The Journal of Experimental Biology 205, 2267–2273 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB4015

Review

Adaptive thermogenesis in hummingbirds

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Accepted 13 May 2002

Summary

The occurrence of non-shivering thermogenesis in birds has long been a controversial issue. Although birds are endothermic vertebrates, sharing with mammals (placental mammals and marsupials) a common ancestor, they do not possess brown adipose tissue or a similar type of tissue, unlike their mammalian counterparts. Some bird species are, however, able to withstand very low ambient temperatures (–70 °C) or undergo periods of heterothermia, and there is now good experimental evidence showing that non-shivering thermogenesis may indeed occur in birds under such conditions. The skeletal muscles of birds, particularly the flight muscles, occupy a significant fraction (approximately 30 %) of the total body mass, and recent results have shown that they are likely to be the main sites for non-shivering thermogenesis. The precise mechanisms involved in adaptive thermogenesis in birds are still not fully understood. The translocation of Ca^{2+} between intracellular compartments and the cytosol mediated by the sarcoplasmic reticulum Ca^{2+}-ATPase, uncoupled from ATP synthesis, is one mechanism whereby chemi-osmotic energy can be converted into heat, and it has been proposed as one of the possible mechanisms underlying non-shivering thermogenesis in birds on the basis of data obtained mainly from ducklings acclimatized to cold conditions. The recent characterization of an uncoupling protein homolog in avian skeletal muscle and the expression of its mRNA at different stages of the torpor/rewarming cycle of hummingbirds indicate that it has the potential to function as an uncoupling protein and could play a thermogenic role during rewarming in these birds.

Key words: non-shivering thermogenesis, brown adipose tissue, Ca^{2+}-ATPase, uncoupling protein, bird, hummingbird.

Introduction

It is well established that, unlike mammals, brown adipose tissue is not present in birds (Johnston, 1971; Saarela et al., 1991). Despite sharing a common ancestor, the evolution of homeothermy from ectothermic diapsid ancestors in mammals (placental mammals and marsupials) and synapsid ancestors in birds is presumed to have occurred independently approximately 310 million years ago (Kumar and Hedges, 1998). Both groups are homeotherms capable of sustaining constant core temperatures above the environmental temperature throughout their lives. Nevertheless, some birds and mammals undergo periods of heterothermy, which may be triggered by internal or environmental cues. In mammals and birds, homeothermy is maintained at the expense of high metabolic rates: both groups have, in general, fivefold greater standard metabolic rates (SMRs) than other vertebrates of the same size.

Factors affecting adaptive thermogenesis

Heat production in response to environmental temperature or diet is commonly referred to as adaptive thermogenesis. Resting energy expenditure changes markedly in response to environmental temperature. Rates of oxygen consumption increase two- to fourfold in rodents during both acute and chronic cold exposure (4 °C) (Hart et al., 1956; Davis et al., 1960). The thermogenic capabilities of birds of the avian cardueline subfamily (goldfinches, house finches, redpolls), for example, are impressive, and in many cases exceed the abilities of mammals. Goldfinches can elevate and sustain a fivefold higher SMR for 6–8 h at winter temperatures of –70 °C (Dawson and Carey, 1976), whereas the same birds will become hypothermic after only a few minutes of cold exposure in spring (Dawson and Carey, 1976). This transformation occurs in the absence changes in the aerobic capacity of the skeletal muscles, which are probably the major thermogenic tissues in birds (Carey et al., 1978; Marsh and Dawson, 1982).

Cold-induced metabolic scope has been examined in a few studies and has been shown to range from three to eight times SMR in birds (Withers, 1977; Prinzinger and Siedle, 1988; Brigham, 1992; Maddocks and Geiser, 1997). Metabolic scope during flight can range from five to ten times SMR, thus exceeding the maximum metabolic rate elicited by cold exposure (Brackenbury, 1984; Marsh and Dawson, 1989). The extraordinary demands of flight in birds have resulted in selection for muscle fiber types with high rates of substrate utilization and may predispose the use of these muscles in birds as thermogenic organs (Block, 1994).

