance swimming and most dives in king penguins) and anaerobic metabolism (extremely deep/long dives). Once at sea, penguins' daily metabolic cost of thermoregulation is reduced by their ability to develop powerful peripheral vasoconstriction, regional hypothermia and a thick subcutaneous fat layer (Dumonteil et al., 1994; Handrich et al., 1997; Enstipp et al., 2017; Lewden et al., 2017a). Sea acclimatization is also characterized by the fuel selection of skeletal muscle towards lipid transport and oxidation, concomitant with an increase in the content and oxidative capacity of mitochondria in skeletal muscle (Teulier et al., 2012; Rey et al., 2016). These biochemical remodeling processes are associated with an increased thermogenic effect of circulating lipids on whole-body metabolism *in vivo* and in mitochondrial skeletal muscle *in vitro* (Talbot et al., 2004; Teulier et al., 2012; Rey et al., 2017). Finally, sea acclimatization improves the diving capacity of penguins, by increasing the transport of oxygen and its storage in skeletal muscles, and allowing the maintenance of muscle enzymes involved in glycolytic pathways (Weber et al., 1974; Badwin, 1988; Ponganis et al., 1999, 2010; Noren et al., 2001; Teulier et al., 2012; Rey et al., 2016).

In addition to its locomotor function, skeletal muscle is the main thermogenic tissue in birds, contributing to 70–80% of the regulatory heat production induced by cold (Hissa, 1988; Duchamp et al., 1999). In birds, highly energetically demanding exercise (e.g. flight migration) and muscle heat production are both mainly fueled by lipid oxidation (Vaillancourt et al., 2005; Weber, 2009; Guglielmo, 2010). Through these inter-connected physiological functions (locomotion and thermogenesis), both exercise and cold training help to promote skeletal muscle aerobic capacity, as well as lipid transport and catabolism. This can lead to cross-training effects, where exercise training also improves thermogenic performances in birds, and vice versa (Schaeffer et al., 2001; Petit and Vézina, 2014; Zhang et al., 2015a,b). Hence both locomotion over long distances and muscle thermogenic mechanisms require aerobic metabolism and thus high mitochondrial oxidative phosphorylation capacity in birds.

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Received 21 July 2020; Accepted 10 September 2020

Nevertheless, oxygen availability is repeatedly and greatly reduced in the skeletal muscles of penguins during diving (Williams et al., 2011). This suggests that the careful management of oxygen stores is essential to develop effective diving capacity in king penguins. This observation also explains why the glycolytic pathway is maintained in the skeletal muscle of sea-acclimatized immature king penguins (Teulier et al., 2012). At the cellular level, oxygen storage is greatly increased by the high myoglobin content in muscles in sea-acclimatized penguins (Weber et al., 1974; Ponganis et al., 1999, 2010; Noren et al., 2001). At the mitochondrial level, the question remains open whether skeletal muscle mitochondria become more 'thermogenic' to sustain heat production or more 'economical' to decrease the need for oxygen in sea-acclimatized king penguins.

The aim of the present study was therefore to determine *in vitro* whether the transition from shore to marine living affects the innate oxidative phosphorylation efficiency of skeletal muscle mitochondria. We measured the oxidative activity of permeabilized fibers and mitochondria isolated from the pectoralis muscles of penguins. We also measured oxidative phosphorylation activity (i.e. oxygen consumption and ATP synthesis) and efficiency (ATP:O ratio) in skeletal muscle mitochondria working at different steady-state rates and respiring on carbohydrate- or lipid-derived substrates (i.e. pyruvate/malate or palmitoyl-L-carnitine/malate, respectively). Results from pre-fledging juveniles were compared with the results of sea-acclimatized immatures returning from a foraging trip.

MATERIALS AND METHODS

Animals

Field experiments were conducted on the Crozet archipelago (Possession Island, 46°25′S, 51°45′E) at the French Alfred Faure Station during four austral summer campaigns (from December to February, 2009–2010, 2010–2011, 2013–2014 and 2014–2015). According to the Agreed Measures for the Preservation of Antarctic and Sub-Antarctic Fauna, the project received ethical approval from the French Committee for Polar Research (IPEV program 131). Pre-fledging juvenile king penguins (*Aptenodytes patagonicus* J. F. Miller 1778) of both sexes (13–16 months old) were captured on the nearby breeding colony of Baie du Marin before they had completed molting, which is a pre-requisite for departing to sea. Captured birds finished their molt in an outside enclosure near the laboratory and constituted the never-immersed group (NI). A second group of birds (immatures of 25–35 months old) of both sexes was caught at the end of their molting period on land. Hence these birds had accomplished a pre-molt foraging trip at the time of their capture, ensuring that they had fully accomplished their acclimatization to marine life. They constituted the sea-acclimatized group (SA). All birds were kept in an outside enclosure and fasted for 10 days before experiments. This protocol tended to minimize confounding effects of potential differences in nutritional status. On completion of the study, all penguins were fed on mackerel (for one week until full recovery) and then released at the site of their capture.

Skeletal muscle fibers

Two successive austral summers (2013–2014 and 2014–2015) were devoted to measure the metabolic rate of skeletal muscle *in vitro*. A total of 25 birds were included in this protocol (13 NI juveniles and 12 SA immatures). Superficial pectoralis muscle was surgically biopsied under general isoflurane anesthesia as described previously (Talbot et al., 2004; Teulier et al., 2012; Rey et al., 2016). The biopsy (100 mg) was freshly used for muscle fiber preparation and bioenergetics analysis. Note that for this protocol SA immatures were significantly heavier than NI juveniles (12.1±0.8 versus 8.2±0.2 kg, respectively; $P<0.05$).

