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# Endothermy in birds: underlying molecular mechanisms

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## SUMMARY

**Endothermy is significant in vertebrate evolution because it changes the relations between animals and their environment. How endothermy has evolved in archosaurs (birds, crocodiles and dinosaurs) is controversial especially because birds do not possess brown adipose tissue, the specialized endothermic tissue of mammals. Internal heat production is facilitated by increased oxidative metabolic capacity, accompanied by the uncoupling of aerobic metabolism from energy (ATP) production. Here we show that the transition from an ectothermic to an endothermic metabolic state in developing chicken embryos occurs by the interaction between increased basal ATP demand ($Na^+/K^+$-ATPase activity and gene expression), increased oxidative capacity and increased uncoupling of mitochondria; this process is controlled by thyroid hormone *via* its effect on PGC1$\alpha$ and adenine nucleotide translocase (ANT) gene expression. Mitochondria become more uncoupled during development, but unlike in mammals, avian uncoupling protein (avUCP) does not uncouple electron transport from oxidative phosphorylation and therefore plays no role in heat production. Instead, ANT is the principal uncoupling protein in birds. The relationship between oxidative capacity and uncoupling indicates that there is a continuum of phenotypes that fall between the extremes of selection for increased heat production and increased aerobic activity, whereas increased cellular ATP demand is a prerequisite for increased oxidative capacity.**

Key words: thermoregulation, mitochondria, uncoupling proteins, PGC1$\alpha$, heat production, $Na^+/K^+$-ATPase, thyroid hormone.

## INTRODUCTION

The evolution of endothermy is one of the most significant events in vertebrate evolution because it fundamentally changes the energetic relations between animals and their environment. In contrast to mammals (Kemp, 2006), the origin and mechanisms that led to endothermy in the Archosauria is controversial and one of the most hotly debated topics in vertebrate evolution (Gillooly et al., 2006; Seebacher, 2003; Seymour et al., 2004). At present there is no agreement even about whether ancestral archosaurs were endotherms or ectotherms (Ruben, 1995; Seymour et al., 2004), although there is little doubt that ectothermy is ancestral to endothermy (Hillenius and Ruben, 2004). Metabolic processes are highly conserved among animals (Smith and Morowitz, 2004) so that the transition from ectothermy to endothermy is likely to involve quantitative changes in pathways rather than *de novo* structures or processes. An exception is brown adipose tissue (BAT), which is present in mammals but not in birds (Cannon and Nedergaard, 2004). Brown adipose tissue is specialized to produced heat, and it is the main site for adaptive thermogenesis. The lack of brown adipose tissue in archosaurs raises the question of what the molecular processes are that lead from an ectothermic to an endothermic metabolic state.

Endothermy in birds and mammals is characterized by metabolic rates that are five to ten times higher compared with ectotherms (Bennett and Ruben, 1979; Else and Hulbert, 1981; Hemmingsen, 1960). Mitochondrial uncoupling and proton leak across the inner mitochondrial membrane determine the proportion of energy that is released as heat (Brand et al., 1991; Porter et al., 1996). Hence, increased leakiness and uncoupling of mitochondrial membranes mean that the supply of ATP for energy consuming cellular processes will also lead to heat production (Brand et al., 1991; Else and Hulbert, 1987; Hulbert and Else, 1990). Uncoupling of the mitochondrial electron transport chain from oxidative phosphorylation (ATP production) can be mediated by anion carrier proteins located in the inner mitochondrial membrane. The most prominent member is uncoupling protein 1 (UCP1) which facilitates non-shivering thermogenesis in BAT of mammals (Cannon and Nedergaard, 2004). However, the role of the single avian UCP (avUCP) is controversial, and whether it functions in heat production is unclear (Ueda et al., 2005; Criscuolo et al., 2006).