Non-shivering thermogenesis

The existence of non-shivering thermogenesis (NST) and its relative importance in birds is still a matter of active debate (Barré et al., 1989; Connolly et al., 1989). Birds meet the bulk of their increased thermogenic requirements in response to cold stress with shivering thermogenesis (West, 1965). In severe cold, however, the integrated pectoral electromyographic activity of European finches (*Carduelis spinus*, *Carduelis chloris*, *Pyrhulla pyrhulla*, *Fringilla montifringilla* and *Coocothraustes coccothraustes*) was independent of the rate of oxygen consumption (heat production), suggesting the existence of NST (Saarela et al., 1995). In fact, there is now strong evidence than NST plays a role in birds during cold-exposure, as in the case of cold-acclimated ducklings (Barré et al., 1985, 1986). It is important to note that in these studies only cold-induced thermogenesis that was not accompanied by an increase of muscular electrical activity was referred to as NST. In addition, a cold-induced increase in electrical activity does not exclude the possibility of a concurrent increase in NST (Janský, 1973). It is assumed, therefore, that under conditions of severe cold both shivering and NST are activated simultaneously and cooperate to ensure thermal homeostasis.

Torpid and hibernating placental mammals rely mostly on NST in brown adipose tissue for rewarming (Smith and Horwitz, 1969). Brown adipose tissue is specialized for heat production as a result of the expression of uncoupling protein 1 (UCP1), a physiological mitochondrial uncoupler (Klingenberg and Huang, 1999). This protein dissipates the H^+ gradient formed across the inner mitochondrial membrane, preventing both the passage of H^+ through the F_1F_o-ATP synthase and the synthesis of ATP from ADP and inorganic phosphate (P_i). As a result, oxidative metabolism is activated, leading to an increase in the rate of heat production (Nicholls and Locke, 1984). Shivering, however, has been considered to be the primary thermogenic mechanism during rewarming in torpid birds or during cold stress (West, 1965; Dawson, 1975; Block, 1994). The capacity for NST has been more carefully investigated in birds over the past few years, and there is now good evidence that it occurs in this group, although it may be limited to a few species. The literature reports data accumulated for a relatively restricted group of birds, but this phenomenon might well be more widespread than previously thought (Bicudo et al., 2001).

Thermogenic mechanisms in birds

Skeletal muscle Ca^{2+}-ATPase

Ca^{2+}-cycling-mediated thermogenesis in skeletal-muscle-like cells is a well-characterized mechanism for NST in certain species of fish (Block, 1994; O'Brien and Block, 1996). The thermogenic potential for Ca^{2+} cycling in mammals is evident from the pathological syndrome of malignant hyperthermia, a dominant genetic disorder of humans and pigs which, in many cases, is due to a mutation in the skeletal muscle ryanodine receptor, the Ca^{2+}-release channel of the sarcoplasmic reticulum (Denborough, 1998). Abnormal Ca^{2+} release, triggered by anesthesia and/or stress, causes intense thermogenesis, which leads to hyperthermia.

Ca^{2+}-ATPase and ryanodine-receptor content increase in the sarcoplasmic reticulum during cold acclimation in birds (Dumonteil et al., 1995), raising the possibility that Ca^{2+} cycling in bird skeletal muscle could also contribute to adaptive thermogenesis, including NST. Within this context, hummingbirds appear to be a good model system for investigating NST among birds. They possess one of the highest mass-specific metabolic rates recorded among vertebrates, requiring an abundant supply of oxygen and energy substrates (Suarez et al., 1986). Their limited amount of adipose tissue impairs the maintenance of euthermic metabolic rate under conditions of food deprivation. To circumvent this, hummingbirds enter torpor on a daily basis to conserve energy and survive throughout the night (Pearson, 1950; Lasiewsky, 1963; Calder, 1994; Bicudo, 1996).

