Ontogeny of muscle bioenergetics in Adélie penguin chicks (*Pygoscelis adeliae*)

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Running title: Muscle bioenergetics in growing Adélie penguin chicks

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The ontogeny of pectoralis muscle bioenergetics was studied in growing Adélie penguin chicks during the first month after hatching and compared with adults using permeabilized fibers and isolated mitochondria. With pyruvate/malate/succinate or palmitoyl-carnitine as substrates, permeabilized fiber respiration markedly increased during chick growth (3-fold) and further rose in adults (1.4-fold). Several markers of muscle fiber oxidative activity (cytochrome oxidase, citrate synthase, hydroxyl-acyl-CoA dehydrogenase) increased 6-19 fold with age together with large rises in intermyofibrillar (IMF) and subsarcolemmal (SS) mitochondrial content (3-5 fold) and oxidative activities (1.5-2.4 fold). The proportion of IMF relative to SS mitochondria increased with chick age but markedly dropped in adults. Differences in oxidative activity between mitochondrial fractions were reduced in adults as compared with hatched chicks. Extrapolation of mitochondrial to muscle respirations revealed similar figures with isolated mitochondria and permeabilized fibers with carbohydrate-derived but not with lipid-derived substrates suggesting diffusion limitations of lipid substrates with permeabilized fibers. Two immunoreactive fusion proteins, Mitofusin 2 (Mfn2) and Optic Atrophy 1 (OPA1), were detected by western blots on mitochondrial extracts and their relative abundance increased with age. Muscle fiber respiration was positively related with Mfn2 and OPA1 relative abundance. Present data showed by two complementary techniques large ontogenic increases in muscle oxidative activity that may enable birds to face thermal emancipation and growth in childhood and marine life in adulthood. The concomitant rise in mitochondrial fusion protein abundance suggests a role of mitochondrial networks in the skeletal muscle processes of bioenergetics that enable penguins to overcome harsh environmental constraints.

Key words: Growth, permeabilized fibers, isolated mitochondria, fusion proteins, mitofusin
INTRODUCTION

Growing in the cold represents a major challenge for most polar endotherms as energy allocation for growth and development is impaired by excessive energy expenditure for thermoregulation. Regulatory heat production is thus classically associated with reduced growth rate (45). In Polar Regions, endotherms face an additional constraint of temporal limitation as the summer period is rather short. This situation is particularly challenging for Adélie penguin chicks (*Pygoscelis adeliae*) that must rapidly grow, build up energy reserves, molt and develop efficient thermogenic mechanisms to succeed in their departure to sea within the short Antarctic summer (10, 44). Such rapid growth requires a maturation of tissue energetic processes able to sustain metabolic activity but this remains unexplored.

Among tissues, the rapid development of skeletal muscle bioenergetics may be of primary importance as skeletal muscle is the major tissue contributing to whole-body energy expenditure and regulatory thermogenesis in birds (15, 16, 33, 34) and is essential for locomotion by walking on land or swimming at sea in adulthood. Mitochondria provide most of the ATP required for metabolic muscle cell processes via oxidative phosphorylation and the rapid response of energy demanding skeletal muscles to metabolic challenges like growth, exercise or cold thermogenesis may depend on the plasticity of their mitochondria (48). Increased metabolic capacity has been documented in skeletal muscle mitochondria from cold-acclimated birds such as ducklings (5, 50) or king penguins (16, 55). No study has so far addressed the ontogeny of muscle mitochondrial bioenergetics in growing penguins.

Two distinct populations of mitochondria exist in skeletal muscles on the basis of their sub cellular localization, morphology and biochemical properties and they constantly adapt to cellular needs. Subsarcolemmal (SS) mitochondria are located just underneath the sarcolemma and have a large, lamellar shape while intermyofibrillar (IMF) mitochondria are smaller, more compact, and inserted between contractile filaments (4, 16, 24, 37, 46). SS mitochondria exhibit lower enzymatic activities, rates of oxidative phosphorylation and ATP synthase activity than IMF mitochondria. It was thus postulated that SS mitochondria may provide energy for membrane-related events like signalling and transport of ions and other substrates, while IMF mitochondria supply ATP for the interaction between myofilaments leading to muscle contraction. In penguins or ducklings, SS mitochondria were more affected by cold acclimation than the IMF ones with regards of their biochemical parameters of protein content (16, 49).

Mitochondria are dynamic organelles that adapt their morphology, number and activity by fusion and fission events to the energetic requirements of the cell (36, 58). Fusion of isolated mitochondria
induces the formation of an extended interconnected mitochondrial network, possibly enabling mitochondria to mix their contents within the network and preventing the local accumulation of defective/abnormal mitochondria which may be advantageous under conditions of high energy demand (48). Various proteins are known to be tightly implicated in these fusion processes. In mammals, mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and optic atrophy protein 1 (OPA1) are required for fusion of both outer and inner mitochondrial membranes (21). Perturbations in mitochondrial fusion processes by skeletal muscle-specific knockout for Mfn1 and Mfn2 in mice have been related to muscle loss and impairment in bioenergetics (12). Conversely, mitochondrial fission or fragmentation will segregate components of the mitochondrial network and excessive fission will generate isolated mitochondria with lower ATP production and lower energetic efficiency than fused ones (36, 48). Accordingly, tissues with high energy requirements (e.g., heart and skeletal muscle) have a predominantly fused mitochondrial network with tightly packed cristae. Fused mitochondria with intermixing of mitochondrial content may thus be preferred when optimal mitochondrial function and increased cellular metabolic capacity is needed such as during rapid growth but experimental data are lacking to support this point.

