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Cold-acclimation-induced non-shivering thermogenesis in birds is associated with upregulation of avian UCP but not with innate uncoupling or altered ATP efficiency

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SUMMARY

Despite their lack of brown adipose tissue, some bird species develop regulatory non-shivering thermogenesis (NST) of skeletal muscle origin in response to cold acclimation. Mechanisms involved in avian NST are still unclear but may involve reduced energetic coupling in skeletal muscle mitochondria through the expression of an avian homologue of mammalian uncoupling proteins. The aim of this work was to investigate whether the expression of avian uncoupling protein (avUCP) would correlate with the capacity for cold-induced muscle NST. Various levels of cold acclimation were obtained by rearing 1-week-old ducklings (*Cairina moschata*) for 4 weeks at three different ambient temperatures (25°C, 11°C or 4°C). Muscle NST was measured by simultaneous recordings of metabolic rate and electromyographic activity (gastrocnemius muscle) at ambient temperatures (T_a) ranging from 27°C to –5°C. The expression of avUCP gene and mitochondrial bioenergetics were also determined in gastrocnemius muscle. Results showed that muscle NST capacity depends on the T_a at which ducklings were acclimated, i.e. the lower the rearing temperature, the higher the capacity for NST. This increased metabolic heat production occurred in parallel with an upregulation of avUCP, which was not associated with a change in mitochondrial membrane conductance. The intensity of mitochondrial oxidative phosphorylation also increased in proportion with the harshness of cold, while the efficiency of ATP generation was equally effective in all three acclimation temperatures. In the absence of mitochondrial uncoupling, these data indicate a clear link between avUCP expression and the capacity of ducklings to adjust their muscular aerobic activity to cold exposure.

Key words: uncoupling protein, mitochondria, skeletal muscle, cold.

INTRODUCTION

With the exception of some species, which become torpid in the cold, most birds maintain a relatively constant and high body temperature in a wide variety of habitats and over a wide range of environmental temperatures. Such ability to survive in the cold depends on a combination of behavioral, thermal insulation and production of metabolic heat (Chaffee and Roberts, 1971). Although shivering thermogenesis was long thought to be the main thermogenic mechanism in birds, it remains that the survival of some avian species also depends on their ability to develop non-shivering thermogenesis (NST) when chronically exposed to cold (Hissa, 1988; Duchamp et al., 1999).

In the absence of both mammalian thermogenic brown adipose tissue and UCP1 (UCP, uncoupling protein) in birds (Johnston, 1971; Barré et al., 1986), there is some evidence that mitochondrial loose coupling might contribute to cold-induced heat production in skeletal muscle (Skulachev and Maslov, 1960; Duchamp et al., 1992; Roussel et al., 1998), i.e. the major tissue contributing to 70% of the observed NST in cold-acclimated ducklings (Duchamp and Barré, 1993). This mitochondrial mechanism has recently gained molecular support from the identification of a functional homologue of mammalian UCP3 in avian skeletal muscles (Raimbault et al., 2001; Vianna et al., 2001; Talbot et al., 2003). This avian uncoupling protein (avUCP) mRNA expression was found upregulated after long-term cold exposure in birds (Raimbault et al., 2001; Toyomizu et al., 2002; Collin et al., 2003a), and its activity was increased after cold-water adaptation in penguins (Talbot et al., 2004). By contrast, the levels of avUCP mRNA remained at baseline following short-term cold exposure (Raimbault et al., 2001; Ueda et al., 2005; Mujahid et al., 2005), when shivering thermogenesis is the main thermogenic mechanism. Interestingly, two independent studies provided data, although scarce, suggesting that the expression of avUCP may depend upon the intensity of the cold temperature at which birds were reared (Collin et al., 2003a; Ueda et al., 2005). However, a metabolic analysis at the bird level and at the mitochondrial level was not reported in those studies.

Therefore, the question remains whether the cold-induced upregulation of the avUCP gene in skeletal muscles correlates with the capacity of NST in cold-acclimated birds. In order to generate such data, we acclimated male muscovy ducklings to different cold ambient temperatures (T_a). Then, we measured NST capacity in whole birds, as well as avUCP gene expression and mitochondrial ATP efficiency and membrane potential in skeletal muscle.

MATERIALS AND METHODS

Animals

Male muscovy ducklings (*Cairina moschata* Linnaeus, pedigree R31, Institut National de la Recherche Agronomique, Paris, France) were obtained from a commercial stockbreeder (Eclosion Grimaud-la-corbière, Roussay, France). Birds were fed *ad libitum* with commercial mash (Moulin Guenard, 645000MI, Vonnas, France) and had free access to water. From the age of 10 days, ducklings were caged for

a period of 26 days at either 4°C, 11°C or 25°C [thermoneutral (TN) temperature] in a constant photoperiod (light:dark 8 h:16 h). Body mass was recorded daily, between 09.30 h and 11.30 h. 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.