In addition to increased uncoupling and proton leak in mitochondria, plasma membranes of endotherms are more permeable to $Na^+$ and $K^+$ ions compared with ectotherms (Hulbert and Else, 1990). In endotherms, therefore, there is a greater demand for a high $Na^+/K^+$-ATPase activity to maintain ion gradients across membranes (Else et al., 1996). The increased $Na^+K^+$-ATPase activity increases cellular ATP demand, which requires greater proton flux to increase ATP synthesis in the mitochondria. This increased proton flux can also increase heat production because of an inefficiency in proton pumping (slip reaction), and because a certain proportion of protons pumped across the membrane will leak back into the mitochondrial matrix *via* passive proton leak and protein-mediated uncoupling of the electron transport chain from oxidative phosphorylation; proton leak and uncoupling can make up 20% of basal metabolic rate (Brand et al., 2005). Hence, endothermy could be defined as the three-way interaction between uncoupling and proton leak in mitochondria, cellular energy demand, and oxidative capacity. One mechanism that could provide a higher level control of these different components is the activity of thyroid hormones (Hayes and Garland, 1995; Hulbert and Else, 1981). Triiodothyronine (T3) regulates UCP-1 gene expression in BAT *via* a thyroid binding element (Rabelo et al., 1995). Furthermore, thyroid hormones can modulate aerobic metabolic capacity by regulating

mitochondrial biogenesis (Nelson et al., 1995) and PGC1α gene expression (Irrcher et al., 2008). The transcriptional coactivator PGC1α is a prominent metabolic regulator in mammals (Finck and Kelly, 2006; Puigserver and Spiegelman, 2003) and it has recently been shown to play a role in metabolic regulation in birds (Walter and Seebacher, 2007).

The aim of this study was to determine the molecular mechanisms that lead from an ectothermic to an endothermic metabolic state. Specifically we tested the hypotheses that the transition to endothermy occurs by concurrent increases in (1) uncoupling of the electron transport chain from oxidative phosphorylation, (2) in cellular ATP demand and (3) in oxidative capacity of mitochondria, and that (4) thyroid hormones regulate the coordination of these events.

## MATERIALS AND METHODS

### Animals

We used chicken embryos as a model system. During development embryos are ectothermic and cannot regulate their body temperature metabolically, but become endothermic soon after hatching (Decuypere, 1984; Tzschentke and Nichelmann, 1999). This provided us a perfect model system to study the development of endothermy. Fertilized chicken eggs (*Gallus gallus* Linnaeus 1758) were obtained from a local supplier (Baiada Poultry, Sydney, Australia). Eggs were incubated at a constant temperature (38°C) and relative humidity (60–67%; Octagon 40 Incubator, Brinsea Products, Sandford, North Somerset, UK). Internal piping occurred on day 19 or 20 and hatching on day 20 or 21 of incubation. After hatching the hatchlings were transferred to an enclosure (air temperature 25–29°C), and provided with heating lamps, water and commercial chicken starter feed *ad libitum*.

Tissue samples (from six to eight birds per time point) were collected 3 days before hatching (referred to as –3), 1 day before hatching (–1), and 8 days after hatching (+8). Six adult chickens (168 days old) were obtained separately from a local supplier as a reference point for the gene expression analysis. Animals were killed by cervical dislocation. Breast muscle (musculus pectoralis) and liver were collected, and tissue was frozen in liquid nitrogen for enzyme analyses or in RNAlater (Ambion, Austin, TX, USA) for gene expression analyses. Mitochondria were extracted from fresh tissue to conduct mitochondrial respiration experiments. All procedures were approved by the University of Sydney Animal Ethics Committee (approval number: L04/7-2006/2/4414).

### Thyroid hormone quantification

To induce hypothyroidism, on day 14 of incubation, chicken embryos were injected in the yolk with 1.5 mg methimazole (Verhoelst et al., 2004) dissolved in physiological saline (hypothyroid group; 12) or a physiological saline solution alone (control group; 12). Tissue samples were collected 1 day before hatching (–1). Embryos were staged using the HH system (Hamburger and Hamilton, 1951) to confirm that both treatment groups were at the same developmental stage.

Before the experiment, we conducted a pilot study to verify the effect of methimazole: blood samples of separate hypothyroid (13) and control (9) groups were taken *via* heart puncture, centrifuged (2500 ***g***, 10 min, 4°C) and the upper layer was frozen at –20°C. Free T4 concentrations were measured by the Endocrinology Unit, Prince of Wales Hospital, Sydney, NSW, Australia. Free T4 concentrations of the methimazole treatment group were under the detection level of 3.9 pmol $l^{-1}$ in 12 samples and 4.5 pmol $l^{-1}$ in one sample. In the control group free T4 concentrations were 11.9±1.25 pmol $l^{-1}$ (mean ± s.e.m.).