The aim of this work was to investigate the ontogeny of bioenergetics in skeletal muscle of growing Adélie penguin chicks and ascertain whether it may be related with modifications of intrinsic mitochondrial activity and modifications of mitochondrial fusion protein expression. To answer these questions, we measured the respiratory activity of either isolated muscle mitochondria or permeabilized muscle fibers preserving intact mitochondrial networks. Further, we aimed at characterizing the mitochondrial fusion proteins expressed in Adélie penguin muscles and investigating whether their expression would be correlated with muscle respiratory activity.

**MATERIALS AND METHODS**

Ethical approval for all procedures was granted by the ethics committee of the French Ethic Committee of the Institut Polaire Paul Emile-Victor (IPEV) and by the Polar Environment Committee of the Terres Australes et Antarctiques Françaises (TAAF). Our experiments conformed to the Code of Ethics of Animal Experimentation in the Antarctic.

**Animals**

Experiments were conducted in Antarctica at French Research Station Dumont D’Urville, Adélie Land (66°40’S – 140°00E) during two summer campaigns. The Adélie penguin colony of Pointe Geologie archipelago is composed of nearly 34,000 pairs of breeding birds (*Pygoscelis adeliae*) every year (27).
A total number of 62 birds were biopsied to perform the experiments and separated according to their age. Chicks were either 1-, 7-, 15- or 30-days old (D1, D7, D15, D30; n = 5, 16, 15 and 8, respectively). These groups were defined according to Adélie penguin biology. After hatching, chicks are protected against climatic hazards by the brood pouch of their incubating parent. This protection occurs until their body size prevents them to be completely covered by the brood pouch. Chicks are thus progressively exposed to harsh climatic conditions in parallel with progressive down growth. Thermal emancipation occurs around 15 days and by 30 days of age, chicks that ensure their thermal autonomy can group into chick crèches enabling both parents to fish at sea. We thus chose five different ages corresponding to different developing steps from hatching time (D1), an immature stage, day 7 (D7), a stage characterized by parental thermal protection and down growth, day 15 (D15), a stage corresponding to the gradual acquisition of thermal emancipation and more frequent standing outside the brood pouch, and day 30 (D30) when chicks are continuously exposed to cold environment and group in crèches. Chick age was determined from nest marks at hatching and daily monitoring on the colony. Adélie penguins mostly lay two eggs and the smallest chick does not usually survive, we therefore only used the second chicks in our experiments to minimize the impact of our experiments on breeding success. Chicks were compared to fully mature adult penguins (Adult, n=18) able to face cold water immersion and endurance swimming exercise at sea. Penguins were weighed before experiments.

Experimental design

All the experiments were performed on superficial pectoralis muscle. Biopsies were surgically performed under general isoflurane anesthesia. A small sample of muscle was immediately frozen in liquid nitrogen and kept at -80°C until molecular analysis. The remainder of the biopsy was used immediately for analysis of bioenergetics.

Permeabilized muscle fiber preparation and respiration

Muscle fiber bioenergetics was investigated in vitro in 20 animals belonging to 4 groups: D7 (n=8), D15 (n=8), D30 (n=2) and adult (n=10) using a method adapted from those described previously by Kuznetsov et al. and Picard et al. Muscle biopsy (50 mg) was immersed in ice-cold isolation solution containing K₂EGTA 7.23 mM, CaK₂EGTA 2.77 mM, Imidazole 20 mM, DTT 0.5 mM, Taurine 20 mM, ATP 5.7 mM, Phosphocreatine 15 mM, MgCl₂ 6.5 mM, K-MES 60 mM, pH 7.2 and muscle strips were dissected to separate muscle fibers. Fiber bundles were transferred in isolation solution containing saponin (50 µg/mL) and mixed gently at 4°C for 30 min. Then, permeabilized fibers were washed 3 times by gentle mixing for 10 min at 4°C in the isolation solution without ATP and phosphocreatine but with KH₂PO₄ 10 mM and free fatty acid BSA (5 mg/mL). Muscle fibers were
weighed and their respiration was monitored with an Oroboros® oxymeter at 38°C, the core temperature in penguins, in a respiration buffer (K-MES 100 mM, KCl 30 mM, KH$_2$PO$_4$ 10 mM, MgCl$_2$ 5 mM, EGTA 1 mM, fatty acid-free bovine serum albumin (FAF-BSA) 0.5%) using various substrates or inhibitors (Pyruvate/Malate (PM) 5/2.5 mM, Succinate 5 mM, Palmitoyl-carnitine/Malate (PCM) 40 µM/2.5 mM). Phosphorylating state of respiration was determined in the presence of ADP (1 mM). Non phosphorylating state was obtained by addition of oligomycin (1.25 µg/mL). The integrity of mitochondria within permeabilized fibers was systematically verified by the absence of stimulation of respiration by cytochrome c (10 µM) addition. Because of the late arrival on field of the Oroboros oxymeter, measurements could not be measured immediately at hatching but only from D7.

