ACTA ABP BIOCHIMICA POLONICA

Vol. 57, No 4/2010
409–412
on-line at: www.actabp.pl

Regular paper

Tissue variation of mitochondrial oxidative phosphorylation efficiency in cold-acclimated ducklings*

Karine Salin[2], Loïc Teulier[1], Benjamin Rey[3], Jean-Louis Rouanet[1], Yann Voituron[2], Claude Duchamp[1] and Damien Roussel[1]✉

[1]Physiologie Intégrative, Cellulaire et Moléculaire, UMR 5123 CNRS, Université de Lyon, Villeurbanne, France; [2]Ecologie des Hydrosystèmes fluviaux, UMR 5023 CNRS, Université de Lyon, Villeurbanne, France; [3]Biométrie et Biologie Evolutive, UMR 5558 CNRS, Université de Lyon, Villeurbanne, France

We investigated the oxidative phosphorylation efficiency of liver and gastrocnemius muscle mitochondria in thermoneutral and cold-acclimated ducklings. The yield of oxidative phosphorylation was lower in muscle than in liver mitochondria, a difference that was associated with a higher proton conductance in muscle mitochondria. Cold exposure did not affect oxidative phosphorylation efficiency or basal proton leak in mitochondria. We conclude that the basal proton conductance of mitochondria may regulate mitochondrial oxidative phosphorylation efficiency, but is not an important contributor to thermogenic processes in cold-acclimated ducklings.

Keywords: thermogenesis, liver, skeletal muscle, mitochondrial oxidative phosphorylation, proton conductance

Received: 28 August, 2010; revised: 04 November, 2010; accepted: 27 November, 2010; available on-line: 29 November, 2010

INTRODUCTION

When chronically exposed to cold, the survival of some species of birds, including chickens, ducklings, and penguins, depends on their ability to develop non-shivering thermogenesis processes (review in Duchamp *et al.*, 1999). In the absence of both mammalian thermogenic brown adipose tissue and UCP1, both skeletal muscle and liver appear as contributors to the non-shivering thermogenesis (Duchamp & Barre, 1993; Bedu *et al.*, 2001). Two separate mechanisms have been proposed to explain how skeletal muscle may contribute to non-shivering thermogenesis. The first one is cytoplasmic and would generate heat from the futile cycling of calcium across the reticulum sarcoplasmic membrane (Dumonteil *et al.*, 1995), consuming ATP and stimulating mitochondrial respiration. The second one is mitochondrial and involves loose coupling of oxidative phosphorylation (Skulachev & Maslov, 1960; Duchamp *et al.*, 1992; Roussel *et al.*, 1998), whereby a regulated leak of protons would partially dissipate the protonmotive force, producing heat instead of ATP. New evidence for this lies in the recent identification in the skeletal muscle of a functional avian uncoupling protein homologous to UCP1 (Raimbault *et al.*, 2001; Vianna *et al.*, 2001; Talbot *et al.*, 2003), whose mRNA expression and activity are up-regulated during cold acclimation in birds (Raimbault *et al.*, 2001; Toyomizu *et al.*, 2002; Collin *et al.*, 2003; Talbot *et al.*, 2004; Rey *et al.*, 2010; Teulier *et al.*, 2010). In liver, heat generation is ill defined and may be a by-product of the increased metabolic activity of the organ rather than involve a specific thermogenic mechanism. Indeed, cold acclimation has been reported to stimulate hepatic energy-demanding pathways such as lipogenesis and glucagon-enhanced gluconeogenesis (Bedu *et al.*, 2001; 2002).