Metabolic rate

Metabolic rate (MR) was measured by indirect calorimetry. We used an open-circuit system as already used in ducklings (Barré et al., 1985). Ducklings were fasted overnight prior to being positioned in a thermostatic chamber ventilated by a constant atmospheric airflow (0.05 m s^{-1}). Variable heat loss by conduction on the ground was minimized by a polypropylene bed. T_a was controlled and measured with copper-constantan thermocouples inside the thermostatic chamber. Air flow rates (14 l min^{-1}) were measured using a Platon volumeter (Domont, France), and converted to standard values [standard temperature and pressure, dry (STPD)]. The fractional concentrations of oxygen were measured using a Servomex 1100 paramagnetic gas analyzer (Taylor Instrument Analytics Ltd, Sussex, UK). Carbon dioxide concentrations were measured using a Servomex 1400 infrared gas analyzer. The O_2 analyzer was calibrated with pure nitrogen gas and atmospheric air assuming an oxygen content of 20.93%. The CO_2 analyzer was calibrated with pure nitrogen gas and a known mixture of 0.502% CO_2. The rates of O_2 consumption and CO_2 production were calculated according to the equation of Depocas and Hart (Depocas and Hart, 1957). The caloric equivalent for O_2 was determined from the respiratory quotient using Lusk tables (Lusk, 1924).

Shivering

Shivering was measured as integrated electromyogram (EMG) activity of the gastrocnemius leg muscle. EMG was recorded using three monopolar electrodes insulated except for the tips (Stabilohm 110, Ni 80% and Cr 20%, 0.12 mm diameter) acutely inserted into the muscle 10 mm apart. The EMG signal was collected by an acquisition interface (MP30B-CE, Biopac System Inc., Santa Barbara, CA, USA) and integrated with Biopac Student Lab Pro v.3.6.7 software (Santa Barbara, CA, USA). Measurement of the integrated signal value was made at rest over long periods of 15 min. Portions of those integrated recordings in which artifacts were observed, which were due to the occasional movements of ducklings, were eliminated by simultaneous monitoring of the bird using a webcam.

Non-shivering thermogenesis

NST was assessed by the simultaneous measurement of MR and EMG at constant T_a ranging from 27°C to –5°C. MR and EMG were determined in overnight-fasted ducklings over 30 min that followed an initial 60 min adjustment period to obtain metabolic steady state and thermal equilibrium at a given T_a. From these relationships, a lower critical temperature (LCT) and a shivering threshold temperature (STT) were determined as the T_a below which the MR and the muscle electrical activity elicited a significant increase, respectively. The NST is given by the MR in the cold, not yet accompanied by shivering activity. Hence, the activity of NST was calculated as the level of MR determined at the STT temperature minus the resting metabolic rate (RMR).

Mitochondrial isolation

Ducklings were fasted overnight prior to being killed by decapitation. Skeletal muscle mitochondria were isolated in an ice-cold isolation buffer containing 100 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 50 mmol l^{-1} Tris-base, and pH 7.4 at 4°C (Roussel et al., 2000). Briefly, gastrocnemius muscle was rapidly dissected and cut up finely with sharp scissors and diluted 1:10 (w/vol.) in isolation medium. The minced tissue was homogenized with a potter-Elvehjem homogenizer (five passages) and centrifuged at 800 ***g*** for 10 min. The pellet was suspended in 40 ml of isolation buffer and was treated with protease (1 mg g^{-1} muscle wet mass) for 5 min in an ice bath. The muscle mixture was diluted 1:2, homogenized with a potter-Elvehjem homogenizer (three passages) and centrifuged at 1000 ***g*** for 10 min. The supernatant containing muscle mitochondria was collected, filtered through cheesecloth and centrifuged at 8700 ***g*** for 10 min. The resulting pellet was washed twice by suspension in the isolation buffer and centrifuged at 8700 ***g*** for 10 min. All steps were carried out at 4°C. The protein concentration of mitochondrial suspensions was determined by a biuret method with bovine serum albumin as standard.

Mitochondrial respiratory parameters and membrane potential

Oxygen consumption was measured with a Clark oxygen electrode (Rank Brothers Ltd, Bottisham, UK), in a closed and stirred glass cell of 1 ml volume, thermostated at 38°C. While the body temperature of ducklings is 40°C (Barré et al., 1985), we made the choice to perform all of the respiration measurements at 38°C in order to generate data comparable with those recently reported in other avian species, especially penguins (Talbot et al., 2003; Talbot et al., 2004; Rey et al., 2008) and chickens (Ueda et al., 2005; Toyomizu et al., 2002). Muscle mitochondria (0.3 mg protein ml^{-1}) were incubated in a respiratory buffer containing 120 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} EGTA, 2 mmol l^{-1} $MgCl_2$, 0.3% bovine serum albumin (w/vol.) and 3 mmol l^{-1} Hepes, pH 7.4. Substrate concentrations were 5 mmol l^{-1} succinate plus 5 μmol l^{-1} rotenone. The active state of respiration (state 3) was initiated by the addition of 500 μmol l^{-1} ADP. The basal non-phosphorylating respiration rate (state 4oligo) was obtained by the addition of 2 μg ml^{-1} of oligomycin. The uncoupled state of respiration (state 3unc) was initiated by the addition of 2 μmol l^{-1} carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Thereafter, myxothiazol (3 μmol l^{-1}) was added to fully inhibit succinate-supported respiration. Then, ascorbate (2 mmol l^{-1}) and N,N,N′,N′-tetramethyl-p-phenylenediamine (500 μmol l^{-1}) were added and the maximal respiration rate associated with isolated cytochrome *c* oxidase activity recorded. The respiratory control ratio (RCR) refers to the ratio of oxygen consumed after adding ADP to that consumed in the presence of oligomycin.