### Gene expression

RNA was extracted from 40–100 mg of liver and muscle samples using TRIreagent (Molecular Research Centre, Cincinnati, OH, USA), following the manufacturer's instructions. RNA quality and concentration were verified using a Bioanalyzer (Agilent Biotechnologies, Palo Alto, CA, USA). One microgram of total RNA was treated with DNAse I (Sigma) and reverse-transcribed using RNAse $H^-$ MMLV reverse transcriptase (Bioscript, Bioline, London, UK) and random hexamer primers (Bioline).

Quantitative RT-PCR was performed on an Applied Biosystems 7500 qRT-PCR machine (Applied Biosystems) according to published protocols (Seebacher et al., 2006). Primer and dual label probes were designed from sequences obtained from GenBank for uncoupling protein (UCP), adenine nucleotide translocase (ANT), peroxisome proliferator activated receptor γ coactivator-1alpha (PGC1α), the $Na^+/K^+$-ATPase subunit α1 (ATP1α1), and the $Na^+/K^+$-ATPase subunit β1 (ATP1β1). We measured mRNA levels of the ATP1 α1 and β1 subunits because the genes for both are expressed in liver and skeletal muscle (Lemas et al., 1994; Lingrel et al., 2003). The RNA polymerase II polypeptide E (POLR2E) was used as housekeeping gene (Table 1). Real-time PCR reactions with dual labelled probes contained 1× Immomix (Bioline), 4.5 mmol $l^{-1}$ $MgCl_2$, 50–900 nmol $l^{-1}$ primer and probe, 1× ROX reference dye

Table 1. Primer and dual-label probes designed from sequences obtained from GenBank

| Accession no. | Primer and probe | Sequences |
|---|---|---|
| NM_204107 | UCP (F) | 5′-CTGATGACAGACAACGTCCC-3′ |
| | UCP (R) | 5′-TTCATGTACCGCGTCTTCAC-3′ |
| AB088686 | ANT (F) | 5′-CTATAGAGCTGCCTACTTTGG-3′ |
| | ANT (R) | 5′-ATGATATCAGCTCCTTTGCG-3′ |
| NM_001006457 | PGC-1α (F) | 5′-GACGTATCGCCTTCTTGCTC-3′ |
| | PGC-1α (R) | 5′-CTCGATCGGGAATATGGAGA-3′ |
| | PGC-1α probe | 5′-(FAM)CCTCAACGCAGGTCTTGTTCCCG(BHQ1)-3′ |
| NM_205521 | ATP1A1 (F) | 5′-TGCTCGACTCAACATTCCTG-3′ |
| | ATP1A1 (R) | 5′-GGGGAATCATTCACACCATC-3′ |
| NM_205520 | ATP1B1 (F) | 5′-CTAAGGCGCCAGAAAATGAG-3′ |
| | ATP1B1 (R) | 5′-GGGTAGTACTGCAGGGCAAA-3′ |
| XM_418224 | POLR2E (F) | 5′-CATGCAGGAGGAGAACATCA-3′ |
| | POLR2E (R) | 5′-TGCAGAAACTGCTCCAGGAT-3′ |
| | POLR2E probe | 5′-(cy5)TGCTGCACCACAATCAGCGCACG(BHQ1)-3′ |

(F), forward; (R), reverse.

(Invitrogen, Carlsbad, CA, USA), and ~50 ng cDNA. The cycle consisted of 95°C for 7 min, 40 cycles of 95°C for 20 s, 60°C for 1 min. Real-time PCR reactions with sybr green contained 1× SensiMixPlus SYBR (Quantace, London, UK), 4.5 mmol $l^{-1}$ $MgCl_2$, 50–900 nmol $l^{-1}$ primer and ~50 ng cDNA. The PCR cycle consisted of 95°C for 7 min, 40 cycles of 95°C for 20 s, 60°C for 1 min. A dissociation curve analysis was performed after the amplification step to verify the presence of only a single PCR product. Relative gene expression of the five target genes at each stage of development and hypothyroid treatment was calculated according to the method of Pfaffl (Pfaffl, 2001), and normalized with POLR2E. Adult chicken samples ($N$=6) were used as a control.