As illustrated above, there are two fundamental ways of generating more heat for thermogenesis purpose, one is to increase the cellular ATP utilization and the other is to reduce the efficiency of mitochondrial ATP synthesis. A central requirement of these two mechanisms is the mitochondrial oxidative phosphorylation process, as mitochondria either supply the cellular energy-consuming mechanisms with ATP or undergo loose coupling, generating more heat per ATP synthesized. In the context of thermal physiology, the coupling efficiency of mitochondrial energy transduction is therefore of interest, because it describes how much energy mitochondria use for each ATP molecule they produce in the course of substrate oxidation and oxidative phosphorylation. Several studies have previously reported values of mitochondrial ADP/O ratio in muscle and liver from cold-acclimated birds, and found either a decrease or no changes when compared to the values found for control birds (Roussel *et al.*, 1998; Chaînier *et al.*, 2000; Toyomizu *et al.*, 2002). However, in those studies the yield of ATP synthesis was calculated from the rates of oxygen consumption in mitochondria under fully phosphorylating active state (state 3, high concentration of ADP). In order to generate data with more physiological relevance, we initiated the study reported here. By measuring oxygen consumption and ATP synthesis at maximal and submaximal oxidative phosphorylation rates, we re-investigated the effect of cold acclimation on the energetic efficiency of skeletal muscle and liver mitochondria from ducklings. We found that the ATP yield of mitochondria was not affected by cold exposure but greatly differed between the muscle and liver mitochondria. This tissue variation was associated with a difference in the membrane proton conductivity of mitochondria.

MATERIALS AND METHODS

Animals. Male muscovy ducklings (*Cairina moschata* L, pedigree R31, Institut National de la Recherche Agronomique, France) were obtained from a commercial

✉e-mail: damroussel@yahoo.fr

★This work is related to that presented in the poster and oral forms at the 16th European Bioenergetics Conference (Warsaw, 2010); abstract in *Biochim Biophys Acta*, **1797** (Suppl): 62 (2010).

stock breeder (Ets Grimaud, France). From one-week of age, ducklings were randomly assigned to thermoneutral control group or cold-acclimated group and reared for 1 month at either 25 °C (thermoneutral group) or 11 °C (cold-acclimated group) under a constant photoperiod (8:16 light/dark cycle) and free access to food and water. Ducklings were fasted overnight prior to being killed by decapitation. The present investigation was conducted in accordance with the guiding principles of the French Department of Animal and Environmental Protection for the care and use of laboratory animals.

Mitochondria isolation. Muscle and liver samples were rapidly dissected, weighed, placed in ice-cold isolation buffer and homogenized with a Potter-Elvehjem homogenizer. Liver mitochondria were isolated in a buffer containing 250 mM sucrose, 2 mM KH_2PO_4, 1 mM EGTA, 20 mM Tris/HCl, pH 7.3 at 4 °C. The liver homogenate was centrifuged at $700 \times g$ for 10 min. The resulting supernatant was centrifuged at $1\,000 \times g$ for 10 min, filtered through cheesecloth and re-centrifuged at $8700 \times$ g for 10 min to pellet mitochondria. Muscle mitochondria were isolated from the internal part of the gastrocnemius muscle in a buffer containing 100 mM sucrose, 50 mM KCl, 5 mM EGTA, 50 mM Tris/HCl, pH 7.4 at 4 °C. The muscle homogenate was centrifuged at $800 \times g$ for 10 min. The pellet containing intermyofibrillar mitochondria was suspended in 40 ml of isolation buffer and then treated with nagarse (1 mg/g muscle wet weight) for 5 min in an ice bath. The mixture was diluted 1:2, homogenized and then centrifuged at $1\,000 \times g$ for 10 min. The supernatant was filtered through cheesecloth and centrifuged at $8\,700 \times g$ for 10 min. Finally, liver and muscle mitochondrial pellets were washed twice by suspension in their respective isolation buffer and centrifugation at $8\,700 \times g$ for 10 min. The protein concentration of mitochondrial suspensions was determined by the biuret method with bovine serum albumin as a standard.