Membrane potential was measured using an electrode sensitive to the potential-dependent probe triphenylmethylphosphonium ($TPMP^+$). Mitochondria (0.3 mg ml^{-1}) were suspended in 2 ml respiratory buffer supplemented with rotenone (5 μmol l^{-1}), oligomycin (2 μg ml^{-1}) and nigericin (60 ng ml^{-1}). The electrode was calibrated by sequential 0.5 μmol l^{-1} additions up to 2 μmol l^{-1} $TPMP^+$, then 5 mmol l^{-1} succinate was added to start the reaction. 2 μmol l^{-1} FCCP was added at the end of the measurement to dissipate the membrane potential and release all TPMP back into the medium for baseline correction. Membrane potentials were calculated as previously described by Brand (Brand, 1995), assuming a TPMP binding correction of 0.35 mg of protein per μl for skeletal muscle mitochondria (Rolfe et al., 1994).

Mitochondrial ATP synthesis and oxidative phosphorylation efficiency

Oxygen consumption and ATP synthesis rates were performed at 38°C in 1.5 ml respiratory buffer supplemented with glucose

($20\,mmol\,l^{-1}$), hexokinase ($1.5\,U\,ml^{-1}$), rotenone ($5\,\mu mol\,l^{-1}$) and succinate ($5\,mmol\,l^{-1}$). The mitochondrial ATP synthesis was initiated by the addition of $500\,\mu mol\,l^{-1}$, $20\,\mu mol\,l^{-1}$ or $5\,\mu mol\,l^{-1}$ ADP. After recording the phosphorylating respiration rate (state 3) for 2 min, four 300 µl samples of mitochondrial suspension were withdrawn from the suspension every 30 s and were quenched in a perchloric acid solution consisting of 10% $HClO_4$ and $25\,mmol\,l^{-1}$ EDTA. After centrifugation of the denatured protein (15,000 ***g*** for 6 min) and neutralization of the resulting supernatant, the ATP production was determined from the glucose-6-phosphate content of samples, which was assayed spectrophotometrically by monitoring the production of NADH in the presence of glucose-6-phosphate dehydrogenase at 340 nm (Lang and Michal, 1974). Briefly, the supernatant was incubated in 1 ml of a reaction buffer consisting of NAD ($0.5\,mmol\,l^{-1}$), triethanolamine-HCl ($50\,mmol\,l^{-1}$), $MgCl_2$ ($7.5\,mmol\,l^{-1}$), EDTA ($3.75\,mmol\,l^{-1}$), pH 7.4. The concentration of glucose-6-phosphate was calculated from the difference between the level of NADH measured before and 1 h after the addition of glucose-6-phosphate dehydrogenase (0.5 U). The rate of mitochondrial ATP production was calculated from the slope of the linear accumulation of glucose-6-phosphate over the sampling time interval (1.5 min). The linearity of glucose-6-phosphate accumulation allowed us to check that the system was in a steady state.

It is important to note that we made sure that the ATP production rates were specific of the mitochondrial ATP synthase activity, by determining oxygen consumption and ATP synthesis rates in the presence of oligomycin ($2\,\mu g\,ml^{-1}$). Over the range of ADP concentrations used, a non-mitochondrial ATP synthesis activity was measurable at $500\,\mu mol\,l^{-1}$ of ADP (TN=57±6 nmol ATP $min^{-1}\,mg^{-1}$ protein; CA11=68±4 nmol ATP $min^{-1}\,mg^{-1}$ protein; CA4=76±2 nmol ATP $min^{-1}\,mg^{-1}$ protein). Even though, this oligomycin-insensitive ATP synthesis production, presumably associated with adenylate kinase activity, was significantly higher in CA4 ducklings than in TN birds ($P<0.05$), it represented approximately 10% of total ATP production in each experimental group. These values were taken into account to calculate the rate of mitochondrial ATP synthesis.