Mitochondrial respiration and isolation

Pectoralis muscle intermyofibrillar (IMF) and subsarcolemmal (SS) mitochondria were isolated following published protocols from our group (4, 16, 50), involving potter homogenization, partial protease digestion, and differential centrifugations in an ice-cold isolation buffer (100 mM sucrose, 50 mM KCl, 5 mM EDTA, 50 mM Tris-base, pH 7.4) with all steps performed at 4°C. Final pellets were re-suspended in 150-400 µL of storage buffer (250 mM sucrose, 1 mM EGTA, and 20 mM Tris base, pH 7.4). Protein concentration of final mitochondrial suspensions was determined by the Biuret method using bovine serum albumin as standard. Oxygen uptake was determined at 38°C in a respiration buffer (120 mM KCl, 5 mM KH$_2$PO$_4$, 2 mM MgCl$_2$, 3 mM Hepes, 1 mM EGTA, 0.3% FAF-BSA, pH 7.4) with a Clark oxygen electrode (Rank Brother LTD). Respiratory substrates were 5 mM pyruvate plus 2.5 mM malate or 30 µM palmitoyl-carnitine plus 1 mM malate. The phosphorylating respiration rate was initiated by the addition of 500 µM ADP. The basal non-phosphorylating respiration rate was obtained by the addition of 1.25 µg.mL$^{-1}$ oligomycin. Cytochrome c oxidase (COX) activity was measured polarographically as described previously (3, 4, 16) on tissue homogenates, isolated mitochondria and aliquots that were sampled at different steps of the isolation procedure to estimate mitochondrial isolation recovery (16). Briefly, the COX activities of the two fractions obtained after the first centrifugation of muscle homogenate, the supernatant containing SS mitochondria and the pellet containing IMF mitochondria, were used to calculate the mitochondrial recovery within the SS versus IMF compartments.

The activities of citrate synthase (CS; E.C. 4.1.3.7) and 3-hydroxy-acyl-CoA dehydrogenase (HAD; E.C. 1.1.1.35) were used respectively as indicators of the capacity for aerobic flux through the tricarboxylic acid cycle and for flux through the β-oxidation pathway in the catabolism of fatty acids and determined by fluorimetric techniques (34). Briefly, activities were determined on muscle samples (10 mg) that were weighed and immediately homogenized in 0.3M phosphate buffer
containing 0.05% bovine serum albumin (pH 7.7) with a glass Potter-Elvehjem homogenizer. Homogenates were frozen and thawed three times to disrupt the mitochondrial membrane. Enzyme activities were determined at 25°C. Enzyme activities were expressed as μmoles of substrate per minute per gram wet mass.

Western blot analysis

Mitochondrial extracts were prepared from frozen pectoralis muscles that were homogenized in a glass Potter homogenizer in 5 mL of sucrose buffer (sucrose 100 mM, KCL 50 mM, EDTA 5 mM, Tris-base 50 mM, pH 7.4 + protease inhibitor cocktail (10 μL/mL, SIGMA P8340)). Cellular membranes were eliminated by two centrifugations (800 g; 10 min; 4°C). Mitochondria were pelleted by two high speed centrifugation (10000 g; 10 min; 4°C) and then resuspended in 70 μL of Ripa buffer containing protease inhibitor cocktail (10 μL/mL). Mitochondrial protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL) and 50 μg of denaturated protein were loaded for western blotting. All samples were separated by electrophoresis on 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes. Monoclonal antibody was used to detect optic atrophy 1 protein (OPA1) (1:1000; 612606, BD Transduction Laboratories). Polyclonal antibody was used to detect mitofusin 2 protein (Mfn2) (1:250; ab50838, Abcam). Preliminary data showed that these antibodies developed against mouse proteins detected proteins of similar sizes in penguin mitochondrial extracts. Ponceau staining was used to ensure consistent loading and transferring. Blots were revealed using the ECL chemiluminescence method (ThermoScientific). Densitometric analysis of specific bands on immunoblots was performed by Scion software (Scion Corporation, USA).

Statistical analysis

All data presented are means ± SE and were analyzed with Statview 5.0 software (Abacus Concept, Berkeley). Results were compared by analysis of variance (ANOVA) followed by post hoc test (Fisher). Differences were considered significant at P <0.05.

RESULTS

Body growth
Despite harsh climatic conditions encountered in the colony during Antarctic summer, chick growth was remarkably rapid since body weight increased more than 25 fold during the first month of life (Table 1).

**Permeabilized muscle fiber respiration rates**

The ontogeny of muscle bioenergetics was first investigated by measuring the oxygen consumption of permeabilized muscle fibers at different ages. Figure 1 reports fiber respiration using either carbohydrate (Figure 1A) or lipid-derived substrates (Figure 1B). When energized with pyruvate/malate/succinate (PMS), a standard carbohydrate substrate that provides electrons to complex I+II of the respiratory chain, fibers exhibited a higher respiratory activity (two fold) than when providing electrons to the same complexes, but with a donor originating from lipid metabolism (palmitoyl-L-carnitine/malate (PCM)). During chick growth, respiratory activity markedly increased after D15 (state 3: +164%; state 4: +151%). Fibers from adult birds showed the highest respiration rate being 3-fold higher than at D15. Phosphorylating respiration rate was always higher than nonphosphorylating respiration although the difference between both rates was smaller than expected (+40-50%, p<0.05) indicating a small respiratory control ratio. This low ratio with pyruvate/malate/succinate appeared to be based on a high stimulatory effect of succinate addition on nonphosphorylating state while no major stimulation of phosphorylating state was observed. Respiration rates with pyruvate/malate only (state 2) were much smaller than rates measured when succinate was added (Figure 1A). Myofiber respiration with ascorbate/TMPD, a substrate of cytochrome c oxidase (COX), increased from D15 (Figure 1C) and was 5-fold higher in adult fibers than at D7 (p<0.05), suggesting a rise in the oxidative potential and mitochondrial abundance within fibers. When expressed by COX activity in tissue homogenates (Table 1), permeabilized myofiber phosphorylating respiration with pyruvate/malate/ascorbate (Figure 1) increased from D7 to adulthood (0.14 ± 0.02 vs 0.08 ± 0.01, P<0.05).