Muscle biopsies were immersed in ice-cold isolation solution [BIOPS; containing (mmol l^{-1}): 2.77 Ca-EGTA, 7.23 EGTA, 20 imidazole, 20 taurine, 50 K-MES, 0.5 DTT, 6.56 $MgCl_2$, 5.77 ATP and 15 phosphocreatine; pH 7.2], and fiber bundles were permeabilized as previously described for penguins (Bourguignon et al., 2017). Muscle fibers were weighed and their respiration was monitored with an Oroboros oxymeter (Oroboros Instruments, Innsbruck, Austria) at 38°C in a hyper-oxygenated respiratory buffer (Mir05: 110 mmol l^{-1} sucrose, 0.5 mmol l^{-1} EGTA, 3 mmol l^{-1} $MgCl_2$, 60 mmol l^{-1} potassium lactobionate, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4, 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 that provides electrons to complex I and complex II of the electron transport system (5 mmol l^{-1} pyruvate/2.5 mmol l^{-1} malate and 5 mmol l^{-1} succinate). The phosphorylating state of respiration was determined in the presence of 1 mmol l^{-1} ADP. The integrity of mitochondria within permeabilized fiber bundles was then verified by adding cytochrome *c* (10 µmol l^{-1}). Finally, uncoupled respiration associated with the maximal activity of the electron transport system was initiated by the addition of 2 µmol l^{-1} carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP).

Skeletal muscle mitochondrial isolation and oxidative activity

Two successive austral summers (2009–2010 and 2010–2011) were devoted to biochemical analysis of muscle mitochondria *in vitro*. Twenty-nine fasted birds (14 NI juveniles and 15 SA immatures) were included in this protocol. Note that during the campaign in 2009–2010, 13 supplementary birds were included in the protocol and re-fed for 4 days (7 NI juveniles and 6 SA immatures). Superficial pectoral muscle was surgically biopsied under general isoflurane anesthesia as described previously (Talbot et al., 2004; Teulier et al., 2012; Rey et al., 2016). The biopsy (∼1 g) was freshly used for mitochondrial extraction and bioenergetics analysis. Note that SA immatures included in this protocol were significantly heavier than NI juveniles (11.8±0.5 versus 8.5±0.2 kg, respectively; $P<0.05$).

Muscle mitochondria were isolated in an ice-cold isolation buffer containing 100 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA and 50 mmol l^{-1} Tris-base (at pH 7.4 and 4°C) by a standard extraction protocol, involving potter homogenization, partial protease digestion and differential centrifugation (Monternier et al., 2014). Mitochondria were pelleted at 8700 ***g*** for 10 min. Mitochondrial proteins were determined by a Biuret method. Oxygen consumption was measured with a Clark oxygen electrode (Rank Brothers Ltd, Cambridge, UK), in a closed and stirred glass cell of 0.5 ml volume, thermostatically controlled to 38°C. Muscle mitochondria (1 mg protein ml^{-1}) were incubated in a respiratory buffer containing 120 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} EGTA, 2 mmol l^{-1} $MgCl_2$, 0.3% bovine serum albumin (w/v) and 3 mmol l^{-1} Hepes at pH 7.4. Substrate concentrations were 5 mmol l^{-1} pyruvate plus 2.5 mmol l^{-1} malate or 40 µmol l^{-1} palmitoyl-L-carnitine plus 2.5 mmol l^{-1} malate. The active state of respiration (state 3) was initiated by the addition of 500 µmol l^{-1} ADP. The basal non-phosphorylating respiration rate was obtained by the addition of 2 µg ml^{-1} oligomycin (state 4_{oligo}). The maximal respiration rate of the electron transport system (state ETS_{max}) was initiated by the addition of 2 µmol l^{-1} FCCP. The respiratory control ratio (RCR)

refers to the ratio of oxygen consumed after adding ADP to that consumed in the presence of oligomycin.

Oxidative phosphorylation and efficiency of skeletal muscle mitochondria

Oxygen consumption and corresponding ATP synthesis rates were performed at 38°C in 1 ml respiratory buffer supplemented with an ADP-generating system consisting of glucose (20 mmol l^{-1}) and hexokinase (1.5 U ml^{-1}) (Teulier et al., 2010; Monternier et al., 2014). Respiration was initiated with either pyruvate/malate (5 mmol l^{-1}/2.5 mmol l^{-1}) or palmitoylcarnitine/malate (40 µmol l^{-1}/2.5 mmol l^{-1}) as respiratory substrates. The different steady-state rates of mitochondrial ATP synthesis were initiated by the addition of 500, 100, 20, 10 or 5 µmol l^{-1} ADP. After recording the phosphorylating respiration rate for 2 min, four 200 µl samples of mitochondrial suspension were withdrawn from the suspension every 30 s and were quenched in a perchloric acid solution (10% $HClO_4$, 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 assayed spectrophotometrically by monitoring the production of NADH in the presence of glucose-6-phosphate dehydrogenase at 340 nm, using a reaction buffer consisting of NAD (0.5 mmol l^{-1}), triethanolamine-HCl (50 mmol l^{-1}), $MgCl_2$ (7.5 mmol l^{-1}) and EDTA (3.75 mmol l^{-1}) at pH 7.4. The concentration of glucose-6-phosphate was calculated from the difference between the level of NADH measured before and 1 h after the addition of glucose-6-phosphate dehydrogenase (0.5 U) from *Leuconostoc mesenteroides*. The rate of mitochondrial ATP production was calculated from the slope of the linear accumulation of glucose-6-phosphate over the sampling time interval (Teulier et al., 2010; Monternier et al., 2014). The linearity of glucose-6-phosphate accumulation allowed us to check that the system was in a steady state.

Mitochondrial enzyme activity

Eighteen frozen mitochondrial preparations were used (8 NI juveniles and 10 SA immatures) to assay enzyme activities. The activities of citrate synthase, NADH ubiquinone reductase (complex I), succinate ubiquinone reductase (complex II), NADH cytochrome *c* reductase (complex I+III), and succinate cytochrome *c* reductase (complex II+III) were measured spectrophotometrically at 38°C following the protocol of Medja et al. (2009). Frozen mitochondria were thawed and diluted 1:20 in 100 mmol l^{-1} phosphate buffer. All assays were performed in duplicate using a final volume of 260 µl. Citrate synthase activity, NADH ubiquinone reductase activity (complex I), and succinate ubiquinone reductase activity (complex II) were measured spectrophotometrically following the protocols of Medja et al. (2009) as described previously (Bourguignon et al., 2017). Briefly, the citrate synthase activity was assayed in reaction medium [100 µmol l^{-1} 5,5′-dithiobis(2-nitrobenzoic acid), 300 µmol l^{-1} acetyl CoA and 100 mmol l^{-1} Tris buffer (pH 8) and supplemented with 500 µmol l^{-1} oxaloacetate] by following the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) by CoASH at 412 nm. Enzyme activity was quantified using an extinction coefficient of 13.6 mmol l^{-1} cm^{-1}. The complex I activity, i.e. the rotenone-sensitive activity of NADH ubiquinone reductase, was assayed in reaction medium [100 µmol l^{-1} decylubiquinone, 3.75 mg ml^{-1} bovine serum albumin and 50 mmol l^{-1} phosphate buffer (pH 7.5) and supplemented with 100 µmol l^{-1} NADH] by following NADH oxidation at 340 nm in the presence or absence of 12.5 µmol l^{-1} rotenone. Enzyme activity was quantified using an extinction coefficient of 6.22 mmol l^{-1} cm^{-1}. The complex II activity, i.e. the activity of succinate ubiquinone reductase, was assayed in reaction medium [20 mmol l^{-1} succinate, 50 µmol l^{-1} 2,6-dichlorophenol, 1 mmol l^{-1} KCN, 100 µmol l^{-1} ATP, 2 mg ml^{-1} bovine serum albumin and 25 mmol l^{-1} phosphate buffer (pH 7.5) and supplemented with 100 µmol l^{-1} decylubiquinone] by following reduction of 2,6-dichlorophenol at 600 nm. Enzyme activity was quantified using an extinction coefficient of 19.2 mmol l^{-1} cm^{-1}.