The cellular and molecular mechanisms underlying the rewarming process in hummingbirds are still not entirely understood. Occupying between 25 and 30 % of body mass, the pectoral muscle of hummingbirds, with a mitochondrial volume density of 25–30 %, is the natural candidate for heat generation in this avian group. The same kind of argument might also hold for adult humans, which, unlike rodents, do not have large, distinct depots of brown adipose tissue. Skeletal muscle, in contrast, constitutes up to 40 % of total body mass in humans and has a significant volume density of mitochondria, prompting several investigators to assume that it is the logical tissue in which to study adaptive thermogenesis. The mechanisms involved are not completely understood but could include effects on mitochondrial function and uncoupling, Ca^{2+} cycling or both (Lowell and Spiegelman, 2000).

During Ca^{2+} transport, the chemical energy derived from ATP hydrolysis is used by the Ca^{2+}-ATPase to pump Ca^{2+} into the sarcoplasmic reticulum, and chemical energy is converted into osmotic energy during this process. After accumulation of Ca^{2+} within the sarcoplasmic reticulum, the Ca^{2+} gradient formed across the membrane promotes the reversal of the catalytic cycle of the enzyme. During this reversal, some of the chemi-osmotic energy is either used to resynthesise some of the ATP previously cleaved (Makinose and Hasselbach, 1971)

or dissipated into the surrounding medium as heat (Block, 1994). Ca^{2+} leaves the sarcoplasmic reticulum through the action of the Ca^{2+}-ATPase both during ATP synthesis from ADP and P_i and during heat production (de Meis, 1998).

An increase in the rate of Ca^{2+} uptake does not necessarily indicate an augmentation of the Ca^{2+}-ATPase activity. It simply represents the net accumulation of Ca^{2+} in the lumen of the sarcoplasmic reticulum: the product of a dynamic equilibrium between what is released and taken up by the sarcoplasmic reticulum as a whole. Ca^{2+}-ATPase itself participates in the uptake of Ca^{2+} as well as in its release, and in both processes the conversion of different forms of energy into heat may occur. According to de Meis et al. (1997), with the release of Ca^{2+} mediated by the Ca^{2+}-ATPase, the accumulated energy derived from the Ca^{2+} electrochemical gradient may be converted into heat, i.e. in this case, Ca^{2+} transport is uncoupled from ATP synthesis.

During non-shivering thermogenesis, most of the heat is derived from resting muscle, but the mechanism of heat production remains unclear. It has been proposed that Ca^{2+} leaks from the sarcoplasmic reticulum, and heat would therefore be derived from the hydrolysis of the extra ATP needed to maintain a low myoplasmic Ca^{2+} concentration (Block, 1994). In this situation, it is assumed that the amount of heat produced during the hydrolysis of an ATP molecule is always the same and is not modified by the formation of the Ca^{2+} gradient, as if the energy released by the ATP hydrolysis were divided into two independent packets, one to be converted into heat and the other to be used for Ca^{2+} transport (Landeira-Fernandez et al., 2000). More recently, microcalorimetric measurements of ATP hydrolysis have shown that, in the presence of a Ca^{2+} gradient, the heat produced during the hydrolysis of each ATP molecule is 2–3 times greater than that measured in sarcoplasmic reticulum vesicles in the absence of a Ca^{2+} gradient (de Meis, 1998). These results indicate that the Ca^{2+}-ATPase isoform found in vertebrate skeletal muscle is able to convert osmotic energy into heat.

Experiments using sarcoplasmic reticulum vesicles from hummingbird (*Eupetomena macroura*) pectoral muscle have shown that the ratio between rates of ATP hydrolysis and ATP synthesis, with the release of Ca^{2+} mediated by the Ca^{2+}-ATPase, is twice that in rabbit skeletal muscle sarcoplasmic reticulum (Vianna et al., 1999), suggesting that, in the presence of a Ca^{2+} gradient, at least theoretically, twice as much energy is being converted into heat in hummingbird pectoral muscle as in rabbit skeletal muscle. Thus, during arousal from torpor, rewarming of the hummingbird body could also take place through NST, an alternative source of heat derived from the hydrolysis of ATP by the sarcoplasmic reticulum Ca^{2+}-ATPase of its pectoral muscle fibers. This mechanism might contribute to some energy conservation during arousal, particularly when energy storage is low before entering into torpor.