### Mitochondrial function

Mitochondria were isolated from 150–200 mg of liver and muscle samples. Tissue was finely chopped on ice and homogenized in nine volumes of ice-cold isolation buffer [140 mmol KCl, 10 mmol EDTA, 5 mmol $MgCl_2$, 20 mmol Hepes, 1% bovine serum albumin (BSA), pH 7.3 at 20°C] by four gentle passes in a Potter-Elvehjem homogenizer. After initial centrifugation (1400 ***g***, 5 min, 4°C) the supernatant was centrifuged (9000 ***g***, 7 min, 4°C), and the resultant pellet was resuspended in assay medium [140 mmol KCl, 5 mmol $NaH_2PO_4$, 20 mmol Hepes, 0.5% bovine serum albumin (BSA), pH 7.3 at 20°C]. Mitochondrial oxygen consumption was measured in duplicate in a volume of 250 µl assay medium at 38°C in a respiration chamber (Mitocell MT200; Strathkelvin Instruments, North Lanarkshire, Scotland) with a microelectrode (model 1302; Strathkelvin Instruments) connected to an oxygen meter (model 782; Strathkelvin Instruments). State 3 respiration was measured in a final concentration of 5 mmol $l^{-1}$ malate to spark the Krebs cycle and 2.6 mmol $l^{-1}$ pyruvate acid as substrate and 0.5 mmol $l^{-1}$ ADP, as previously described (Johnston et al., 1994). State 4+ respiratory rate was obtained after adding 2 µl of 1 µg µl$^{-1}$ oligomycin dissolved in ethanol to inhibit the Fo/F1 ATPase. Free fatty acids (FFAs) cause uncoupling of oxidative phosphorylation (Borst et al., 1962), and we measured maximal uncoupled respiration by adding palmitate (148 µmol $l^{-1}$). The relative contributions of uncoupling protein (UCP) and the adenine nucleotide translocase (ANT) in uncoupling respiration was determined by adding 1 mmol $l^{-1}$ guanosine diphosphate (GDP) to block uncoupling protein (UCP) and 5 µmol $l^{-1}$ carboxyatractylate (CATr), which is a specific inhibitor of ANT. All stated concentrations are final concentrations. Before we undertook the experiments we conducted pilot experiments to determine optimal concentrations of palmitate and CATr. We confirmed that the effect of CATr was independent from the effect of GDP, so that we could analyse the effect of CATr and GDP sequentially in the same sample. Furthermore, we verified that there were no differences in the electron pumping efficiency during development in liver [means ± s.e.m. (nmol $O_2$ $min^{-1}$ $mg^{-1}$ protein) day –3: 20.10±2.19; day –1: 21.08±3.95; day +8: 32.60±7.13; one-way ANOVA $F_{2,8}$=2.04, $P$=0.21] and muscle (day –3: 115.56±2.41; day –1: 104.34±6.83; day +8: 103.88±10.90; one-way ANOVA $F_{2,13}$=0.93, $P$=0.42) mitochondria by inducing maximal electron transport chain capacity by adding 2 µmol $l^{-1}$ carbonyl-cyanid $p$-[trifluoromethoxy]-phenyl-hydrazone (FCCP), an artificial proton translocator. Protein concentrations of the mitochondrial solutions were measured by the bicinchoninic acid assay (BCA, Sigma-Aldrich, USA) according to the manufacturer's instructions.

### $Na^+/K^+$-ATPase activity

$Na^+/K^+$-ATPase activity was determined using a modified protocol of Esmann and Skou (Esmann and Skou, 1988). Liver and muscle tissue were finely diced on ice and homogenized in nine volumes of ice-cold homogenization buffer (250 mmol $l^{-1}$ sucrose, 5 mmol $l^{-1}$ EDTA and 20 mmol $l^{-1}$ imidazole, pH 7.4 at 20°C). To achieve maximal $Na^+/K^+$-ATPase activity, samples were mixed under constant vortexing with two parts 1.2 mg $ml^{-1}$ sodium deoxycholate (DOC) dissolved in homogenization buffer following a 15 min incubation period at room temperature. DOC-treated samples (50 µl) were pre-incubated (10 min, 38°C) in assay medium (30 mmol $l^{-1}$ histidine, 128 mmol $l^{-1}$ NaCl, 4 mmol $l^{-1}$ $MgCl_2$ and either 1 mmol $l^{-1}$ ouabain or 20 mmol $l^{-1}$ KCl, pH 7.4 at 20°C) to allow thermal equilibration and binding of ouabain to $Na^+/K^+$-ATPase. The reaction was initiated by adding 3 mmol $l^{-1}$ ATP to each sample, and samples were incubated for 5 min. One ml 0.8 mol $l^{-1}$ perchloric acid was used to stop the reaction. Samples were centrifuged (1200 ***g***, 15 min at 4°C) and inorganic phosphate concentrations were quantified *via* the ammonium molybdate assay (Bonting et al., 1961). $Na^+/K^+$-ATPase activity was calculated as the difference in inorganic phosphate liberated in the presence and absence of 1 mmol $l^{-1}$ ouabain. All assays were performed in duplicate.