The rotenone-sensitive activity of mitochondrial NADH cytochrome *c* reductase (complexes I+III) was assayed in reaction medium (100 µmol l^{-1} cytochrome *c*, 1 mg ml^{-1} bovine serum albumin, 1 mmol l^{-1} potassium cyanide and 50 mmol l^{-1} phosphate buffer; pH 7.5) by following reduction of cytochrome *c* at 550 nm in the presence or absence of 12.5 µmol l^{-1} rotenone. After 4 min of incubation, the reaction was started by adding 200 µmol l^{-1} NADH. Enzyme activity was quantified using an extinction coefficient of 18.5 mmol l^{-1} cm^{-1} (Medja et al., 2009).

The activity of succinate cytochrome *c* reductase (complexes II+III) was assayed in reaction medium (20 mmol l^{-1} succinate, 2 mg ml^{-1} bovine serum albumin, 1 mmol l^{-1} potassium cyanide, 100 µmol l^{-1} ATP and 20 mmol l^{-1} potassium phosphate buffer; pH 7.5). After 4 min of incubation, the reaction was started by adding 100 µmol l^{-1} cytochrome *c* and the reduction of cytochrome *c* was followed at 550 nm over 4 min. Enzyme activity was quantified using an extinction coefficient of 18.5 mmol l^{-1} cm^{-1} (Medja et al., 2009).

Statistical analysis

Two-way repeated-measures ANOVA followed by protected least significant difference tests were performed to estimate the effects of groups and ADP addition on mitochondrial ATP:O ratios (SigmaPlot 12.0; https://systatsoftware.com/products/sigmaplot/). Mitochondrial and enzymatic parameters were tested with ANOVA for independent values, followed by protected least significant difference tests (StatView version 4.5 software; https://statview.software.informer.com/4.5/). There were no significant differences between summer campaigns, and data in similar conditions were pooled. Data are presented as means±s.e.m. with significance considered at $P<0.05$.

RESULTS

Oxidative activities of skeletal muscle fibers and isolated mitochondria

Table 1 shows the respiratory characteristics of muscle mitochondria isolated from NI or SA penguins. Rates of oxygen consumption during ADP-stimulated phosphorylating (state 3) and FCCP-induced uncoupled (state ETS_{max}) states were significantly lower (−32% on average) in SA immatures than in NI juveniles when using either pyruvate or palmitoyl-L-carnitine as respiratory substrates. Compared with the values in NI juveniles, the rate of non-phosphorylating respiration (state 4_{oligo}) in SA penguins was 26% lower with pyruvate, but no different with palmitoyl-L-carnitine (Table 1). The respiratory control ratio (RCR), i.e. the state 3 to state 4_{oligo} ratio, was significantly lower in SA immatures than in NI juveniles with palmitoyl-L-carnitine/malate, but not significantly different between groups with pyruvate/malate (Table 1). Table 1 also shows that the rate of ADP-induced maximal phosphorylating respiration was lower in mitochondria energized with palmitoyl-L-carnitine/malate than with pyruvate/

Journal of Experimental Biology (2020) 223, jeb233684. doi:10.1242/jeb.233684

Table 1. Respiratory characteristics of muscle mitochondria isolated from never-immersed juvenile and sea-acclimatized immature king penguins

Respiratory parameters	Pyruvate–malate		Palmitoyl-L-carnitine–malate	
	NI	SA	NI	SA
State 3	112.4±9.7	79.4±4.0*	72.4±6.0‡	56.1±2.9*‡
State 4_{oligo}	13.0±1.3	9.6±0.9*	13.5±0.9	14.2±1.1‡
State ETS_{max}	135.8±11.0	90.3±5.9*	118.1±10.7	83.3±7.3*
RCR	9.1±0.6	9.1±0.9	5.5±0.3‡	4.2±0.2*‡

The rates of respiration (state 3, state 4_{oligo}, state ETS_{max}) were determined at 38°C and are expressed in nanomoles of oxygen per minute per milligram of protein. State 3, ADP-stimulated phosphorylating respiration; state 4_{oligo}, basal non-phosphorylating respiration measured in the presence of 2 µg ml^{-1} oligomycin; state ETS_{max}, FCCP-induced maximal uncoupling respiration measured in the presence of 2 mmol l^{-1} FCCP; RCR, respiratory control ratio, calculated as the state 3 to state 4_{oligo} ratio. See Materials and Methods for more details. Values are means±s.e.m. from N=14 never-immersed (NI) juveniles and N=15 sea-acclimatized (SA) immatures. *P<0.05, significantly different from NI juveniles; ‡P<0.05, significantly different from pyruvate–malate within the same experimental group.

malate. In contrast, FCCP-inducted maximal or oligomycin-induced non-phosphorylating respiration rates were not significantly different when mitochondria were respiring on either respiratory substrate (Table 1). Consequently, the RCR values were significantly lower with palmitoyl-L-carnitine/malate than with pyruvate/malate (Table 1).

In contrast, the oxygen consumption expressed per gram of skeletal muscle was significantly higher in SA immatures than in NI juveniles with all of the following respiratory substrates: pyruvate/malate/succinate (Fig. 1A), pyruvate/malate (Fig. 1B) and palmitoyl-L-carnitine/malate (Fig. 1C).