Uncoupling protein homologs

Although uncoupling protein 1 (UCP1) occurs exclusively in brown adipose tissue, a large array of similar uncoupling proteins was recently found in various tissues of mice; e.g. UCP2, which is expressed ubiquitously (Fleury et al., 1997), and UCP3, which is expressed primarily in skeletal muscle (Boss et al., 1997). When expressed in yeast mitochondria or reconstituted into liposomes, both UCP2 and UCP3 catalyze H^+ flux (Jaburek et al., 1999). The physiological role of these newly described uncoupling proteins is controversial and not

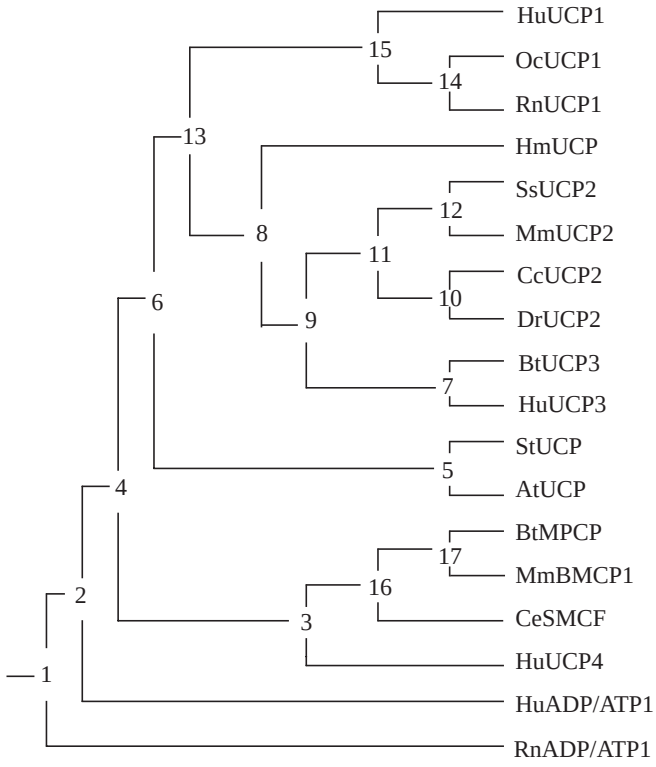

Fig. 1. Evolutionary tree of uncoupling proteins (UCPs) and other mitochondrial carriers. The unrooted evolutionary tree represents inferred genetic distances (the numerical values indicate the number of base substitutions that change the amino acid sequences) between UCP sequences obtained from GeneBank (Protpars program from PHYLIP package) (Felsenstein, 1988). Abbreviations, species and accession numbers are as follows: HuUCP1 is *Homo sapiens* (human) UCP1, P25874; OcUCP1 is *Oryctolagus cuniculus* (rabbit) UCP1, P14271; RnUCP1 is *Rattus norvegicus* (rat) UCP1, P04633; HmUCP is *Eupetomena macroura* (hummingbird) UCP, AF255729; SsUCP2 is *Sus scrofa* (pig) UCP2, AAD05201; MmUCP2 is *Mus musculus* (mouse) UCP2, P70406; CcUCP2 is *Cyprinus carpio* (carp) UCP2, CAB46248; DrUCP2 is *Danio rerio* (zebrafish) UCP2, CAB46268; BtUCP3 is *Bos taurus* (cow) UCP3, AAC61762; HuUCP3 is *H. sapiens* (human) UCP3, P55916; StUCP is *Solanum tuberosum* (potato) UCP, CAA72107; AtUCP is *Arabidopsis thaliana* (thale cress) UCP, CAA77109; BtMPCP is *B. taurus* (cow) mitochondrial carrier protein, P12234; MmBMCP1 is *M. musculus* (mouse) brain mitochondrial carrier protein, AAD03674; CeSMCF is *Caenorhabditis elegans* (worm) similar mitochondrial carrier family, AAB54239; HuUCP4 is *H. sapiens* (human) UCP4, AAD16995; HuADP/ATP1 is *H. sapiens* (human) ADP/ATP translocator, P12235; RnADP/ATP1 is *R. norgevicus* (rat) ADP/ATP carrier, Q05962. Reproduced from Vianna et al. (2001) with permission from the American Physiological Society.