Mitochondrial respiration and ATP synthesis. Respiration was measured in a glass cell of 1.5 ml volume fitted with a Clark oxygen electrode (Rank Brothers Ltd, France), at 38 °C and calibrated with air-saturated respiratory buffer (120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 2 mM $MgCl_2$, 0.3% bovine serum albumin (w/v) and 3 mM Hepes, pH 7.4). Liver mitochondria (1 mg of protein/ml) and muscle mitochondria (0.3 mg of protein/ml) were incubated in the respiratory buffer supplemented with 20 mM glucose, 1.5 U/ml hexokinase, 5 μM rotenone and 5 mM succinate. Different steady states of phosphorylation were obtained by adding increasing amounts of ADP from 5 to 100 μM. The respiration rate was recorded for 2 min and four 300-μl aliquots of mitochondrial suspension were then withdrawn every 30 s and immediately quenched in perchloric acid solution (10% $HClO_4$/25 mM EDTA). After centrifugation of the denatured protein ($15\,000 \times g$ for 5 min) and neutralization of the resulting supernatant with KOH (2 M KOH/0.3 M Mops), the ATP production was determined from the samples' glucose-6-phosphate content, which was measured enzymatically by spectrophotometry according to (Bergmeyer, 1974).

It is important to note that we made sure that the rates we measured were specific of the mitochondrial ATP synthase activity, by determining oxygen consumption and ATP synthesis rates in the presence of oligomycin (2 μg/ml). Over the range of ADP concentrations used, an oligomycin-insensitive ATP synthesis activity was measurable at 100 μM ADP in skeletal muscle mitochondria purified from both experimental groups (37 ± 12 nmol ATP/min.mg of protein and 35 ± 3 nmol ATP/min·mg of protein in control and cold-acclimated birds, respectively) and in liver mitochondria from cold-acclimated ducklings (5 ± 1 nmol ATP/min·mg of protein). These values were taken into account to calculate the rate of mitochondrial ATP synthesis.

Mitochondrial proton leak. Respiration and membrane potential were measured simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe triphenyl-methyl-phosphonium ($TPMP^+$), respectively. Liver mitochondria (1 mg of protein/ml) and muscle mitochondria (0.35 mg of protein/ml) were suspended in respiratory buffer supplemented with 65 ng/ml nigericin, 1 μg/ml oligomycin and 5 μM rotenone. The TPMP electrode was calibrated by sequential 0.5 μM additions up to 2 μM $TPMP^+$, and then succinate was added to start the reaction. Respiration and membrane potential were progressively inhibited through successive steady states induced by additions of malonate. Membrane potential was calculated as described in (Brand, 1995), assuming a TPMP binding correction of 0.42 and 0.35 $(\mu l/mg \text{ of protein})^{-1}$ for liver and skeletal muscle mitochondria, respectively (Rolfe *et al.*, 1994).

Statistical analysis. The statistical significance of observed differences was assessed using two-way ANOVA. Differences between means were subsequently tested by Scheffe's test.

RESULTS AND DISCUSSION

The relationships between the rates of ATP synthesis and oxygen consumption were linear in both liver and muscle mitochondria (Fig. 1A). The maximal ATP synthesis rate in muscle mitochondria (highest points to the right of the linear relationships) was significantly increased by 14% ($P < 0.01$) in cold-acclimated ducklings compared to the control birds, while the corresponding rate of oxygen consumption was slightly increased but failed to reach statistical significance (+11%, $P = 0.06$). In contrast, maximal rates of ATP synthesis and oxygen consumption were not affected by cold in liver mitochondria. Nevertheless, the relationship concerning the cold-acclimated group was superimposed on that of the thermoneutral group in both liver and skeletal muscle mitochondria (Fig. 1A). Hence, 4 weeks of cold exposure did not alter the amount of ATP synthesized from ADP per amount of oxygen consumed. This is better shown in Fig. 1B where the ATP/O ratio is plotted *versus* the oxygen consumption rate. Indeed, at any given rate of oxygen uptake, ATP/O was the same in the cold-acclimated and thermoneutral groups. The present observation that cold acclimation specifically increased the maximal capacity of skeletal muscle mitochondria to synthesize ATP with no alterations in the efficiency of oxidative phosphorylation reinforce previously published data (Roussel *et al.*, 1998; Teulier *et al.*, 2010).