PCR analysis

Total RNA were extracted from muscle samples (70 mg) using Tri Reagent (Euromedex, Sauffelweyersheim, France). Concentration and purity were checked by measuring optimal density at 260 nm and 280 nm. mRNA relative abundance was determined by semi-quantitative reverse transcription polymerase chain reaction using β-actin as an internal standard. For each sample, a RT was performed from 1 µg of total RNA with 100 U of M-MLV Reverse Transcriptase (Promega, Charbonnières, France), 5 µl of M-MLV RT 5× buffer, 20 U of RNasin Ribonuclease Inhibitor, 12 pmol of deoxynucleoside triphosphate and 1 µg of oligo dT, in a final volume of 25 µl. After chilling, 2.5 µl was used for PCR reactions by adding 47.5 µl of PCR mix containing 5 µl 10× EurobioTaq PCR buffer (Lesulis, France), 6 pmol $MgCl_2$, 8 pmol deoxynucleosides triphosphate, 2.5 U Taq Platinium (InVitrogen, Cergy-Pontoise, France), 22.5 pmol of corresponding antisense (3′-ATG AAC ATC ACC ACG TTC CA-5′ for avUCP gene and 3′-GGG TGT TGA AGG TCT CAA ACA-5′ for β-actin gene) and sense primers (5′-GTG GAT GCC TAC AGG ACC AT-3′ for avUCP gene and 5′-GAC GAG GCC CAG AGC AAG AGA-3′ for β-actin gene). The PCR conditions were: 2 min at 94°C followed by 26 and 20 cycles for avUCP and β-actin, respectively (1 cycle=1 min at 94°C, 1 min at 60°C, 1 min at 72°C). These cycle numbers were chosen after we performed preliminary tests in which a range of cycles (25, 27, 29 and 31 cycles for avUCP and 17, 19, 20, 21 and 23 cycles for β-actin) had confirmed that these conditions were in the exponential phase of PCR and that there was no saturation limitation. PCR was ended by 10 min at 72°C. avUCP and β-actin products of the three experimental conditions were simultaneously separated on 2% agarose gel prestained with ethidium bromide. Relative bands intensities and the ratio of each target to β-actin were determined with Kodak Digital Science 1D 2.0 software (Kodak Scientific Imaging System, New Haven, CT, USA).

Statistical analysis

The relationships between integrated EMG activity and T_a, or MR and T_a were expressed by two linear regression lines (Scholander et al., 1950; Barré et al., 1985) that intersect at the STT or at the LCT, respectively. To draw these regression lines, we statistically determined by a paired analysis of variance (ANOVA) test at which T_a EMG activity or MR became significantly different from basal values, respectively. These values and those measured at lower T_a were then integrated in a second linear regression line distinct from the basal linear regression line.

Values are means ± s.e.m. When not detailed, the statistical significance of observed variations was assessed using one-way ANOVA. Differences between means were subsequently tested by Sheffe's test.

RESULTS

Body mass and growth rate

Ducklings were weighed every day in order to determine the effect of cold exposure on their growth rate. Long-term cold exposure slowed the growth rate of ducklings (Fig. 1) despite an increased food intake in the cold (Benistant et al., 1998; Chaînier et al., 2000). From day 17, body mass of cold-acclimated (CA) ducklings was significantly less ($P<0.05$) than that of TN ducklings, and the difference between CA and TN ducklings increased with increasing age. Thus, after 26 days of cold exposure at 11°C (CA11) or 4°C (CA4) body mass was significantly lowered by –15% and –38%, respectively, as compared with corresponding control birds.

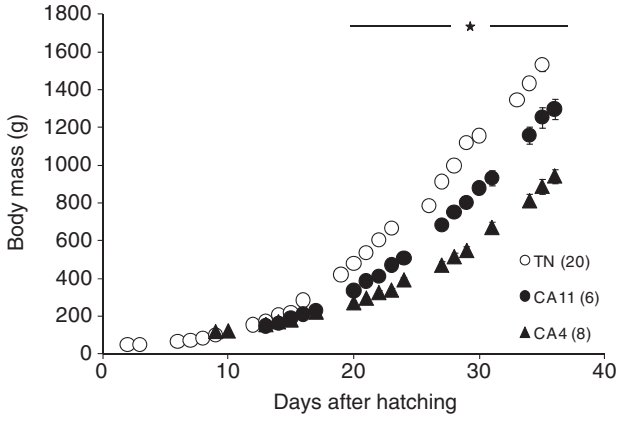

Fig. 1. Effect of cold acclimation on growth rate in ducklings. From the age of 10 days, thermoneutral control ducklings (TN, open symbols) were reared at 25°C, while cold-acclimated (CA) birds (filled symbols) were either reared at 11°C (filled circles) or at 4°C (filled triangles). Values are means ± s.e.m. for (*N*) ducklings per group as specified in the graph. *$P<0.01$, body mass was significantly different between the three groups. When not visible, bar errors are within symbols.