**Tissue mitochondrial protein content and COX activity**

In growing chicks, the protein yield of isolated mitochondria per gram pectoral muscle markedly increased with age in both muscle mitochondrial subpopulations (Table 1). Subsarcolemmal (SS) mitochondria protein yield increased between D1 and D7 (+108%; p<0.05), remained stable until D30 and then rose again between D30 and Adult (+200%; p<0.05). Intermyofibrillar (IMF) mitochondria protein yield increased to a larger extend between D1 and D7 (+220%; p<0.05) than between D7 and D30 (+73%; p<0.05) and between D30 and Adults (+46%; p<0.05). Altogether, both the SS (8-fold) and IMF (8.2-fold) mitochondrial yield per g muscle markedly increased from hatching to adulthood. The increase in mitochondrial yield with age was associated with a large rise in COX activity of muscle
homogenates (Table 1) between D1 and D7 (+146%; p<0.05) and a smaller rise until D30 (+32% vs D7; +227% vs D1). COX activity was further increased in adult pectoralis muscles (+458% vs D1; +72% vs D30; p<0.05). Changes in COX activity occurred in parallel with striking rises in CS and HAD activities. CS activity increased 8.7-fold between hatching and 15-days of age (p<0.05) and then more than doubled between 15 days and adults. CS activity in adult pectoralis muscle was 19-fold higher than in newly hatched chicks. HAD activity increased 8.7-fold between hatching and 15-days of age (p<0.05) and then doubled between 15 days and adults. HAD activity in adult pectoralis muscle was 13-fold higher than at hatching.

In isolated mitochondria, COX activity was twice as high in the IMF as in the SS fraction in chick pectoralis muscle (e.g. 0.9 ± 0.07 vs 0.45 ± 0.02 µatom O.(min.mg protein)⁻¹ at hatching in IMF and SS mitochondria, respectively) but the difference was much less marked in adult muscle (1.35 ± 0.04 vs 1.09 ± 0.05 µatom O.(min.mg protein)⁻¹ in IMF and SS mitochondria, respectively). Apparent mitochondrial COX activity rose with increasing age in the isolates but this could reflect in part a greater mitochondrial purity in the isolates with increasing age. COX activity was used to estimate mitochondrial isolation recovery that varied with age, ranging between 6 to 25% for the IMF fraction and between 12 to 23% for the SS fraction. Taking isolation recoveries and mitochondrial yields into account, tissue mitochondrial content was calculated. Chick growth was associated with an increase in the mitochondrial content of both muscle fractions but the rise mainly occurred during the first week and mainly affected the IMF fraction (Table 1). This led to a gradual increase in the proportion of the IMF fraction in pectoralis muscle with an IMF/SS ratio doubling (+164%) between hatching and 1 month of age. In adults, the mitochondrial content of both fractions was further increased but this rise affected more the SS (+200% vs D30, p<0.05) than the IMF (+40% vs D30) fraction. Consequently the IMF/SS ratio markedly dropped in adult pectoralis muscles and the SS fraction accounted for one fourth of total pectoralis muscle mitochondria.

Mitochondrial respiration rates

Figure 2 shows the mitochondrial respiration rates measured with either carbohydrate (PM) or lipid-derived (PCM) substrates. With both substrates, the respiration rate of IMF mitochondria was higher than that of SS mitochondria but the differences between populations were reduced in adults as compared with hatched chicks (e.g. phosphorylating rate of IMF with PM was 3.8-fold higher than that of SS at hatching but only 2.1 higher in adults, Figure 2A & 2B). In both populations phosphorylating respiration rate was always higher (10-fold in IMF and 6-fold in SS) than nonphosphorylating rate (p<0.05) in a respiration buffer rich in FAF-BSA (0.3%). In IMF mitochondria, except at D7 with PM (Figure 2A), nonphosphorylating respiration increased (p<0.05) from D15 and
respiration rates at D30 were similar to those attained in adults that were 81% (PM, Figure 2A) and 57% (PCM, Figure 2C) higher than at hatching (p<0.05). Phosphorylating respiration of IMF mitochondria followed a similar ontogenic profile with both substrates, with a transient rise at D7 and then a marked increase from D15. The respiration rates attained in adults were 74% (PM, Figure 2A) and 120% (PCM, Figure 2C) higher than at hatching (p<0.05). Respiration of SS mitochondria followed a different ontogenic profile. With both substrates (Figure 2B and 2D), nonphosphorylating respiration decreased during the first 2 weeks post-hatching and then gradually increased but the respiration rate in adults did not exceed that measured at hatching. Phosphorylating respiration of SS mitochondria gradually increased with lipid-derived substrates (Figure 2D) during the first month post-hatching (+76% between D1 and D30; p<0.05) while there was a non-significant trend with carbohydrate substrates. With both substrates, the phosphorylating respiration rates of SS mitochondria were markedly increased in adults being nearly doubled as compared with hatching. When expressed per mitochondrial COX activity (Table 1), mitochondrial phosphorylating respiration with pyruvate/malate slightly increased from hatching to adulthood (0.17 ± 0.01 vs 0.22 ± 0.02 in IMF and 0.08 ± 0.01 vs 0.11 ± 0.01 in SS from hatched chicks and adults, respectively, P<0.05).

Extrapolation of mitochondrial to pectoral muscle fiber respiration

When mitochondrial respiration was extrapolated to pectoralis muscle taking into account both mitochondrial respiration (Figure 2) and muscle total (IMF+SS) mitochondrial content (Table 1), an ontogenic pattern closely similar to that observed with permeabilized fibers was found (Figure 3). With a carbohydrate substrate, respiration rate increased between D1 and D7 (phosphorylating: +168%; non phosphorylating: +158%; p<0.05), remained stable until D15 and then increased again between D15 and D30 and between D30 and Adult (respectively phosphorylating: +52% and +80%; non phosphorylating: +17% and +75%; p<0.05). With a lipid derived substrate, phosphorylating respiration increased between D1 and D7 (+131%), between D7 and D15 (+12%), and then between D15 and D30 and D30 and adult (respectively +34% and +90%). The phosphorylating respirations calculated in this way (Figure 3) were in the same order of magnitude as those measured with permeabilized fibers (Figure 1) with carbohydrate-derived substrates but not with lipid-derived substrates. With palmitoyl-carnitine, indeed, extrapolated respiratory rates exceeded fiber data by a factor 2-2.5. With both substrates, extrapolated nonphosphorylating respirations were markedly lower than those of permeabilized fibers, indicating a better coupling state of isolated mitochondria.