Moreover, liver mitochondria displayed higher oxidative phosphorylation efficiency than skeletal muscle mitochondria (Fig. 1). Indeed, the linear relation in liver mitochondria was shifted to the left compared to muscle mitochondria, indicating that to produce a given amount of ATP, less oxygen was consumed by liver mitochondria (Fig. 1A). This remains true even though Fig. 1B shows that at maximal rates of ATP synthesis and oxygen consumption (the highest points to the right of the relationships), ATP/O was slightly higher in skeletal

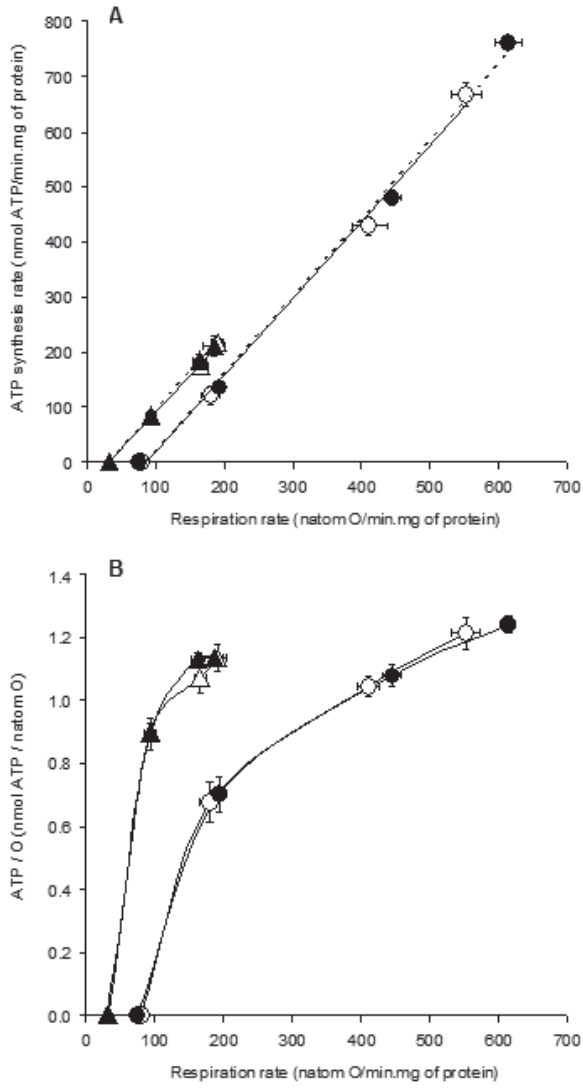

Figure 1. Effects of cold acclimation on oxidative phosphorylation efficiency
Liver (triangles) and skeletal muscle mitochondria (circles) from thermoneutral (open symbols) and cold-acclimated ducklings (filled symbols) were incubated in respiratory buffer supplemented with 20 mM glucose and 1.5 U/ml hexokinase, as described in Materials and Methods. Oxygen consumption and ATP synthesis rates were measured in the presence of increasing concentrations of ADP (0–100 μM). Values are means ± S.E.M. of four to six independent preparations.