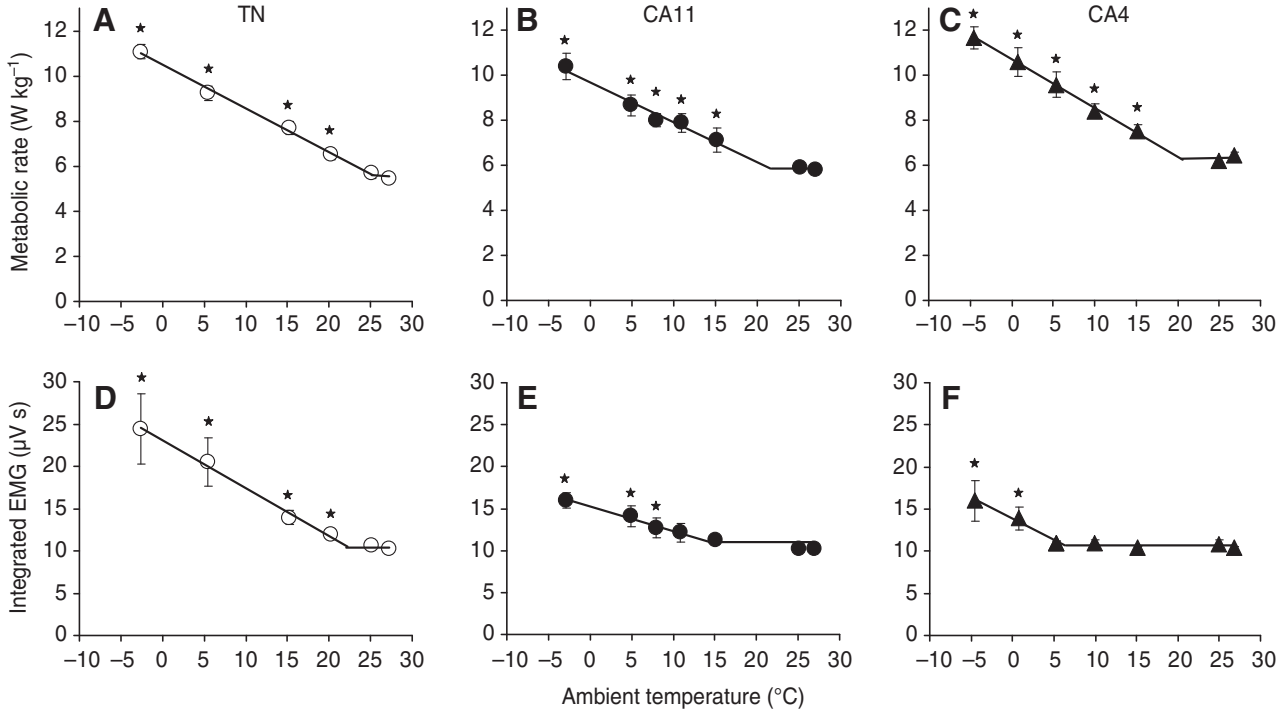

Fig. 2. The relationship between whole-animal metabolic rate (MR; W kg^{-1}) or integrated muscle electrical activity (EMG; μV s) and ambient temperature (T_a; °C) in thermoneutral control ducklings (TN; open symbols) and in birds that were cold acclimated at 11°C (CA11; filled circles) or 4°C (CA4; filled triangles). Regression lines for MR *versus* T_a in: (A) TN ducklings in cold T_a y=–0.19x+10.48 (R^2=0.99) and in TN zone y=5.57 intersect at 25.3°C [the lower critical temperature (LCT)]; (B) CA11 ducklings in cold T_a y=–0.18x+9.72 (R^2=0.97) and in TN zone y=5.85 intersect at 22.4°C; and (C) CA4 ducklings in cold T_a y=–0.22x+10.72 (R^2=0.99) and in TN zone y=6.33 intersect at 19.5°C. Regression lines for integrated electromyograms (EMG) activity *versus* T_a in: (D) TN ducklings in cold T_a y=–0.06x+2.31 (R^2=0.99) intersect basal level line (y=1.00) at 22.9°C [the shivering threshold temperature (STT)]; (E) CA11 ducklings in cold T_a y=–0.03x+1.52 (R^2=0.97) intersect basal level line (y=1.10) at 13.0°C; and (F) CA4 ducklings in cold T_a y=–0.05x+1.39 (R^2=0.98) intersect basal level line (y=1.08) at 6.0°C. Values are means ± s.e.m. of 5–8 ducklings per group. *P<0.05 significantly different from values measured in the TN zone (T_a=27°C). The magnitude of non-shivering thermogenesis (NST) is here given by the level of MR in the cold, not yet accompanied by an increase in muscle electrical activity due to shivering. Hence, the NST capacity is the MR calculated at the STT temperature minus the resting metabolic rate.

Metabolic rate

The relationship between MR and T_a was expressed by two linear regression lines that intersect at the LCT according to Scholander et al. (Scholander et al., 1950). Below this critical temperature there was a significant linear increase in MR as T_a decreased (Fig. 2A–C). The LCT was significantly decreased in CA4 ducklings as compared with TN birds, and was slightly, but not significantly, lowered (P=0.3) in CA11 birds (Table 1). In all three experimental groups, MR remained constant over T_a ranging from 25°C to 27°C. The RMR was calculated within this temperature range, considered to be in the TN zone. RMR was higher in cold-acclimated ducklings (CA11=8.2±0.3 W, CA4=7.9±0.5 W, P<0.05) than in TN (6.1±0.4 W) ducklings. When body mass differences were taken into account, the RMR remained significantly higher in CA4 ducklings (Table 1). Note that respiratory quotient values were not significantly different between groups (TN=0.75±0.02; CA11=0.76±0.02; CA4=0.77±0.01).

Shivering

Integrated EMG activity showed a continuous basal activity of 10–11 μV s in all three duckling groups. EMG activity was significantly increased in the cold from an T_a of 20°C in TN ducklings, 8°C in CA11 ducklings and 0°C in CA4 ducklings (Fig. 2D–F). The STT are reported in Table 1. In both cold-acclimated groups, the STT was significantly lower than the LCT

Table 1. Effect of cold acclimation upon critical temperatures, resting metabolic rate and non-shivering thermogenic capacity

	Control ducklings Reared at 25°C (N=8)	Cold-acclimated (CA) ducklings	
		Reared at 11°C (N=5)	Reared at 4°C (N=6)
LCT (°C)	25.3±0.8	22.4±2.5	19.5±1.2*
STT (°C)	22.9±1.6	13.0±1.7*,‡	6.0±1.4*,†,‡
RMR (W kg^{-1})	5.6±0.2	5.8±0.3	6.3±0.1*
NST (W kg^{-1})	0.7±0.2	1.7±0.5*	2.8±0.2*,†

Values are means ± s.e.m. The number of independent determinations is given in parentheses. The lower critical temperature (LCT), the shivering threshold temperature (STT), the resting metabolic rate (RMR) and the capacity for non-shivering thermogenesis (NST) were measured as described in the Materials and methods. *P<0.05, significantly different from thermoneutral (TN); †P<0.05, significantly different from CA11; ‡P<0.05, significantly different from LCT within the same group.