yet defined, particularly because they have been found in tissues whose primary function is not thermogenic, e.g. spleen, kidney and brain. Given, however, the apparent widespread distribution of uncoupling proteins among eukaryotes and the lack of brown adipose tissue in birds, hummingbirds again become an interesting model system in which to test the hypothesis that body heat might also be generated in birds through the mediation of an uncoupling protein.

Raimbault et al. (2001) have recently cloned an uncoupling protein homolog (avUCP) from chicken (*Gallus gallus*) skeletal muscle, and they suggest that this avUCP may be involved in facultative muscle thermogenesis. They claim, however, that it was not possible to obtain functional data, and the uncoupling activity of avUCP could not therefore be demonstrated. However, the unique expression of this protein in skeletal muscle of chicken, and its upregulation after cold-acclimation in ducklings or following treatment with glucagon, inducing muscle NST or in association with diet-induced thermogenesis, support, according to these authors, a role for avUCP in energy expenditure in birds.

Hummingbird uncoupling protein homolog

Vianna et al. (2001) were able to clone and functionally characterize a novel uncoupling protein homolog in the swallow-tailed hummingbird *Eupetomena macroura*. They used reverse transcriptase/polymerase chain reaction (RT-PCR) of total RNA from *E. macroura* pectoral muscle to amplify a cDNA fragment flanked by conserved domains in the uncoupling proteins. This fragment has approximately 73 % homology to rat UCP3, approximately 70 % homology to rat UCP2 and approximately 63 % homology to rat UCP1. Racemic amplification of DNA ends (RACE) was used to isolate the 5′ and 3′ ends. The deduced amino acid sequence of the hummingbird uncoupling protein (HmUCP) consists of 304 amino acid residues, with a molecular mass of 32.8 kDa. The predicted amino acid sequence of HmUCP has higher identity to human UCP3 and rat UCP2 isoforms, approximately 72 % and approximately 70 %, respectively, than to rat and human UCP1 (approximately 55 %). Analysis infers an unrooted phylogeny, and one most parsimonious tree was found in this case. This indicated that HmUCP, UCP2 and UCP3 of placental mammals and fish UCPs form a group that is closely related in terms of inferred genetic distance (Fig. 1).

HmUCP was expressed in *Saccharomyces cerevisiae* and used in functional studies. A pYES2 plasmid containing the 12CA5 epitope-tagged HmUCP was used to transform yeast cells. The western blot revealed a single protein of approximately 34 kDa, compatible with the predicted molecular mass, in the enriched mitochondria fraction. In these cells, transiently expressed HmUCP decreased the 3′,3-dihexyl-oxacarbocyanine iodide ($DiOC_6$) uptake as measured by flow cytometry by a similar degree as did rat UCP1, demonstrating that HmUCP is capable of lowering the mitochondrial membrane potential (Fig. 2). The analysis of HmUCP mRNA expression in various tissues indicated a high level of expression in the pectoral muscle. Heart and liver both show slightly lower levels of expressions, followed by lung and kidneys. Brain was the only tissue tested in which HmUCP mRNA was not detected (Vianna et al., 2001).

Vianna et al. (2001) also tested whether HmUCP mRNA levels change under various physiological conditions. Hummingbirds were studied in conditions of euthermy, torpor and rewarming. At each phase, various tissues were processed for northern blotting. A typical profile of the rate of oxygen consumption by a *E. macroura* for these phases is shown in Fig. 3A.