Expression of fusion proteins in growing chicks and adults

Two immunoreactive mitochondrial fusion proteins, mitofusin 2 protein (Mfn2) and optic atrophy 1 protein (OPA1), were detected by western-blots in penguin muscle mitochondria (Figure 4). Mfn2...
penguin analog appeared as a duplicated band around 86 kDa. The relative abundance of both bands decreased between D1 and D7 (-51%; p<0.05) then increased back to the initial level until D30 (+125% between D7 and D30; p<0.05) and further rose between D30 and Adult (+48%; p<0.05). The relative abundance of Mfn2 bands was therefore 73% higher in muscle mitochondria from adult penguins than in those at hatching. OPA1 penguin analogs appeared as 4 to 5 bands around 90-110 kDa. The relative abundance of all bands (without discriminating individuals bands) was measured and data showed a slight rise (+30%) during the first month of growth and a further increase in adults (+27%; p<0.05). The overall relative abundance of all OPA1 bands was therefore 90% higher in muscle mitochondria from adult penguins than in those at hatching. Homogeneity of protein gel loading was assessed by ponceau staining and the relative mean intensity of each lane was not significantly different between ages (Figure 4).

Correlation between respiratory activity of muscle fibers and fusion protein relative abundance in muscle mitochondria

As shown in Figure 5, muscle fiber phosphorylating respiratory activity with a carbohydrate substrate was significantly related with the relative abundance of either Mfn2 (n=11; R²=0.76; p<0.05) or OPA1 (n=11; R²=0.59; p<0.05) penguin analogs. This suggested that the degree of expression of the penguin analogs of these two fusion proteins in pectoral muscle may play a role in the energetic activity of permeabilized muscle fibers i.e. an experimental model preserving the integrity of mitochondrial networks.

DISCUSSION

The present study showed for the first time the ontogeny in muscle bioenergetics in growing Adélie chicks using permeabilized muscle fibers and isolated mitochondria. Data underlined the marked increase in skeletal muscle oxidative activity from hatching to adulthood, the differential regulation of mitochondrial muscle fractions and the parallel rise in mitochondrial fusion protein expression.

Comparison of permeabilized fibers and isolated mitochondria

Muscle bioenergetics was investigated using two complementary approaches, permeabilized myofibres, where the sarcolemme is selectively permeabilized leaving the mitochondrial networks intact within their native cytoarchitectural environment (29, 52), and isolated mitochondria, where organelles are extracted by mechanical homogenization and differential centrifugations. Both approaches differ in the morphological aspects of mitochondria that present either branched
elongated structures of heterogeneous size and shape in intact muscle fibers (32) or quasi-spherical units of relatively homogeneous size and shape when isolated (4, 41). Recovery was low with isolated mitochondria but within the range of published data in penguin muscle (16). Low recovery in newly-hatched chicks might potentially rely on small fibers more difficult to homogenize and to more fragile organelles possibly lost during the isolation process. Both methods showed similar ontogenic changes in Adélie penguin pectoralis muscle with clear increases in respiratory activity and COX activity with age. Tissue state 3 respiratory capacity measured in permeabilized myofibers (Figure 1) increased to a greater extent than biochemically measured COX activity in tissue homogenates (Table 1), as shown by the higher phosphorylating state with pyruvate/malate/ascorbate to COX ratio in adults than at D7 (0.14 ± 0.02 vs 0.08 ± 0.01), supporting the notion of increased respiratory capacity per mitochondrion during penguin growth and development. A similar trend was also observed with isolated mitochondria. Fiber respirations compared with rates extrapolated from mitochondrial data taking recovery into account (Figure 1 and 3) with substrates derived from carbohydrates. However, with more complex substrates such as palmitoyl-carnitine or ascorbate-TMPD, higher respirations were obtained with isolated mitochondria than with permeabilized fibers as already observed by others using ascorbate-TMPD in rats (41). From differences between both techniques, it was postulated that the isolation-induced fragmentation of mitochondria may have altered some key functions because of the loss of soluble matrix enzymes from the mitochondrial matrix during isolation-induced fragmentation-resealing (41, 43). Present data cannot support nor refute this hypothesis but suggested that permeabilized fibers may encounter substrate delivery limitations to deep mitochondrial fractions, particularly those located within myofibrils, while isolated organelles have full access to added substrates. Accordingly, in very young chicks, i.e. when fiber size is expected to be small, phosphorylating respiration estimated from total mitochondrial content matched that measured with permeabilized fibers with palmitoyl-carnitine as substrate (difference of only 13% at hatching). In adults, by contrast, muscle respiration estimated from isolated mitochondria exceeded 2.5-fold the value of permeabilized fibers. It cannot be excluded that within permeabilized fibers, some lipophilic substrates such as PC might be used by other cellular enzymes or trapped in subcellular compartments thus reducing the availability to mitochondrial carnitine palmitoyltransferases. With simpler substrates such as those derived from carbohydrates, diffusion limitation may not occur as respiratory rates were similar in both cases. Another difference between both techniques was the higher coupling state of oxidative phosphorylation of isolated mitochondria, as assessed by phosphorylating to non phosphorylating respiration ratio, than that of permeabilized fibers, as already observed by others (41). The high respiratory control ratio of isolated mitochondria is likely related with the abundance of FAF-BSA used to trap most of uncoupling fatty acids in mitochondrial preparations while BSA will hardly attain mitochondria within fibers. A rather low
respiratory control ratio of mitochondria within fibers is consistent with the high contribution of
proton leak (nearly one-half) to resting respiration reported in perfused skeletal muscle (47).