Mitochondrial enzyme activities

When measured in isolated mitochondrial pellets, the activities of citrate synthase (an enzyme of the citric acid cycle) and cytochrome *c* oxidase (complex IV of the electron transport chain) were not significantly different between experimental groups (Fig. 2A). However, the activities of NADH-ubiquinone (complex I of the electron transport chain) and NADH-cytochrome *c* reductase (complexes I+III of the electron transport chain) were significantly higher (+49% on average) in SA immatures than in NI juveniles (Fig. 2A). Succinate-ubiquinone reductase (complex II of the electron transport chain) was significantly higher (+26%) in SA immatures than in NI juveniles (Fig. 2A). Despite this trend, the activity of succinate-cytochrome *c* reductase (complexes II+III of the electron transport chain) was not statistically different (P=0.09). When taking into account the higher mitochondrial content of skeletal muscle in SA immatures (Rey et al., 2016), then all enzyme activity expressed per gram of skeletal muscle was significantly higher in SA immatures than in NI juveniles (Fig. 2B).

Mitochondrial oxidative phosphorylation activity and efficiency

Figure 3 shows the linear relationship between the rate of ATP synthesis and oxygen consumption in pectoral muscle mitochondria respiring on pyruvate (Fig. 3A) or palmitoyl-L-carnitine (Fig. 3B) and working at different steady-state rates of ATP production. These results came from penguins that had been fasted for 10 days. Rates of ATP synthesis and oxygen consumption with pyruvate/malate were reduced by −24 and −34%, respectively, in SA immatures compared with these values in NI juveniles (Fig. 3A). The differences between experimental groups were significant for both ATP production ($F_{1,26}$=6.0; P<0.05) and respiration ($F_{1,26}$=14.6; P<0.001). There were no significant differences in mitochondrial fluxes (ATP synthesis or oxygen consumption) between NI and SA penguins with palmitoyl-L-carnitine/malate (Fig. 3B). Regardless of the respiratory substrate, a two-way ANOVA indicated that the slope values of the linear relationships were significantly improved by sea acclimatization ($F_{1,51}$=5.5; P<0.05; Fig. 3A,B). However, differences failed to reach statistical significance for each of the respiratory substrates, whether mitochondria were respiring on pyruvate/malate ($F_{1,26}$=3.9; P=0.06; Fig. 3A) or palmitoyl-L-carnitine/malate ($F_{1,25}$=1.7; P=0.21; Fig. 3B). The non-phosphorylating respiration rates, the lowest point to the left of the linear relationships, were significantly different between experimental groups with palmitoyl-L-carnitine/malate ($F_{1,25}$=21.7; P<0.0001), but failed to reach statistical significance with pyruvate/malate ($F_{1,26}$=3.3; P=0.08). Figure 3D and E show that at any given rate of oxygen uptake, the efficiency of mitochondria (ATP:O) was higher in SA immatures than in NI juveniles, regardless of the respiratory substrate used. When all steady-state rates were considered, the effective coupling efficiency was improved by an average of 28 and 45% in fasted SA penguins compared with fasted NI birds, when mitochondria were respiring on pyruvate/malate ($F_{1,26}$=4.1; P=0.05) and palmitoyl-L-carnitine/malate ($F_{1,25}$=9.6; P<0.01), respectively.

Nutritional status can affect the relationship between the rates of ATP synthesis and oxygen consumption (Bourguignon et al., 2017), so we also measured the oxidative phosphorylation efficiency of mitochondria respiring on palmitoyl-L-carnitine/malate in fed penguins (Fig. 3C,F). The maximal and sub-maximal rates of ATP synthesis shown in Fig. 3C, i.e. the two highest points to the right of the linear relationship, were significantly higher in fed SA immatures than in fed NI juveniles. However, the corresponding oxygen consumption rates were not significantly different between the experimental groups. In contrast to fasted birds, the slopes of the linear relationships were not significantly different between fed SA and NI birds (Fig. 3C), but the basal non-phosphorylating respiration rates (the intercepts with the *x*-axis) were significantly lower in fed SA immatures than in fed NI juveniles (Fig. 3C). This resulted in the linear relationship of the fed SA immatures being significantly shifted to the left compared with that of fed NI juveniles. This result indicates that to produce a given amount of ATP, less oxygen was consumed by mitochondria in SA birds. This can be better illustrated when effective ATP:O ratios are plotted against oxygen consumption rates (Fig. 3F). As with the fasted birds described above, the effective ATP:O was on average higher at any given rate of oxygen consumption in fed SA immatures than in fed NI juveniles. When all steady-state rates were considered, the effective coupling efficiency of mitochondria respiring on palmitoyl-L-carnitine/malate was improved by an average of 63% in fed SA immatures compared with that in fed NI juveniles ($F_{1,11}$=7.1; P<0.05). Taken together, these results indicate that sea acclimatization increases the coupling efficiency of oxidative phosphorylation in the mitochondria of pectoralis muscles of immature penguins, regardless of their nutritional status.

Muscle ATP synthesis

Taking into account the mitochondria content (Rey et al., 2016), the ATP synthesis rate in skeletal muscle was significantly higher in SA immatures than in pre-fledging NI juveniles, irrespective of the respiratory substrate used and regardless of the nutritional status (Fig. 4).