(Table 1), indicating that within this range of T_a, regulatory thermogenesis was independent of shivering. Table 1 clearly showed that the capacity for NST was dependent on the harshness of cold, i.e. the lower the rearing ambient temperature, the higher the capacity for NST. At the shivering threshold, ducklings acclimated at 11°C or at 4°C displayed a capacity for NST that amounted to 29% and 44% above the RMR, respectively.

avUCP expression

As shown in Fig. 3, the relative abundance of avUCP transcript in gastrocnemius muscle was significantly higher in cold-acclimated groups (CA11, +56%, $P<0.05$, and CA4, +95%, $P<0.05$) than in the TN control group.

Mitochondrial metabolism

Table 2 reports respiratory characteristics of skeletal muscle mitochondria isolated from control or cold-acclimated ducklings. Rates of oxygen consumption during maximal ADP-stimulated (state 3), resting (state 4oligo) and FCCP-induced uncoupled (state 3unc) states were significantly increased by 37% on average in CA4 ducklings. Interestingly, these rates of oxygen consumption were less affected in CA11 birds. Indeed, FCCP-induced maximal oxygen consumption rate was 22% higher in CA11 ducklings than in TN birds whereas a slight but not statistically significant 15% increase ($P=0.06$) was noted in state 3 respiration. Finally, the state 3-to-state 4oligo ratio (TN control=5.9±0.5; CA11=6.3±0.3; CA4=5.9±0.5) and the maximal oxygen consumption of mitochondrial cytochrome *c* oxidase were not significantly altered by cold acclimation (Table 2).

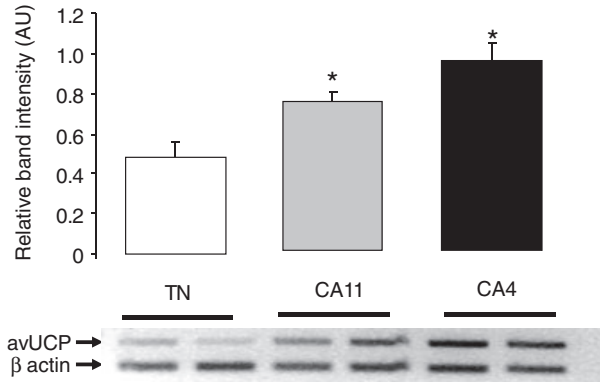

Fig. 3. Relative abundance of avian uncoupling protein (avUCP) mRNA in gastrocnemius muscle from thermoneutral (TN) control ducklings (white bar), and birds that were cold acclimated at 11°C (CA11) (gray bar) or 4°C (CA4) (black bar). Transcript levels were standardized with mRNA level of β-actin gene. Values are expressed in arbitrary units (AU) as means ± s.e.m. for four independent skeletal muscle preparations. A representative gel is shown. *$P<0.05$ compared with TN controls.

In non-phosphorylating conditions, the mitochondrial membrane potential was significantly higher in CA4 ducklings (207±4 mV, $P<0.05$) than in TN control (193±4 mV) or CA11 (195±2 mV) birds. This result indicates that the higher rate of resting oxygen consumption (state 4oligo) observed in CA4 ducklings (Table 2) was mainly due to an increase in the oxidation of succinate rather than a change in inner membrane proton conductance.

The relationships between the rates of ATP synthesis and oxygen consumption were linear in muscle mitochondria working at different steady-state rates of ATP production, regardless of experimental group (Fig. 4). In accordance with the respiratory parameters reported above, maximal rates of ATP synthesis and oxygen consumption, the highest points to the right of the linear relationships, tended to be higher in CA11 ducklings (+22% and +14%, respectively), and significantly ($P<0.05$) increased in CA4 ducklings (+55% and +41%, respectively) when compared with TN birds. However, the mitochondrial oxidative phosphorylation efficiency (ATP/O) calculated from the ratio between the ATP synthesized and the oxygen consumed during state 3, in the presence of 500 μmol l^{-1} ADP (arrows in Fig. 4) were not significantly different between groups, displaying a mean value of 1.31±0.03. This is truly underlined by the fact that the linear relationships concerning both cold-acclimated groups were superimposed to that of the TN group, showing that 26 days of cold exposure did not alter the thermodynamic efficiency of mitochondrial ATP synthesis. Hence, at any given rate of oxygen uptake, between maximal phosphorylating respiration (state 3) and basal non-phosphorylating respiration (state 4), ATP synthesis rate was the same in cold-acclimated and TN ducklings.