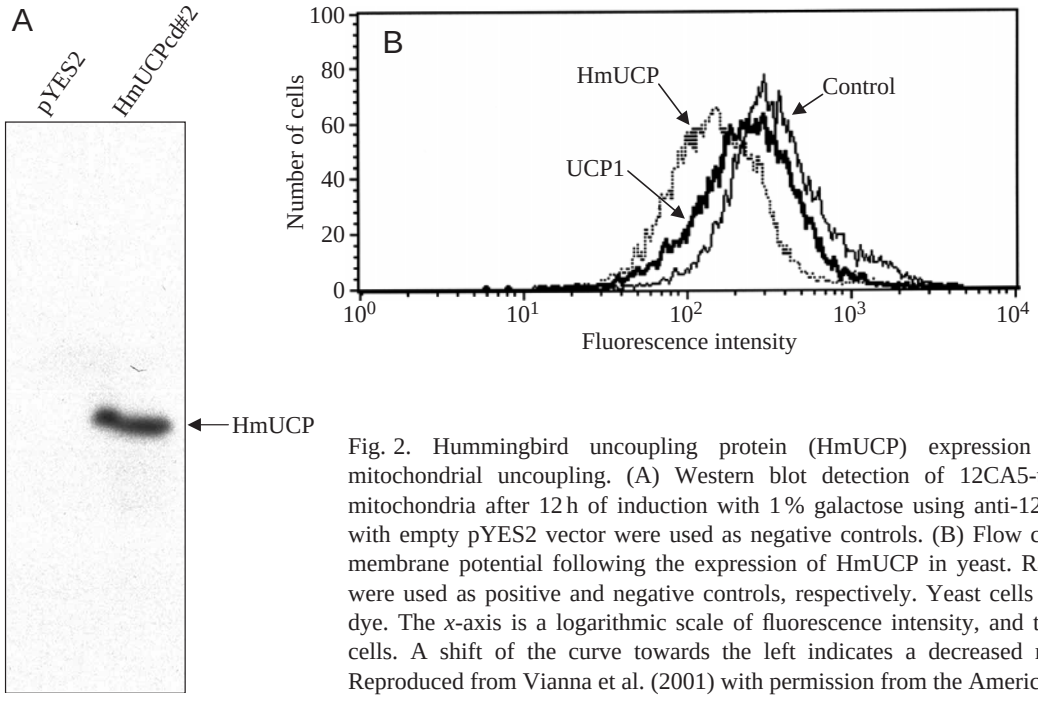

Fig. 2. Hummingbird uncoupling protein (HmUCP) expression in yeast and HmUCP-induced mitochondrial uncoupling. (A) Western blot detection of 12CA5-tagged HmUCP in isolated yeast mitochondria after 12 h of induction with 1 % galactose using anti-12CA5 antibodies. Cells transformed with empty pYES2 vector were used as negative controls. (B) Flow cytometry analysis of mitochondrial membrane potential following the expression of HmUCP in yeast. Rat UCP1 and empty pYES2 vector were used as positive and negative controls, respectively. Yeast cells were incubated in $DiOC_6$-sensitive dye. The *x*-axis is a logarithmic scale of fluorescence intensity, and the *y*-axis represents the number of cells. A shift of the curve towards the left indicates a decreased mitochondrial membrane potential. Reproduced from Vianna et al. (2001) with permission from the American Physiological Society.