Journal of Experimental Biology (2020) 223, jeb233684. doi:10.1242/jeb.233684

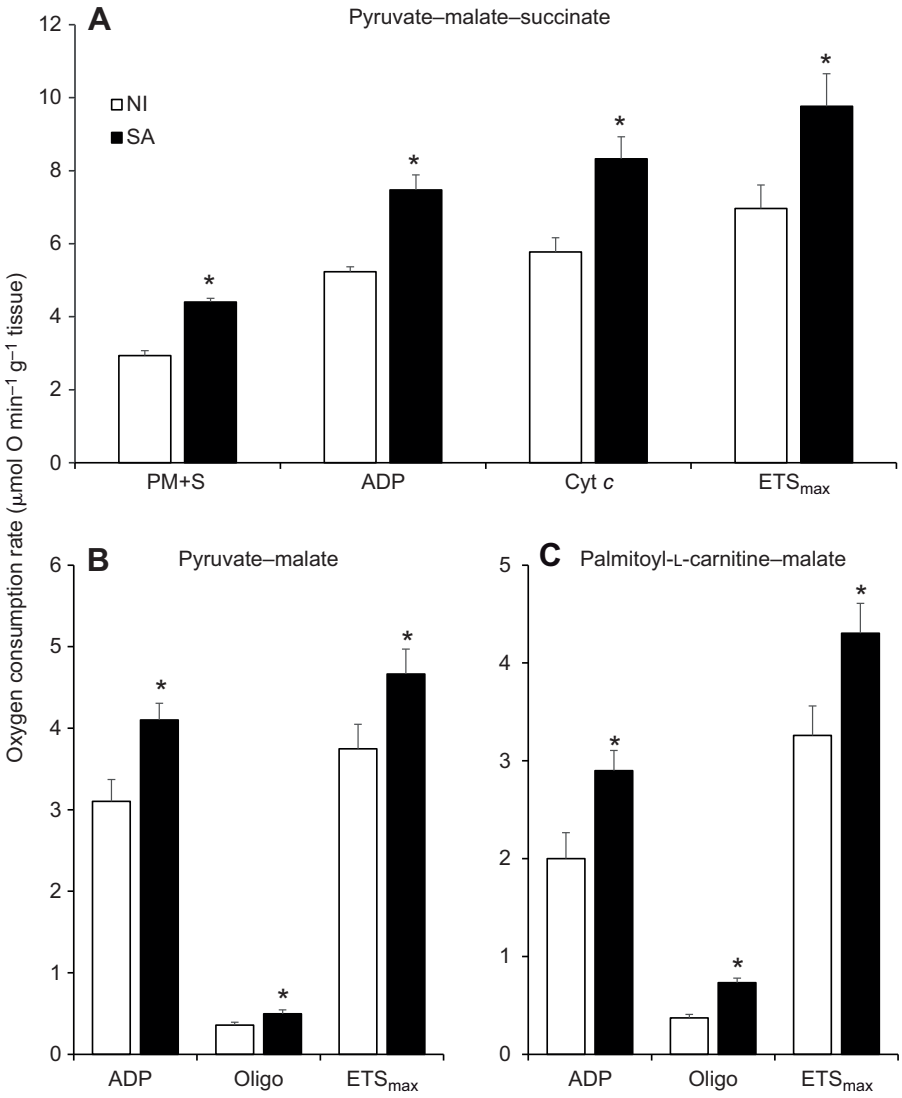

Fig. 1. Effect of sea acclimatization on oxidative activities of king penguin pectoralis muscle. (A) Respiration rates of permeabilized fibers from pectoralis muscle of pre-fledging juveniles [never-immersed (NI), white bars; *N*=13] or sea-acclimatized (SA) immatures (black bars; *N*=12) were measured at 38°C. Fibers were energized with pyruvate–malate–succinate (PM+S). Phosphorylating respiration was initiated with 1 mmol l^{-1} ADP. Mitochondrial integrity within fibers was tested by the addition of 10 µmol l^{-1} cytochrome *c* (Cyt *c*). Maximal oxidative activity of the electron transport system was measured following the addition of 2 µmol l^{-1} FCCP (ETS_{max}). Values are means±s.e.m. from *N*=12–13 independent muscle fiber preparations. Oxidative activity of pectoralis muscle with pyruvate–malate (B) or palmitoyl-L-carnitine–malate (C) were calculated by multiplying the mitochondrial respiration rates reported in Table 1 with the mitochondrial content expressed in milligrams of protein per gram of skeletal muscle and reported in Rey et al. (2016). Phosphorylating respiration was initiated with 500 µmol l^{-1} ADP. Basal non-phosphorylating respiration was obtained by adding 2 µg ml^{-1} oligomycin (Oligo). Maximal oxidative activity of the electron transport system was measured following the addition of 2 µmol l^{-1} FCCP (ETS_{max}). Values are means±s.e.m. from *N*=14–15 independent mitochondrial preparations. **P*<0.05, significantly different from NI juveniles.

DISCUSSION

The fundamental result of the present study is that sea acclimatization increases the innate coupling efficiency of oxidative phosphorylation in skeletal muscle mitochondria *in vitro*. This phenotypic adjustment of mitochondrial energy transduction processes could trigger the economical management of oxygen and fuel substrates in skeletal muscles. Hence the improvement of mitochondrial coupling efficiency could be one of the physiological mechanisms underlying the development of diving ability in juvenile penguins during their early life at sea (Ponganis et al., 1999; Orgeret et al., 2016).

Deep diving performance and mitochondrial efficiency

King penguins (*A. patagonicus*) and emperor penguins (*Aptenodytes forsteri*), of the genus *Aptenodytes*, are the largest of all diving birds and thus have great breath-holding capacities, being able to dive to depths greater than 340 and 560 m, and for duration greater than 7 and 22 min, respectively (Kooyman et al., 1992; Wienecke et al., 2007; Pütz and Cherel, 2005). During their first year at sea, juvenile penguins of this genus improve their diving performance (i.e. the depth and duration of dives), but remain less efficient divers compared with the outstanding diving capacity of adults (Ponganis et al., 1999; Orgeret et al., 2016; Enstipp et al., 2017; Labrousse et al., 2019). The exceptional diving abilities of the *Aptenodytes* genus are partly due to their high capacity to transport, extract and store oxygen in their skeletal muscles (Ponganis et al., 2011). Indeed, sea-acclimatized adult emperor and king penguins show increased hematocrit and thus an increased capacity of their blood to transport oxygen to tissues (Ponganis et al., 1999; Rey et al., 2016). They also exhibit higher myoglobin content in their skeletal muscles than chicks or pre-fledging juveniles (Weber et al., 1974; Ponganis et al., 1999; Noren et al., 2001; Ponganis et al., 2010). The present study has shown that the improvement of muscle mitochondrial coupling efficiency in sea-acclimatized penguins could also contribute to extending aerobic dive limits, by triggering the economical management of oxygen in skeletal muscles. Indeed, an increase in the innate coupling efficiency of oxidative phosphorylation indicates that less oxygen and energy substrates are used by mitochondria to produce ATP and sustain skeletal muscle activity. Such an ‘economical’ phenotype of mitochondrial energy transduction processes would decrease the energy costs of skeletal muscle work by minimizing the rate of oxygen consumption in working skeletal muscles. This result is in line with the low energy costs of transport and foraging reported in *Aptenodytes* penguins (Kooyman et al., 1992; Culik et al., 1996; Nagy et al., 2001; Froget et al., 2004) and the rather low rate of