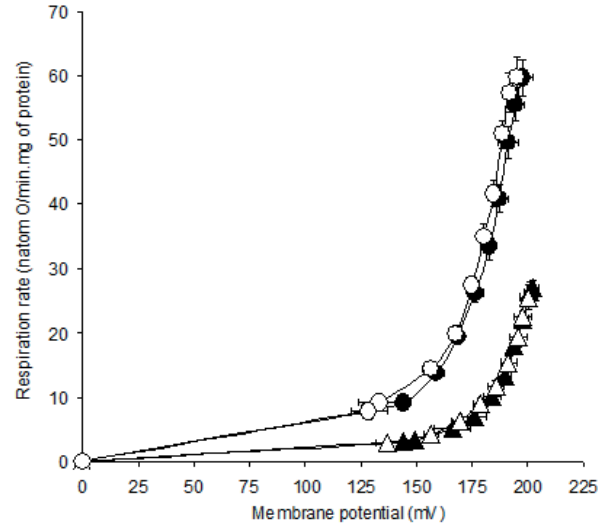

Figure 2. Effect of cold acclimation on basal proton leak
Liver (triangles) and skeletal muscle mitochondria (circles) from thermoneutral (open symbols) and cold-acclimated ducklings (filled symbols) were incubated in respiration buffer supplemented with 65 ng/ml nigericin and 1 μg/ml oligomycin, as described in Materials and Methods. Oxygen consumption rate and membrane potential were measured in the presence of increasing concentrations of malonate (0–3 mM). Values are means ± S.E.M. of seven independent preparations.

muscle (+8%) than in liver mitochondria from both thermoneutral (1.21 ± 0.05 *versus* 1.12 ± 0.05, P=0.27) and cold-acclimated ducklings (1.24 ± 0.02 *versus* 1.14 ± 0.01, $P < 0.05$). However, it remains that for the same fluxes, i.e., at any given rate of oxygen consumption, ATP/O was higher in liver mitochondria (Fig. 1B), indicating that liver mitochondria are set to minimize the cost of ATP production thereby optimizing ATP synthesis for cellular processes. In turn, it appears that mitochondria are more "thermogenic" in the skeletal muscle than in the liver, a characteristic that would be further pronounced in resting skeletal muscle, when cellular ATP demand and mitochondrial phosphorylation activity are low (Fig. 1B).

As suggested from Fig. 2 in (Roussel *et al.*, 2002), a difference in the membrane proton conductivity between liver and muscle mitochondria might explain such a difference in ATP yield. The reason for this being that in the effective ATP/O values reported in Fig. 1B, a fraction of the oxygen consumed is associated with proton leaks in the inner membrane and not directly involved in the production of ATP (Stuart *et al.*, 2001). We therefore measured the activity of basal proton leak in both liver and skeletal muscle mitochondria from thermoneutral and cold-acclimated ducklings (Fig. 2). It clearly appeared that the liver inner mitochondrial membrane displayed a lower leak than the skeletal muscle inner mitochondrial membrane. Indeed, at any given membrane potential, the rate of oxygen consumed to counteract the proton leak (mitochondria respiring in the presence of oligomycin) was lower in the liver than in skeletal muscle mitochondria. Interestingly, cold exposure did not alter the basal proton leak of either mitochondrial type (Fig. 2), which may explain why the mitochondrial ATP yield was not different in thermoneutral and cold-acclimated ducklings. Interestingly, it was found previously that cold exposure altered the basal proton leak in muscle and liver mitochondria from rats (Mollica *et al.*, 2005), underlining some species specificities in mitochondrial adaptations toward cold exposure. Finally, it is important to keep in mind that the basal proton leak measured in the present study was not related to the activity of avian uncoupling protein, which requires specific activators (free fatty acids and superoxides) and inhibitor (GDP) to function (Talbot *et al.*, 2003). Alternatively, it has been suggested that the basal proton conductance of mitochondria might depend on the ADP/ATP carrier content (Brand *et al.*, 2005). Since skeletal muscle is well known to have a higher content of the ADP/ATP carrier than liver mitochondria (Rossignol *et al.*, 2000; Mollica *et al.*, 2005; Hulbert *et al.*, 2006), the lower coupling of oxidative phosphorylation reported in duckling skeletal muscle mitochondria would be explained by their higher content of ADP/ATP carrier. This assumption deserves further investigation.