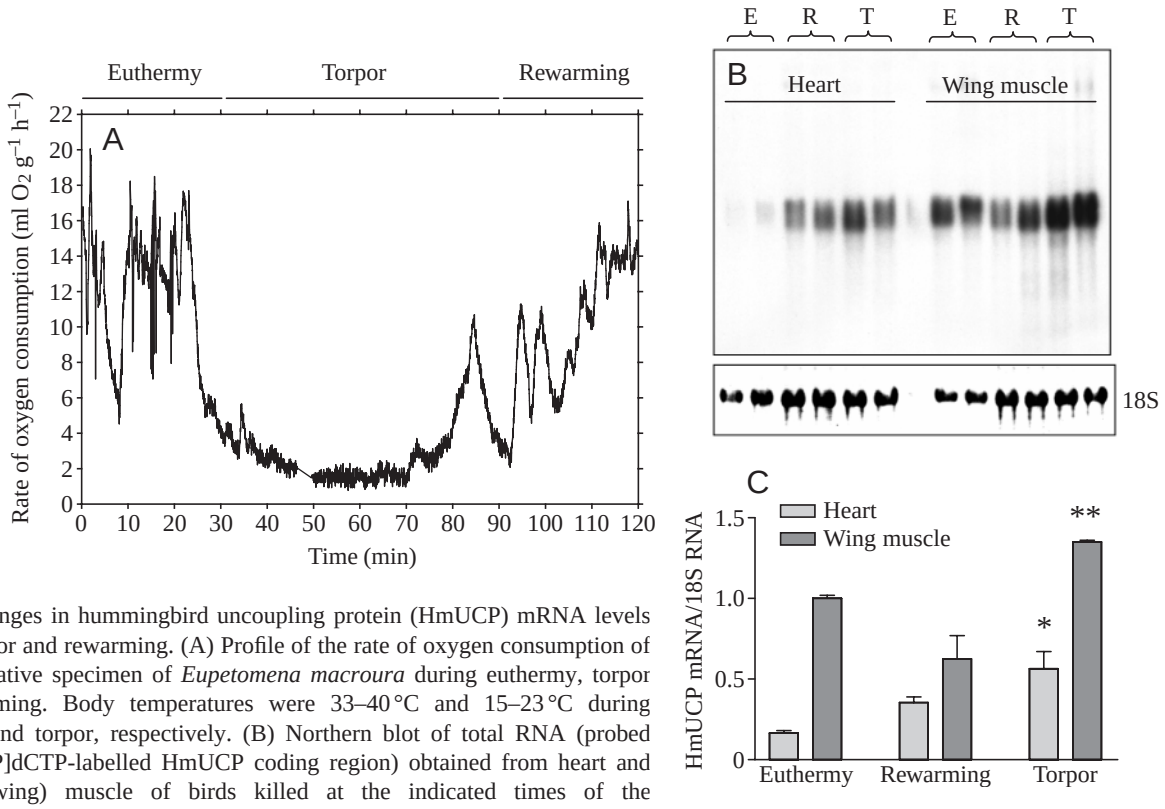

Fig. 3. Changes in hummingbird uncoupling protein (HmUCP) mRNA levels during torpor and rewarming. (A) Profile of the rate of oxygen consumption of a representative specimen of *Eupetomena macroura* during euthermy, torpor and rewarming. Body temperatures were 33–40 °C and 15–23 °C during euthermy and torpor, respectively. (B) Northern blot of total RNA (probed with [α-^{32}P]dCTP-labelled HmUCP coding region) obtained from heart and pectoral (wing) muscle of birds killed at the indicated times of the torpor/rewarming cycle. E, euthermy; T, torpor; R, rewarming. The ribosomal (18S) RNA was stained with Methylene Blue. (C) Ratio between HmUCP mRNA and ribosomal RNA obtained after densitometry. Values are the mean + range. Two animals were used per activity state. **P*<0.05 compared with euthermy (E); ***P*<0.05 compared with rewarming (R) (ANOVA). Reproduced from Vianna et al. (2001) with permission from the American Physiological Society.

Northern blot analysis indicated that, in pectoral muscle and heart, HmUCP mRNA levels changed significantly during the torpor/rewarming process. Accordingly, the heart of torpid animals showed an approximately 3.4-fold increase in HmUCP mRNA levels compared with euthermic animals. During the rewarming phase, the induction of the HmUCP mRNA levels was only 2.2-fold compared with euthermic animals. In the pectoral muscle, a tissue that has fivefold higher levels of HmUCP mRNA than heart, similar, although less marked, changes were detected. It is interesting, however, that both heart and pectoral muscle exhibited their highest levels of HmUCP during torpor. These characteristics show remarkable similarity to the ground squirrel, a hibernating mammal in which UCP2 and UCP3 mRNA levels are also maximal during hibernation (Boyer et al., 1998). Unique to the hummingbird, however, was the approximately 3.4-fold upregulation of HmUCP in the heart. The high level of expression of HmUCP in the heart differs from placental mammals and, given the relatively large size of the heart of hummingbirds (2.0–2.4 % compared with approximately 0.6 % of body mass in mammals), its presence might have physiological relevance. Heart rates recorded for hummingbirds are of the order of 1250 beats min^{-1} (Lasiewski, 1964), which is among the highest heart rates recorded for endotherms. It is conceivable that the high workload and rate of ATP turnover observed in the hummingbird's heart might be associated with elevated HmUCP mRNA levels.