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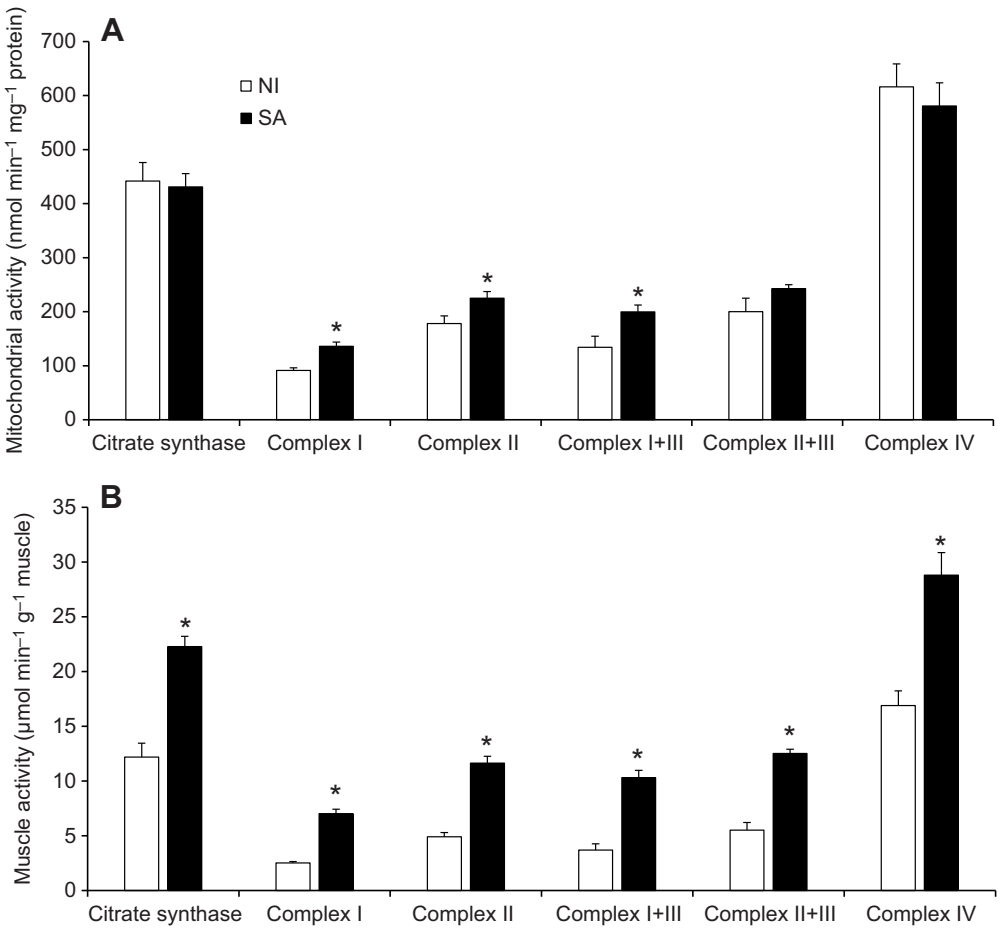

Fig. 2. Effect of sea acclimatization on mitochondrial enzyme activities. (A) Activities of citrate synthase, NADH-ubiquinone reductase (complex I), succinate-ubiquinone reductase (complex II), NADH-cytochrome *c* reductase (complex I+III), succinate-cytochrome *c* reductase (complex II+III) and cytochrome *c* oxidase (complex IV) were measured at 38°C in mitochondria isolated from pectoralis muscle of pre-fledging juveniles (NI, white bars; *N*=7) or sea-acclimatized immatures (SA, black bars; *N*=9). Values are means± s.e.m. from *N*=7–9 independent mitochondrial preparations. (B) Mitochondrial enzyme activities per gram of muscle were calculated by taking into account the mean mitochondrial content of skeletal muscle reported in pre-fledging juvenile and immature king penguins (Rey et al., 2016). *P<0.05, significantly different from NI juveniles.

muscle oxygen consumption estimated to occur in emperor penguins during diving (Williams et al., 2011).

Are adjustments in mitochondrial coupling efficiency under-estimated?

In the present work, mitochondria and muscle fibers were assayed under conditions that allow an optimal activity of mitochondrial energy conversion system (i.e. unlimited oxygen and oxidative substrates). Although mitochondria exhibit a high affinity for oxygen (Gnaiger et al., 1998), P_{O_2} in muscle tissue of deep divers can reach a very low level during dives (Williams et al., 2011; Ponganis et al., 2011), which in turn might limit mitochondrial activity. How low physiological level of oxygen can affect the mitochondrial coupling efficiency of penguin skeletal muscle remains unknown. Nevertheless, it has been reported that oxidative phosphorylation of rat liver mitochondria becomes more efficient at low oxygen levels (Gnaiger et al., 2000; Solien et al., 2005). Furthermore, a reduction in mitochondrial oxidative activity, whether induced by chemicals (e.g. cyanide or nitric oxide), hypothermia or physiological constraint (e.g. long-term fasting), improves the efficiency of ATP synthesis (Clerc et al., 2007; Monternier et al., 2014; Roussel et al., 2018). From these data, we can hypothesize that the increased mitochondrial coupling efficiency reported in sea-acclimatized immatures under our experimental conditions *in vitro* could be further improved during dives *in vivo*. This hypothesis deserves further investigation. Another aspect of the present work is the fact that fasted SA immatures were on average heavier than fasted NI juveniles. This observation indicates that SA birds had not finished their molt at the time of the experiment, while NI juveniles had. In turn, this suggests that SA immatures were fasting for a shorter time period before their capture compared with NI juveniles. As mitochondrial efficiency improvement depends on fasting duration (Bourguignon et al., 2017), the increased mitochondrial coupling efficiency reported in fasted SA immatures might be under-estimated. Note that muscle mitochondria were also better coupled in fed SA immatures than in fed NI juveniles, hence regardless of nutritional status, sea acclimatization did increase the coupling efficiency of oxidative phosphorylation in the pectoralis muscle of king penguins.

Adenine nucleotide translocase- and uncoupling protein-mediated uncoupling activity in more coupled mitochondria: a contradictory phenomenon?