DISCUSSION

The current work clearly indicates that the capacity for NST depends on the T_a at which the ducklings were acclimated. This increase in metabolic heat production was paralleled by an upregulation of avUCP, linking the expression of skeletal muscle avUCP with the capacity for non-shivering heat production. In support of this data, two independent studies have previously reported that after 7 days, avUCP mRNA levels were increased by 20% or 60% in chickens reared at 20°C (Collin et al., 2003a) or at 4°C (Ueda et al., 2005). In the former study, cold-induced over-expression of avUCP in skeletal muscle was associated with a concomitant increase in mass-specific MR of the birds. In addition,

Table 2. Respiratory parameters of skeletal muscle mitochondria respiring on succinate

Mitochondrial oxygen consumption	Control ducklings Reared at 25°C	Cold-acclimated (CA) ducklings	
		Reared at 11°C	Reared at 4°C
State 3	359±26	414±24	495±32*
State 4oligo	62±3	66±2	86±5*,†
State 3unc	378±22	459±24*	502±32*
Cytochrome *c* oxidase	1010±84	1153±59	1189±107

Rates of oxygen consumption are expressed in nmol O min^{-1} mg^{-1} protein and were measured in isolated muscle mitochondria in the presence of 5 mmol l^{-1} succinate plus 5 μmol l^{-1} rotenone as described in the Materials and methods. State 3, ADP-stimulated respiration measured in the presence of 500 μmol l^{-1} ADP; state 4oligo, basal non-phosphorylating respiration measured in the presence of 2 μg ml^{-1} oligomycin; state 3unc, FCCP-induced maximal respiration measured in the presence of 2 μmol l^{-1}, cytochrome *c* oxidase, oxygen consumption measured with 2 mmol l^{-1} ascorbate/0.5 mmol l^{-1} TMPD in the presence of oligomycin, 3 μmol l^{-1} myxothiazol and FCCP. Values are means ± s.e.m. for six animals per group. *$P<0.05$ significantly different from control conditions. †$P<0.05$ significantly different from 11°C cold-acclimated ducklings.

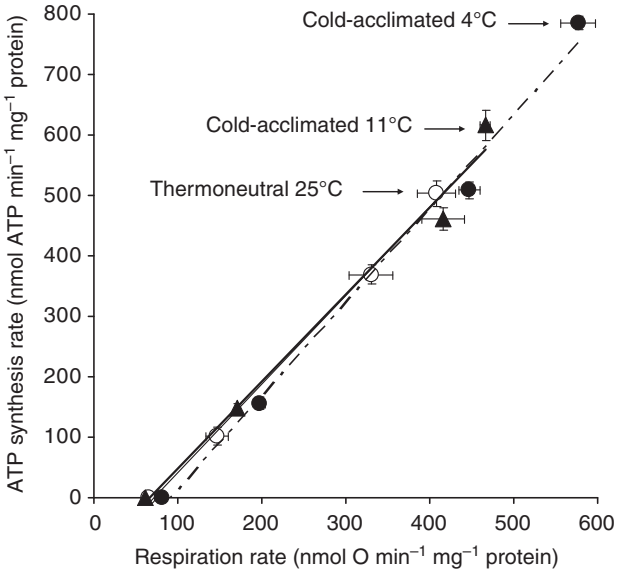

Fig. 4. Effect of cold acclimation on oxidative phosphorylation efficiency. The skeletal muscle mitochondria from thermoneutral (TN) control (open symbols) or cold-acclimated (CA) ducklings (closed symbols) were incubated in respiratory buffer supplemented with 20 mmol l^{-1} glucose, 1.5 U ml^{-1} hexokinase, 5 mmol l^{-1} succinate and 5 μmol l^{-1} rotenone as described in the Materials and methods. Oxygen consumption and ATP synthesis rates were measured in the presence of increasing concentrations of ADP (from 0, 5, 20 and 500 μmol l^{-1}). Values are means ± s.e.m. of six independent preparations.

the expression of avUCP was also upregulated in chronically glucagon-treated ducklings (Raimbault et al., 2001), an avian model showing increased NST (Barré et al., 1987). Altogether, these results would sustain the early hypothesis regarding the function of avUCP that this protein is involved in the regulation of adaptive muscle thermogenesis (Raimbault et al., 2001).

avUCP belongs to the UCP class of the mitochondrial transporter family, in which UCP1, the prime UCP discovered some 30 years ago, is clearly involved in mammalian brown adipose tissue NST. As its homologous mammalian UCP (Echtay et al., 2002), avUCP increases mitochondrial inner membrane conductance when activated in native mitochondria (Talbot et al., 2003) or when ectopically expressed in yeast (Vianna et al., 2001; Criscuolo et al., 2005). This uncoupling activity suggests that avUCP may have a physiological thermogenic activity through the regulation of the efficiency of mitochondrial oxidative phosphorylation. The oxidative phosphorylation represents the biochemical process that couples the transfer of electrons from substrates to oxygen with the production of ATP, i.e. the main form of useable energy that organisms can allocate to fitness-related entities including maintenance, growth, reproduction, foraging and ultimately survival. The energy released when electrons are passed down the respiratory chain is used to pump protons across the membrane, creating an electrochemical proton gradient that is then used by ATP synthase to synthesize ATP. By increasing membrane proton conductance, avUCP should decrease the efficiency of the oxidative phosphorylation process, thereby diverting the energy from ATP synthesis to thermogenesis. In line with this idea, avUCP level increased in a situation where energy efficiency is decreased such as in triiodothyronine-treated chickens (Collin et al., 2003b) whereas it was reported to be decreased in hypothyroid chickens (Collin et al., 2003b) or in long-term fasted penguins (Rey et al., 2008) when energy efficiency would increase. Interestingly, a chicken line selected for its low efficiency of food utilization for growth displayed a higher level of avUCP mRNA in their skeletal muscle than a chicken line divergently selected for its high food efficiency (Raimbault et al., 2001; Ojano-Dirain et al., 2007).