The slope values of the linear relationships shown in Fig. 1A describe the amount of extra oxygen that mitochondria have to consume in order to sustain an additional ATP production imposed by a change in the activity of the phosphorylating system, and so independently of the activity of ATP synthesis-independent proton gradient-consuming processes (e.g., proton leak). As such, these values would be close to, if any a measure of, the mechanistic stoichiometry of mitochondrial oxidative phosphorylation. Notwithstanding, these slopes displayed an average value of 1.38 ± 0.02 and were not significantly different between liver and skeletal muscle mitochondria or between control and cold-acclimated ducklings. Surprisingly, this is a good estimate of the maximal P/O ratio for succinate if we assume that duckling mitochondrial ATP synthase is comprised of three $\alpha\beta$ pairs, hence three catalytic sites, in its catalytic sector F_1, and ten c-subunits in its membrane embedded sector, F_0 (Stock *et al.*, 1999), which would predict an H^+/ATP ratio of 10/3 and an overall coupling stoichiometry of 4.33 (Brand, 2005).

In conclusion, the basal proton conductance of mitochondria may be a determinant of the effective ATP/O ratio but it does not play an important role in the development of non-shivering thermogenesis in cold-acclimated ducklings.

REFERENCES

Bedu E, Cohen-Adad F, Dallevet G, Garin D, Barre H, Duchamp C (2001) Effect of cold-acclimation on oxygen uptake and glucose production of perfused duckling liver. *Comp Biochem Physiol* **128A:** 851–861.

Bedu E, Chainier F, Sibille B, Meister R, Dallevet G, Garin D, Duchamp C (2002) Increased lipogenesis in isolated hepatocytes from cold-acclimated ducklings. *Am J Physiol* **283:** R1245–R1253.

Bergmeyer HU (1974) In *Methods of enzymatic analysis*. Verlag Chemie Weinheim Academic Press, Inc., New York.

Brand MD (1995) Measurement of mitochondrial proton motive force. In *Bioenergetics — a practical approach*, Brown GC, Cooper CE, eds, pp 39–62. IRL Press, Oxford.

Brand MD (2005) The efficiency and plasticity of mitochondrial energy transduction. *Biochem Soc Trans* **33:** 897–904.

Brand MD, Pakay JL, Ocloo A, Kokoszka J, Wallace DC, Brookes PS, Cornwall EJ (2005) The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *Biochem J* **392:** 353–362.

Chainier F, Roussel D, Georges B, Meister R, Rouanet JL, Duchamp C, Barré H (2000) Cold acclimation or grapeseed oil feeding affects phospholipid composition and mitochondrial function in duckling skeletal muscle. *Lipids* **35:** 1099–1106.

Collin A, Buyse J, van As P, Darras VM, Malheiros RD, Moraes VMB, Reyns GÉ, Taouis M, Decuypere E (2003) Cold-induced enhancement of avian uncoupling protein expression, heat production, and triiodothyronine concentrations in broiler chicks. *Gen Comp Endocrinol* **130:** 70–77.

Duchamp C, Barre H (1993) Skeletal-muscle as the major site of non-shivering thermogenesis in cold-acclimated ducklings. *Am J Physiol* **265:** R1076–R1083.

Duchamp C, Marmonier F, Denjean F, Lachuer J, Eldershaw TPD, Rouanet JL, Morales A, Meister R, Benistant C, Roussel D *et al.* (1999) Regulatory, cellular and molecular aspects of avian muscle nonshivering thermogenesis. *Ornis Fenn* **76:** 151–165.

Duchamp C, Cohen Adad F, Rouanet JL, Barre H (1992) Histochemical arguments for muscular nonshivering thermogenesis in muscovy ducklings. *J Physiol* **457:** 27–45.

Dumonteil E, Barre H, Meissner G (1995) Expression of sarcoplasmic-reticulum Ca^{2+} transport proteins in cold-acclimating ducklings. *Am J Physiol* **269:** C955–C960.