Sea acclimatization is, however, also characterized by the increased thermogenic effect of lipids on the whole body *in vivo* (Teulier et al., 2012) and in skeletal muscle mitochondria *in vitro* (Rey et al., 2017). This adaptive thermogenesis is associated with the fuel selection of skeletal muscle towards lipid transport and oxidation, concomitant with an increase in the content of mitochondria (Teulier et al., 2012; Rey et al., 2016) and in mitochondrial respiratory chain complex activity in skeletal muscle (present study). At the level of mitochondria, the present study showed that sea acclimatization is not associated with innate uncoupling, i.e. a more ‘thermogenic’ phenotype of skeletal muscle mitochondria. Instead, it showed that skeletal muscle of

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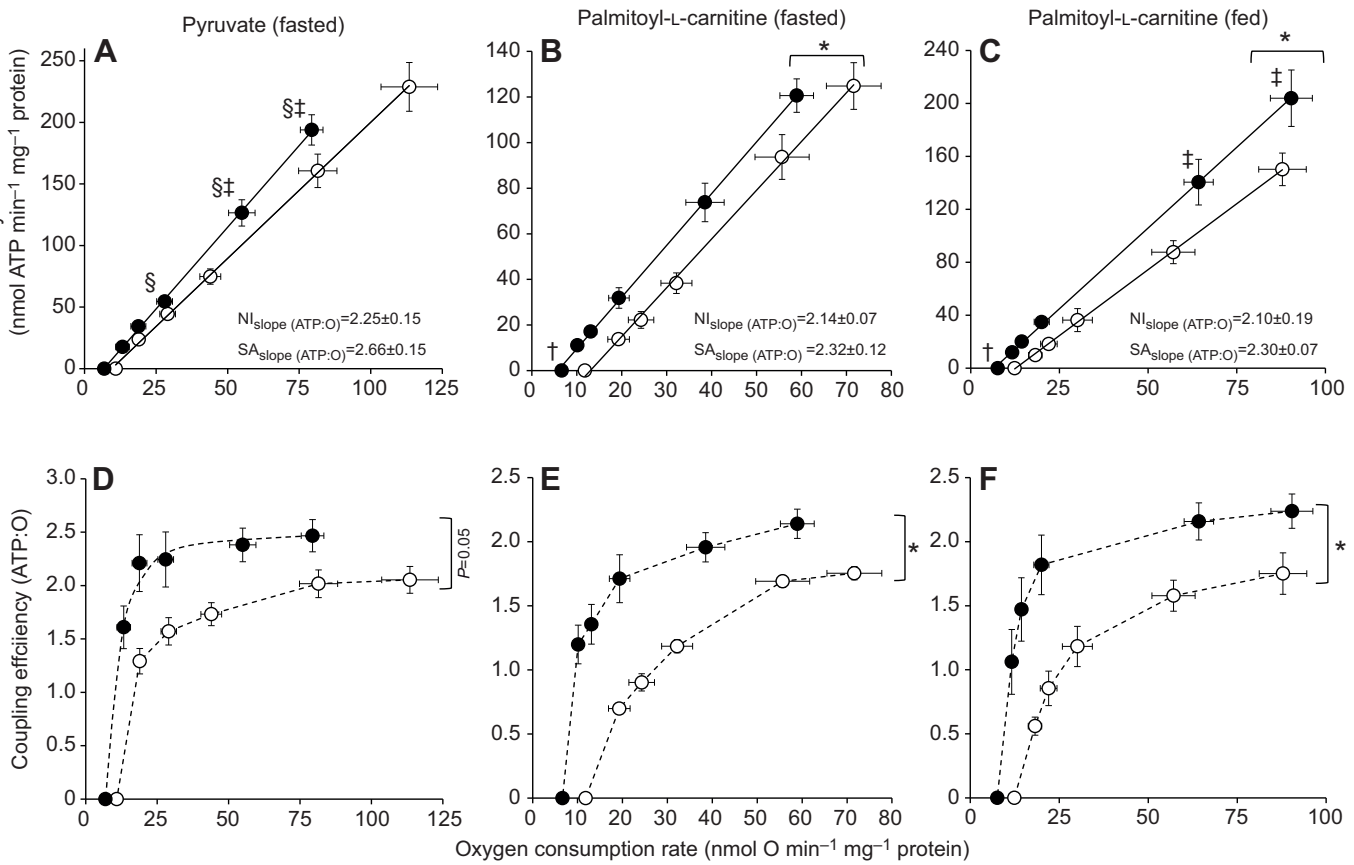

Fig. 3. Effect of sea acclimatization on mitochondrial oxidative phosphorylation efficiency. The rates of oxygen consumption and ATP synthesis were measured in isolated mitochondria from pectoralis muscle of pre-fledging juveniles (NI, open symbols) or sea-acclimatized immatures (SA, filled symbols). Mitochondria were isolated from skeletal muscle of fasted (A,B,D,E: *N*=13–14) or fed penguins (C,F: *N*=6–7), and were respiring on either pyruvate–malate (A,D) or palmitoylcarnitine–malate (B,C,E,F). (A–C) Relationship between ATP synthesis and oxygen consumption. The oxygen consumption (§) and ATP synthesis rates (‡) are significantly different from pre-fledging juveniles ($P<0.05$). *Significantly shifted to the left in sea-acclimatized immatures (see Results for more details). (D–F) Relationship between mitochondrial efficiency (ATP:O ratio) and mitochondrial respiratory activity. *The overall kinetic is significantly different from pre-fledging juveniles ($P<0.05$).

sea-acclimatized penguins exhibited a higher mitochondrial ATP efficiency, which could be partly explained by a 30–50% decrease in mitochondrial proton leak activity (Talbot et al., 2004). However, sea acclimatization does induce greater uncoupling activities of mitochondrial adenine nucleotide translocase (ANT) and avian uncoupling protein (avUCP) in the presence of their natural stimulators, i.e. free fatty acids and/or reactive oxygen species (Talbot et al., 2004). It has been calculated that ANT might catalyse at least half of the basal proton conductance of mitochondria (Brand et al., 2005), which in turn accounts for 20–25% of basal heat production in animals (Rolfe and Brand, 1996; Stuart et al., 2001). It has been reported that cold-induced metabolic heat production was correlated with up-regulation of avUCP in the skeletal muscle of ducklings (Teulier et al., 2010). However, the up-regulation of avUCP in cold-acclimated ducklings was not associated with innate altered ATP efficiency (Teulier et al., 2010). Therefore, the fact that skeletal muscle mitochondria of sea-acclimatized penguins shifted towards a more coupled and less ‘thermogenic’ phenotype (present study), but also show an increased ANT- and avUCP-mediated fatty acid uncoupling activity (Talbot et al., 2004) may not be contradictory. Depending on the cellular abundance of natural stimulators such as free fatty acids and reactive oxygen species, the functional consequences may be different.