However, there are some arguments raising some doubts about such physiological thermogenic functions of avUCP. From a molecular point of view it appears that by virtue of its high sequence homology (~70%) and its mRNA expression in skeletal muscles (Raimbault et al., 2001; Vianna et al., 2001; Talbot et al., 2003), avUCP closely resembles mammalian UCP3, the biological function of which is clearly not heat production. From a cellular point of view, heterologous expression of UCP3 in yeast can induce a mitochondrial uncoupling phenotype that is mainly an artifact, being neither inhibited by nucleotides nor activated by the known activators fatty acids and superoxide (Harper et al., 2002). In this condition, uncoupling data reported in yeast expressing avUCP might be interpreted with caution (Vianna et al., 2001; Criscuolo et al., 2005). From a biochemical point of view, avUCP clearly displays a purine nucleotide-sensitive uncoupling activity in non-phosphorylating mitochondria (Talbot et al., 2003). However, like the mammalian UCPs (Echtay et al., 2002), avUCP requires activation by superoxide before its proton conductance can be seen (Talbot et al., 2003), which suggests that avUCP would not be activated in phosphorylating mitochondria, when ATP is synthesized and superoxide production is largely decreased. This is in accordance with previous results showing an increased ATP production in fully phosphorylating active skeletal muscle mitochondria (in the presence of high concentration of adenine nucleotides) isolated from cold-acclimated ducklings (Roussel et al., 1998). Nonetheless, it is obvious that the proton leak reactions and the ATP synthase compete for the same driving force. Hence, the question arises whether the avUCP-induced proton conductance would significantly increase under submaximal activity of mitochondria, when flux through the ATP synthase decreases. To test this hypothesis, we measured oxygen consumption and ATP synthesis at submaximal oxidative phosphorylation rates. In our experimental conditions, we found no alteration of the energetic efficiency of skeletal muscle mitochondria (Fig. 4). Hence, avUCP may not be involved in heat production through mitochondrial uncoupling, like the mammalian UCP1 in brown adipose tissue. Nevertheless, histochemical arguments have previously suggested that muscle mitochondria were loose-coupled *in situ* in all fibers of cold-acclimated ducklings (Duchamp et al., 1992). Therefore, we can not rule out that intracellular components (pH, fatty acids, reactive oxygen species) might change during cold exposure and ultimately activate the avUCP within cells. This hypothesis will require further investigation.

The present study shows that the intensity of mitochondrial oxidative phosphorylation rate increased in proportion with the harshness of cold at which ducklings were exposed, while the coupling efficiency and the innate membrane conductance of mitochondria remained unaffected. Hence, skeletal muscle NST would rather be sustained either by increasing ATP breakdown through the futile cycling of calcium across the sarcoplasmic reticulum membrane, for example (Dumonteil et al., 1995), or by decreasing mitochondrial ATP efficiency through the oxidation of $FADH_2$-linked substrates such as fatty acyl-carnitine (Clerc et al., 2007). The latter hypothesis would link avUCP gene expression with the mobilization of fat reserves and the increased potential of skeletal muscle to oxidize fatty acid when birds are exposed to low temperatures (Barré et al., 1986; Benistant et al., 1998; Bedu et al., 2002). Further insight, avUCP can also have a role in the control of radical oxidative species production (Abe et al., 2006; Rey et

al., 2010b) when skeletal muscle mitochondria oxidize fatty acids (St Pierre et al., 2002; Seifert et al., 2008). In recent years, increasing evidence suggests that avUCP seems to be related to oxidative stress, which triggers the mitochondrial activity (Talbot et al., 2003; Talbot et al., 2004) and possibly the gene expression of avUCP in skeletal muscle (Rey et al., 2010a).

In conclusion, the present study reports that avUCP gene expression correlates with the capacity of skeletal muscle for NST. In the absence of mitochondrial loose coupling, avUCP is broadly related to an increased capacity in mitochondrial oxidative phosphorylation (Walter and Seebacher, 2009). Whether avUCP plays a role in heat production through mitochondrial loose coupling when mitochondria produced ATP *in situ* within muscle cells remains unclear and still awaits clear demonstration.

LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	analysis of variance
avUCP	avian uncoupling protein
CA4	cold-acclimated at 4°C
CA11	cold-acclimated at 11°C
EMG	electromyogram
LCT	lower critical temperature
MR	metabolic rate
NST	non-shivering thermogenesis
RMR	resting metabolic rate
STT	shivering threshold temperature
T_a	ambient temperature

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