Hulbert AJ, Turner N, Hinde J, Else P, Guderley H (2006) How might you compare mitochondria from different tissues and different species? *J Comp Physiol B* **176:** 93–105.

Mollica AP, Lionetti L, Crescenzo R, Tasso R, Barletta A, Liverini G, Iossa S (2005) Cold exposure differently influences mitochondrial energy efficiency in rat liver and skeletal muscle. *FEBS Lett* **579:** 1978–1982.

Raimbault S, Dridi S, Denjean F, Lachuer J, Couplan E, Bouillaud F, Bordas A, Duchamp C, Taouis M, Ricquier D (2001) An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds. *Biochem J* **353:** 441–444.

Rey B, Roussel D, Romestaing C, Belouze M, Rouanet JL, Desplaches D, Sibille B, Servais S, Duchamp C (2010) Up-regulation of avian uncoupling protein in cold-acclimated and hyperthyroid ducklings prevents reactive oxygen species production by skeletal muscle mitochondria. *BMC Physiol* **10:** 5.

Rolfe DFS, Hulbert AJ, Brand MD (1994) Characteristics of mitochondrial proton leak and control of oxidative-phosphorylation in the major oxygen-consuming tissues of the rat. *Biochim Biophys Acta* **1188:** 405–416.

Rossignol R, Letellier T, Malgat M, Rocher C, Mazat JP (2000) Tissue variation in the control of oxidative phosphorylation**:** implication for mitochondrial diseases. *Biochem J* **347:** 45–53.

Roussel D, Rouanet JL, Duchamp C, Barre H (1998) Effects of cold acclimation and palmitate on energy coupling in duckling skeletal muscle mitochondria. *FEBS Lett* **439:** 258–262.

Roussel D, Harding M, Runswick MJ, Walker JE, Brand MD (2002) Does any yeast mitochondrial carrier have a native uncoupling protein function? *J Bioenerg Biomembr* **34:** 165–176.

Skulachev VP, Maslov SP (1960) The role of nonphosphorylating oxidation in temperature regulation. *Biochemistry* (Moscow) **25:** 826–832.

Stock D, Leslie AGW, Walker JE (1999) Molecular architecture of the rotary motor in ATP synthase. *Science* **286:** 1700–1705.

Stuart JA, Cadenas S, Jekabsons MB, Roussel D, Brand MD (2001) Mitochondrial proton leak and the uncoupling protein 1 homologues. *Biochim Biophys Acta* **1504:** 144–158.

Talbot DA, Hanuise N, Rey B, Rouanet JL, Duchamp C, Brand MD (2003) Superoxide activates a GDP-sensitive proton conductance in skeletal muscle mitochondria from king penguin (*Aptenodytes patagonicus*). *Biochem Biophys Res Commun* **312:** 983–988.

Talbot DA, Duchamp C, Rey B, Hanuise N, Rouanet JL, Sibille B, Brand MD (2004) Uncoupling protein and ATP/ADP carrier increase mitochondrial proton conductance after cold adaptation of king penguins. *J Physiol* **558:** 123–135.

Teulier L, Rouanet JL, Letexier D, Romestaing C, Belouze M, Rey B, Duchamp C, Roussel D (2010) Cold-acclimation-induced non-shivering thermogenesis in birds is associated with upregulation of avian UCP but not with innate uncoupling or altered ATP efficiency. *J Exp Biol* **213:** 2476–2482.

Toyomizu M, Ueda M, Sato S, Seki Y, Sato K, Akiba Y (2002) Cold-induced mitochondrial uncoupling and expression of chicken UCP and ANT mRNA in chicken skeletal muscle. *FEBS Lett* **529:** 313–318.

Vianna CR, Hagen T, Zhang CY, Bachman E, Boss O, Gereben B, Moriscot AS, Lowell BB, Bicudo J, Bianco AC (2001) Cloning and functional characterization of an uncoupling protein homolog in hummingbirds. *Physiol Genomics* **5:** 137–145.