Molecular hypothesis on the mechanism connecting coupled and uncoupled energy conversion in mitochondria

On the one hand, ANT is a major transport protein of the inner mitochondrial membrane, which exchanges mitochondrial ATP for cytosolic ADP, sustaining cellular energy metabolism with ATP. However, ANT has long been known to mediate proton leaks across the inner mitochondrial membrane in the presence of free fatty acids (Andreyev et al., 1989; Skulachev, 1991), an uncoupling activity that is also dependent on the ANT content in mitochondria (Schönfeld, 1990; Roussel et al., 2000; Brand et al., 2005). In penguins, sea acclimatization increases the ANT content in skeletal muscle mitochondria (Talbot et al., 2004), which thus contributes to enhance the maximal capacity of ATP production in skeletal muscle (as shown in the present study), but also the uncoupling activity of fatty acids in isolated mitochondria (Talbot et al., 2004; Rey et al., 2017). ANT behaves like a two-faced ‘Janus’, catalysing either the ADP–ATP exchange to provide ATP when cellular energy demand is high, or proton leakage in resting cells that uncouples energy conversion in mitochondria and produces heat instead (Bertholet et al., 2019).

On the other hand, the uncoupling activity of avUCP requires activation by both free fatty acids and reactive oxygen species (Talbot et al., 2004; Rey et al., 2010) as UCPs in mammals (Echtay et al., 2002). From this mode of activation, we hypothesize that

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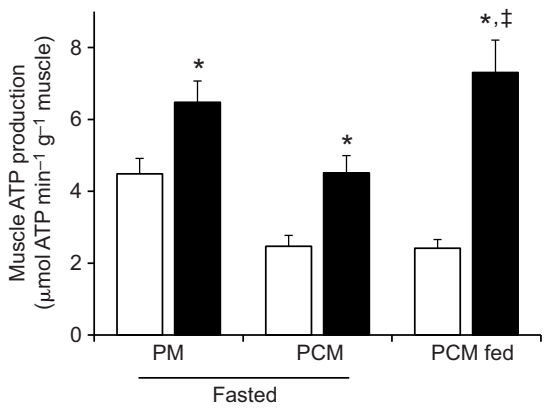

Fig. 4. Effect of sea acclimatization on skeletal muscle ATP production. Maximal rates of ATP synthesis in pectoralis muscle were calculated by multiplying the maximal ATP synthesis rate measured in isolated mitochondria (the highest points on the right of the linear relationship shown in Fig. 3) with the mitochondrial content of pectoralis muscle expressed in milligrams of mitochondrial protein per gram of skeletal muscle and reported in Rey et al. (2016). PM, pyruvate–malate; PCM, palmitoylcarnitine–malate. Mitochondria were isolated from pectoralis muscle of pre-fledging juveniles (white bars) or sea-acclimatized immatures (black bars). Birds were either fasted (N=13–14) or fed (N=6–7); see Materials and Methods for more details. Values are means ±s.e.m.; *P<0.05, significantly different from corresponding pre-fledging king penguins; ‡P<0.05, significantly different from corresponding fasted birds.

these uncoupling activities would be essentially acting in resting birds during post-dive periods when skeletal muscle activity is low and pro-oxidative events may occur (e.g. a sharp increase in the production of reactive oxygen species). In support of this, repeated dives induce repeated severe hypoxemic conditions in the locomotor muscles of penguins (Williams et al., 2011). This condition promotes succinate accumulation within mitochondria (Hochachka et al., 1975), and thereafter a burst of reactive oxygen species production that is associated with its rapid oxidation during post-dive reperfusion of tissues (Dröse, 2013). Again, the phosphorylating activity of mitochondria negatively regulates the ANT-mediated fatty acid-induced proton leak (Roussel et al., 1998, 2000; Bertholet et al., 2019) and the release of reactive oxygen species from mitochondria (Goncalves et al., 2015; Roussel et al., 2019), which would in turn prevent the activation of UCPs. These properties indicate that the uncoupling activities of ANT and avUCP would be at a minimum under high phosphorylating activity when mitochondria are fully coupled and cellular energy demand is high, but maximum in a resting state when mitochondria are loosely coupled and cellular energy demand is low. Although this hypothesis remains to be demonstrated, it is worth noting that in free-ranging king penguins, metabolic rate and thermogenesis increase during the first hour following the end of a diving bout with concomitant body re-warming (Handrich et al., 1997; Froget et al., 2004; Schmidt et al., 2006). ANT- and/or avUCP-related thermogenic activation within muscle fibers could then contribute to the rapid re-warming observed during the extended resting period of penguins at the end of a foraging bout, thus counteracting diving-associated hypothermia.

Conclusion

The most striking result of the present study is the 30–45% improvement in mitochondrial coupling efficiency in skeletal muscle reported in sea-acclimatized immatures compared with pre-fledging never-immersed juveniles. Hence sea acclimatization favors the development of economical management of oxygen by skeletal muscle mitochondria. This mitochondrial phenotype would in turn extend oxygen autonomy in skeletal muscle and thus the aerobic diving limit, by decreasing the oxygen needed to produce the ATP used by muscle work. The present study highlights some of the physiological mechanisms underlying the improvement in diving performance during the early marine life of king penguins.

Acknowledgements

We are grateful to anonymous reviewers for their help in improving the manuscript, and to Yves Handrich (IPEV program 394) and members of IPEV Program 119 for their help in the field.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

Funding

This study was supported financially and logistically by the French Polar Institute Paul-Emile Victor (IPEV research program no. 131 ‘Physionergy’), and received logistic support from the French Southern Territories Administration (Terres Australes et Antarctiques Françaises, TAAF). V.M., P.-A.M., A.B., G.T. were financially supported by the French Polar Institute (IPEV).

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