Increase in skeletal muscle oxidative capacity during post-hatching ontogenesis

Pectoralis muscle oxidative capacity, as assessed by COX activity of permeabilized fibers (Figure 1) or
homogenates (Table 1), was increased nearly 6-fold between hatching and adulthood. This was
associated with striking rises in the activities of both CS and HAD (Table 1). This ontogenic pattern is
similar to that described in other bird species including barnacle geese (8) or red-winged blackbirds
(34). In precocial eider ducklings, by contrast, muscle oxidative activity was much higher at hatching
than in adulthood in relation with a large increase in activity rapidly occurring in the peri-hatching
period (20). It follows that in rather altricial Adélie penguin chicks, pectoralis muscle is rather
metabolically immature at hatching, in agreement with poor thermoregulatory ability at that stage
(10), but rapidly acquires higher COX activity within the first week post-hatching (+146%). By 2-wks of
age, i.e. at a time where chicks acquire thermal emancipation and improved thermoregulatory ability
(10), pectoralis COX activity was increased 3-fold. Similarly, marked increases in aerobic and β-
oxidative activities of skeletal muscles coincided with the ontogeny of thermoregulatory ability in
red-winged blackbirds (34) or increased thermogenic capacity in cold-exposed birds (1, 3, 16). The
development of endothermy in Adélie penguin chicks may therefore depend on the biochemical
maturation of skeletal muscles, and especially pectoralis muscles devoid of locomotor function on
land. In adults, pectoralis muscle oxidative activity was further increased as compared with growing
chicks (Table 1) possibly in relation with further developmental maturation and the living conditions
of adults that face the energetic challenges of cold water immersion, increased contractile activity
during swimming at sea and hypoxic hunting dives. Increases in skeletal muscle oxidative potential
have been documented in relation with the passage from shore to marine life in juvenile king
penguins (56) and with endurance exercise training or intermittent tissue hypoxia in a number of
organisms (18, 23, 25, 28).

Ontogenic changes in mitochondrial characteristics

Present data showed for the first time that the large rise in pectoralis muscle oxidative activity during
post-hatching ontogenesis relies on changes in mitochondrial abundance and functional
characteristics. Since mitochondrial COX activities measured in Adélie penguin muscle were in the
range of published data in birds (4, 16, 19) while muscle COX activity per unit weight of tissue was
much higher, especially in adult penguins, than in other birds, a large increase in inner membrane
area and a marked mitochondrial biogenesis must take place during post-hatching ontogenesis. It
should be large enough to overcome the expected increase in fiber size and myofibrils content that
takes place during growth as shown in chickens and that leads to an age-related decreased density of
mitochondria in muscle sections (6). As expected, IMF and SS mitochondria differed in their
biochemical and functional characteristics as already noted in birds and mammals (4, 14, 16, 24, 37,
46) with the SS mitochondria exhibiting lower enzymatic activities and rates of oxidative
phosphorylation at all ages. Recent findings also showed that SS and IMF mitochondria also differed
in size and shape (42). Such regional differences have been related to distinct local energy-consuming
mechanisms, namely muscle contraction for IMF mitochondria and the active transport of
metabolites through the sarcolemma for SS mitochondria (31). Present data showed that the
ontogenic profile differed between mitochondrial fractions with chick growth mainly affecting the
IMF fraction (Table 1) and resulting in a gradual increase (+164%) in the IMF/SS ratio in pectoralis
muscle. Such specific effect would be consistent with the expected increases in muscle fiber size and
myofibril content during chick growth that may require a parallel rise in local ATP supply for the
myofibril interactions during contraction. This would result in an improved shivering thermogenesis
with age in Adélie penguin chicks, as already documented in growing king penguin chicks (17), and in
increased thermoregulatory ability (10). In adults, by contrast, the IMF/SS ratio markedly dropped
because of the higher abundance of SS mitochondria (+200% vs D30) suggesting specific roles of SS
mitochondria fraction during marine life. Interestingly, the age-related rises in myofiber and isolated
mitochondria respiration occurred in parallel with increasing expression of an avian uncoupling
protein (14b) suggesting processes favoring mitochondrial heat generation during development in
Adélie penguin pectoralis muscle. Mechanisms controlling muscle bioenergetics in Adélie penguin
chicks are unknown but may possibly involve thyroid hormones that control mitochondrial
biogenesis and oxidative metabolism in mammals and birds (53, 57) and showing parallel
development during the ontogenesis of thermoregulation and muscle oxidative capacity in altricial
birds (34, 35, 53, 57). Transcriptional regulators such as peroxisome proliferator-activated receptor
coactivator-1α (PGC-1α) that control mitochondrial biogenesis and oxidative metabolism (30), with
specific effects on SS mitochondria at modest muscle overexpression (7), may also be involved.