An additional feature that contrasts HmUCP with UCPs of placental mammals is the high level of expression in liver, at levels similar to those in the heart. In placental mammals, this is not the case. UCP2 expression is restricted to Kupffer cells (Larrouy et al., 1997) and occurs only in hepatocytes of mice with fatty liver (Cortez-Pinto et al., 1999). Hummingbird hepatocytes contain approximately 20 % fat compared with normal mouse hepatocytes, which contain approximately 0.2 % (Bicudo, 1996), and Vianna et al. (2001) speculate that fat-induced UCP2 expression in placental mammals is similar to a mechanism also present in birds, linking food availability and uncoupling of H^+ entry from ATP synthesis.

On the basis of the uncoupling activity of UCP2 and UCP3, it has been proposed that, as with UCP1, they too could contribute to adaptive thermogenesis. However, the physiological role of UCP2 and UCP3 is still highly controversial, with several studies supporting or rejecting their thermogenic role (Ricquier and Bouillaud, 2000; Nedergaard et al., 2001). Despite its proton conductance capacity, UCP3 expression in skeletal muscle does not change in response to 48 h of cold exposure, a condition known to increase UCP1 mRNA levels three- to fourfold. Furthermore, UCP2 and UCP3 mRNA levels increase during starvation, a situation known to decrease energy expenditure (Samec et al., 1998). However, the recent finding of increased thermogenesis in transgenic mice overexpressing UCP3

(Clapham et al., 2000) strongly favors a role for UCP3 in adaptive thermogenesis.

Even if the primary role of UCP2 and UCP3 in placental mammals is not adaptive thermogenesis, this might not necessarily be the case for HmUCP. Placental mammals have brown adipose tissue whose temperature can rise 3–5 °C within minutes of adrenergic stimulation (Branco et al., 1999). However, unlike placental mammals, birds do not possess brown adipose tissue or a similarly thermogenic tissue and seem to possess only one UCP1 homolog. The presence of HmUCP mRNA in the pectoral muscle and heart and its upregulation during torpor suggest a thermogenic role during rewarming. The presence of HmUCP in the skeletal muscle may also be relevant for shivering thermogenesis by decreasing the thermodynamic efficiency of mitochondrial ATP synthesis. Shivering-induced ATP breakdown stimulates mitochondrial oxidation and ADP phosphorylation which, in the presence of HmUCP, will dissipate greater amounts of heat.

Concluding remarks

It has been proposed that hummingbirds, besides demonstrating skeletal muscle shivering thermogenesis, may also achieve non-shivering thermogenesis through two different mechanisms. One source of heat is based on Ca^{2+} release from the sarcoplasmic reticulum, mediated by the Ca^{2+}-ATPase, in the presence of a Ca^{2+} gradient. This process is uncoupled from ATP synthesis, and the accumulated energy derived from the Ca^{2+} electrochemical gradient is converted into heat. The second thermogenic source appears to be related to the existence of a novel UCP homolog, termed HmUCP, in the flight muscles of hummingbirds. Although the exact function of this novel UCP homolog is still a matter for further research, the finding that the UCPs of fish and birds and UCP2 and UCP3 of placental mammals are located on a single arm of the phylogenetic tree, when analyzed for genetic distances (Fig. 1), opens the possibility of an early evolutionary origin for UCP2 and UCP3. This scenario suggests that UCP1 was adapted for its specific function in placental mammals, which possess brown adipose tissue, as a later evolutionary event. Given the apparent widespread distribution of UCPs, one might speculate about the existence of a common early ancestral gene for UCPs. However, UCPs may have been the object of selection in different taxa, leading to convergent evolution with respect to thermogenesis.

J.E.P.W.B. was supported by a grant from the Brazilian National Research Council (CNPq).

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