Expression of immunoreactive fusion proteins in penguin skeletal muscle relates to fiber energetics

Present data described for the first time the expression of penguin analogs of mitochondrial fusion
proteins Mfn2 and OPA1 that promote mitochondrial tethering and outer mitochondrial membrane
fusion and regulate mitochondrial metabolism in a number of organisms (60). Immunoreactive
proteins of similar sizes have indeed been detected in penguin skeletal muscle using antibodies
directed against mammalian proteins indicating some conservation of these proteins across Classes.
The relative abundance of immunoreactive Mfn2 and OPA1 increased in penguin muscle
mitochondria in conditions of high energy expenditure such as during chick growth in the cold or
adult life in cold sea. This is in keeping with the increased Mfn2 gene expression already reported in skeletal muscle of cold-exposed rats and that was shown to be mediated by PGC-1α (54). If penguin immunoreactive Mfn2 and OPA1 exhibit similar roles in fusion processes, the ontogenic up-regulation of these proteins between D1 and adulthood (+72% and +94% for Mfn2 and OPA1, respectively, Figure 4) suggests changes in mitochondrial muscle networks with potential increased fusion. The positive correlation between respiratory activity of permeabilized fibers, i.e. a model with preserved mitochondrial networks, and mitochondrial fusion protein relative abundance (Figure 5) suggests a link between mitochondrial networks and muscle bioenergetics in penguins. Morphologically, increased fusion with development is consistent with previous ultramicroscopic observations of elongated IMF and dense SS mitochondria patches in pectoralis muscle from juvenile penguin chicks (4, 16, 24, 37, 46). This is in keeping with growing evidences showing that increasing mitochondrial fusion maintains a tubular mitochondrial network and optimizes mitochondrial function (2, 11, 21). Such phenomenon may be of critical interest during post-hatching growth and development. During embryo development, mitochondrial fusion involving Mfn1 and Mfn2 appeared as an essential process for embryonic development, mitochondrial remodelling, muscle bioenergetics and animal thermogenesis (12, 13, 26, 38). The increased functional properties of SS mitochondria and the reduced IMF/SS ratio in pectoralis muscle of adult Adélie penguins (Table 1) could therefore result from the organisation of muscle mitochondria into an extended interconnected mitochondrial network, possibly enabling mitochondria to mix their contents within the network that may be advantageous under conditions of high energy demand (12, 21, 36, 48, 58) as faced by these birds during their marine life. Further since penguins face hypothermia during diving at sea (22), an increased expression of mitochondrial fusion proteins may contribute to maintain mitochondrial function and cell survival during cold stress as observed with cellular models in vitro (59). The drop in Mfn2 relative abundance concomitant to a marked rise in mitochondrial protein content during the first week of penguin life could be interpreted as a required fission to enable an enhanced dynamic mitochondriogenesis at that stage. However, increasing mitochondrial network complexity (increasing mitochondrial fusion) is not consistently associated with higher respiratory capacity, as for instance phosphorylating respiration did not differ between highly glycolytic muscle where mitochondrial structure is more punctate and very aerobic muscle where mitochondrial structure is more tubular/reticular in nature (40). This suggests that mitochondrial networking may not be the only process regulating respiration and that intrinsic properties of mitochondria (40) also play important roles. Alternatively, it cannot be excluded that the increased abundance of fusion proteins with age and in adults might also contribute to create more sarcoplasmic reticulum - mitochondrial tethers, as this occurs in mice striated muscle during postnatal development (9). Connections between sarcoplasmic reticulum and mitochondria in active pectoralis muscle of penguins at sea may
provide a powerful local control mechanism for integrating Ca\(^{2+}\) release/reuptake and ATP utilization during muscle contraction with ATP production, muscle bioenergetics and lipid synthesis (51).

**Perspectives**

On a technical point of view, present data showed that neither isolated mitochondria nor permeabilized fibers are ideal tools to investigate muscle bioenergetics but the combination of both approaches is of primary interest as noted by others (41) and documented here to clarify the ontogenic changes in mitochondrial function in Adélie penguins. Nevertheless the permeabilized fiber technique benefits from the very low amount of biological material requested to investigate muscle bioenergetics opening a wide area of new biological models that could not be addressed with isolated mitochondria approaches. On a biological point of view, present data suggest a potential role of fusion proteins in the ontogeny of muscle bioenergetics. Changes in fusion protein abundance suggest that even in a very constrained and organized cellular environment such as that occurring in myocytes with well-organized contractile machinery, mitochondrial networks dynamics may occur. This is in keeping with recent observations of morphological interactions between mitochondrial membranes in adult mouse skeletal muscle (42). Future studies must address the concomitant changes in fission proteins and the impact of mitochondrial interactions and network dynamic within skeletal muscles. The mechanisms controlling such ontogenic changes in fusion and possibly fission proteins also warrant further investigations.

The rapid ontogeny of skeletal muscle energetics is consistent with the brevity of the breeding season in Antarctica that imposes a true race against time to Adélie penguin chicks. Any parameter such as climatic conditions and food availability, that affect rapid chick growth, skeletal muscle development and ontogenesis of bioenergetics, is likely to be detrimental for juveniles during the growing period on land and later at departure to sea. The first weeks post-hatching, characterized by marked changes in skeletal muscle bioenergetics (present study) and development of adipose tissue (44), are therefore of critical importance for Adélie penguin survival. Noteworthy is the observation that mortality is mainly observed during that early period in tight link with changes in environment and food supply. The capacities to rapidly develop skeletal muscle bioenergetics may represent metabolic adaptations contributing to select birds able to overcome the energetic challenges of Antarctic life on land and later at sea. The molecular events triggering skeletal muscle development and the ontogenic rise in myofiber respiration deserve to be more fully investigated.

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We are grateful to the members of the scientific missions in Terre Adélie and to the French Polar Research Institute for their technical and logistical assistance.

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REFERENCES


FIGURE LEGENDS

**Figure 1.** Respiration rates of permeabilized muscle fibers from pectoralis muscle of growing Adélie penguin chicks from 7 to 30 days old and in adults. Fiber mitochondria were energized with either: (A) 5 mM pyruvate, 2.5 mM malate and 5 mM succinate (PMS) or (B) 40 µM palmitoyl-L-carnitine and 2.5 mM malate (PCM) or (C) 2.5 mM ascorbate, 0.25 mM TMPD and 2.5 µM FCCP. Phosphorylating respiration was initiated with 1 mM ADP. Non-phosphorylating respiration was obtained by addition of 1.25 µg.mL⁻¹ oligomycin. The respiratory rate measured with pyruvate/malate as substrates before addition of succinate is also shown (state 2 PM). Data are means ± SE; n=8 (D7 and D15); n=2 (D30); n=10 (Adult); nd, not determined. Data with different superscript letters are significantly different at p<0.05.

**Figure 2.** Respiration rates of intermyofibrillar mitochondria (IMF) (A, C) and subsarcolemmal mitochondria (SS) (B, D) isolated from pectoralis muscle of growing Adélie penguin chicks from 1 to 30 days old and adults. Mitochondria were energized with either (A, B) 5 mM pyruvate, 2.5 mM malate (PM) or (C, D) 40µM palmitoyl-L-carnitine and 2.5 mM malate (PCM). Phosphorylating respiration was stimulated with 1 mM ADP. Non-phosphorylating respiration was obtained by addition of 1.25 µg.mL⁻¹ oligomycin. Data are means ± SE; n=5 (D1); n=8 (D7); n=7 (D15); n=6 (D30); n=11 (Adult). Data with different superscript letters are significantly different at p<0.05.

**Figure 3.** Muscle content and respiration rates of total pectoralis muscle mitochondria (IMF+SS) in growing Adélie penguin chicks from 1 to 30 days old and adults. Data were calculated from mitochondrial yields (A) and recoveries (Table 1). Mitochondria were energized with either (B) 5 mM pyruvate and 2.5 mM malate (PM) or (C) 40µM palmitoyl-L-carnitine and 2.5 mM malate (PCM). Phosphorylating respiration was stimulated with 1 mM ADP. Non-phosphorylating respiration was obtained by addition of 1.25 µg.mL⁻¹ oligomycin. Data are means ± SE; n=5 (D1); n=8 (D7); n=7 (D15); n=6 (D30); n=11 (Adult). Data with different superscript letters are significantly different at p<0.05.

**Figure 4.** Relative abundance of immunoreactive mitofusine 2 protein (Mfn2 ; 86 kDa) and optic atrophy 1 protein (OPA1 ; 90-110 kDa) analyzed by western-blots in mitochondria isolated from growing chicks from 1 to 30 days old and adults. Gel loading was assessed by ponceau staining and
the relative mean intensity of each lane is presented under the blots. Data are means ± s.e.m.; n=3 (D1) and n=5 (D7, D15, D30 and Adult). Data with different superscript letters are significantly different at p<0.05. Noncontiguous lanes of the same gel were rearranged in the appropriate ontogenic order.

Figure 5. Correlation between respiratory activity of muscle fibers and the relative abundance of immunoreactive mitochondrial Mfn2 and OPA1 in growing chicks from 7 to 30 days old and adults. Analysis was restricted to birds where both respiration and relative protein abundance were determined (n=11; ◇ D7; ◆ D15; ▲ D30; ● Adult; p<0.05). Correlation was significant in both cases (p<0.05).
Table 1. *Body weight, tissue and mitochondrial enzymatic activities and mitochondrial protein content in pectoralis muscle of Adélie penguin chicks or adults.*

<table>
<thead>
<tr>
<th></th>
<th>Chicks</th>
<th>Adults</th>
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<tbody>
<tr>
<td></td>
<td>0-1 day</td>
<td>7 days</td>
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<tr>
<td>Body weight, kg</td>
<td>0.094 ± 0.004</td>
<td>0.39 ± 0.05</td>
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<tr>
<td>Mitochondrial yield, mg/g</td>
<td>IMF 0.54 ± 0.07</td>
<td>1.7 ± 0.2</td>
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<tr>
<td></td>
<td>SS 0.25 ± 0.03</td>
<td>0.5 ± 0.1</td>
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<tr>
<td>Homogenate</td>
<td>COX, µAtom O/(min x g tissue)</td>
<td>8.4 ± 0.2</td>
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<tr>
<td></td>
<td>CS, µmole / (min x g tissue)</td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>HAD, µmole / (min x g tissue)</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>COX, µAtom O/(min x mg protein)</td>
<td>0.90 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>IMF 0.45 ± 0.02</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>Mitochondrial recovery, %</td>
<td>IMF 6.8 ± 0.8</td>
<td>10.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>SS 12.6 ± 1.6</td>
<td>14.1 ± 1.6</td>
</tr>
<tr>
<td>Mitochondrial protein content, mg/g tissue</td>
<td>IMF 7.9 ± 0.5</td>
<td>16.5 ± 1.1</td>
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<tr>
<td></td>
<td>SS 2.0 ± 0.1</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>IMF/SS ratio 3.9 ± 0.2</td>
<td>4.7 ± 0.5</td>
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COX, cytochrome c oxidase. CS, citrate synthase. HAD, 3-hydroxyacyl-CoA-dehydrogenase. IMF, intermyofibrillar. SS, subsarcolemmal mitochondria.

Mitochondrial yield was calculated from protein concentrations in final mitochondrial suspension and muscle mass. Muscle mitochondrial content was calculated from mitochondrial yields and extraction recoveries. Data are means ± SE calculated from n = 5 (0-1 day old), n = 8-16 (7-days old), n = 7-15 (15-days old), n = 6-8 (30-days old) and n = 11-18 in adults. For CS and HAD activities, n were 4, 4 and 5 in 0-1-day-old, 15-days-old and adult birds, respectively. nd, not determined. For a given parameter, mean values with different letters were significantly different at p<0.05.
Figure 1.

A) Pyruvate/malate/succinate

- State 2 PM
- Non-phosphorylating state
- Phosphorylating state

B) Palmitoyl-carnitine/malate

C) Ascorbate/TMPD

Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.