### Statistical analysis

Data are presented as means ± s.e.m. Gene expression data for different stages of development were analyzed by one-way ANOVA followed by LSD pairwise comparison. Mitochondrial respirations rates were analyzed by multivariate repeated measures ANOVA in which respiration rates with additions of different drugs were used as the repeated measure, and developmental stages as the between subject factors. Additionally, changes in mitochondrial respiration rates following different drug treatments were analyzed between stages of development by one-way ANOVA followed by pairwise comparisons. We compared $Na^+/K^+$-ATPase activity between stages of development by one-way ANOVA, and hypothyroid treatments to control by $t$-tests for independent samples. All data were tested for normality and homogeneity of variance using Levene's test. If a data set failed Levene's test the data were ln transformed. Significance was considered as $P$<0.05 unless stated otherwise.

We used published values of state 3 and state 4 mitochondrial oxygen consumption and $Na^+/K^+$-ATPase activity for comparisons with our chicken developmental series. To minimize confounding effects of different thermal sensitivities, we avoided using data from cold climate ectotherms, and we used data from animals that were assayed within 10°C of the chicken incubation temperature (38°C) and adjusted rates to 38°C assuming a $Q_{10}$ value of 2.

## RESULTS

### Mitochondrial oxidative capacity

Oxygen consumption of mitochondria can be partitioned into oxygen consumed in the process of ATP production (state 3 respiration rates), and oxygen consumed as a result of proton leak or uncoupling of the transmembrane proton gradient (state 4+). State 3 respiration rates increased significantly 8 days after hatching in skeletal muscle ($F_{2,21}$=10.76, $P$<0.002; Fig. 1B) but not in liver ($F_{2,22}$=0.6, $P$>0.5; Fig. 1A). Similarly, state 4+ respiration increased significantly with development in skeletal muscle ($F_{2,21}$=7.74, $P$<0.004; Fig. 1B) but not in liver ($F_{2,22}$=0.3, $P$>0.7; Fig. 1A). There was an interaction between stages of development and respiration rates in skeletal muscle ($F_{2,21}$=8.095, $P$<0.003) but not in liver ($F_{2,22}$=0.673, $P$>0.5).

### Uncoupling of mitochondrial respiration

Palmitate stimulation significantly increased muscle and liver mitochondrial state 4+ (uncoupled) respiration rates (skeletal muscle:

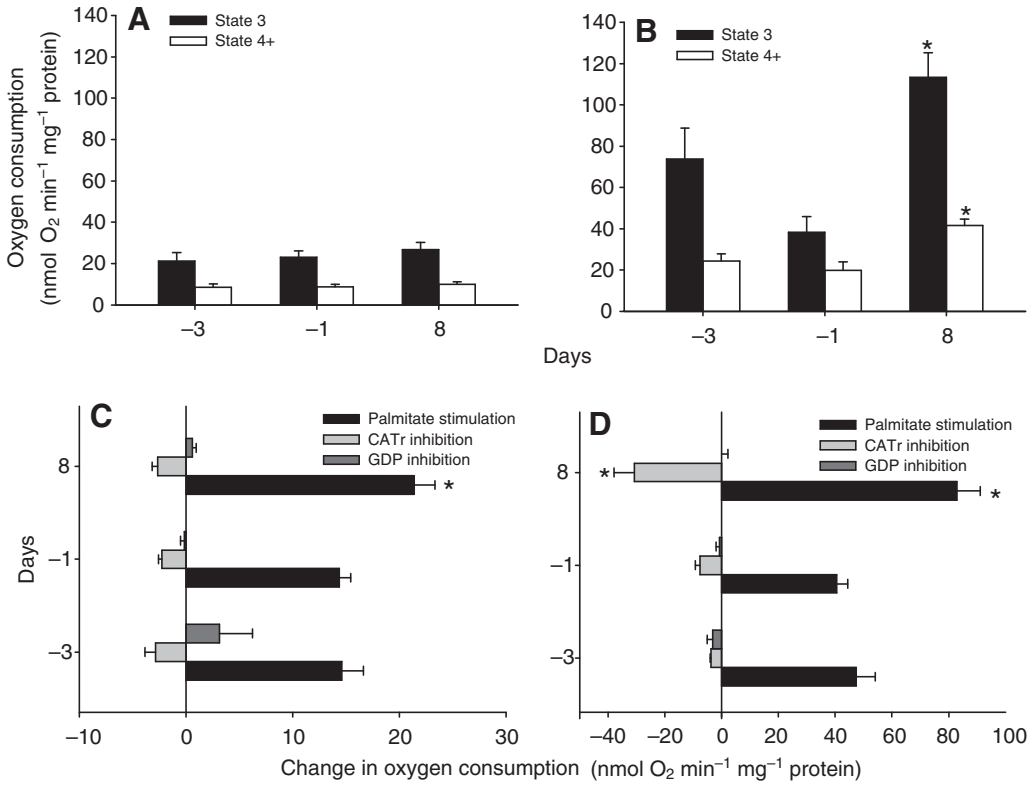


Fig. 1. Mitochondrial respiration rates and uncoupling (means ± s.e.m.) in (A) liver and (B) skeletal muscle. State 3 (ADP-stimulated, black columns) and state 4+ (presence of oligomycin, white columns) rates were measured during development. ATP production (state 3) as well as the proton leak (state 4+) increased significantly 8 days after hatching in skeletal muscle but both parameters remained constant in liver. Mitochondrial uncoupling in (C) liver and (D) skeletal muscle in the presence of oligomycin stimulated by free fatty acid (addition of palmitate, black columns) significantly increased after hatching. In the presence of the ANT blocker carboxyatractylate (CATr), palmitate-stimulated oxygen consumption significantly decreased in liver and skeletal muscle (light grey columns). The proportion of ANT-induced mitochondrial uncoupling increased significantly 8 days after hatching in skeletal muscle but not in liver. Addition of the UCP blocker GDP (dark grey columns) had no effect in either tissue.

$F_{3,19}$=22,247, $P$<0.0001, Fig. 1D; liver: $F_{3,20}$=84.816, $P$<0.0001; Fig. 1C). Additionally, maximal palmitate stimulated uncoupling increased significantly during development in liver ($F_{2,22}$=5.49, $P$<0.02; Fig. 1C) and skeletal muscle ($F_{2,21}$=8.83, $P$<0.002; Fig. 1D). To test the hypothesis that avian UCP (avUCP) has a similar function in uncoupling mitochondria as UCP1 in mammals, we added the UCP inhibitor GDP after fatty acid induced uncoupling. There was no effect of GDP (skeletal muscle: $F_{3,19}$=22,247, $P$>0.09, Fig. 1D; liver: $F_{3,20}$=84.816, $P$>0.3; Fig. 1C), which indicates that UCP is not involved in uncoupling of respiration and metabolic heat production in birds.

Blocking ANT significantly reduced muscle and liver mitochondrial uncoupled respiration rates (skeletal muscle: $F_{3,19}$=22,247, $P$<0.0001, liver: $F_{3,20}$=84.816, $P$<0.0001). Furthermore the uncoupling fraction mediated *via* ANT significantly increased during development in skeletal muscle ($F_{2,21}$=10.48, $P$<0.0007; Fig. 1D) but not in liver ($F_{2,22}$=0.22, $P$>0.7; Fig. 1C). Liver ANT gene expression was significantly elevated after hatching (+8) compared with prenatal stages of development ($F_{2,17}$=4.17, $P$<0.05; Fig. 2B). However, ANT gene expression did not change during development in skeletal muscle indicating a post-transcriptional mode of regulation ($F_{2,17}$=1.35, $P$>0.2; Fig. 2A). By contrast, avUCP mRNA levels increased significantly after hatching in skeletal muscle ($F_{2,17}$=38.62, $P$<0.001; Fig. 2G) but avUCP RNA levels were undetectable in liver.

### Energy demands during development

In liver, expression of the ATP1α1 was significantly elevated after hatching (+8) compared with earlier developmental stages ($F_{2,15}$=8.81, $P$<0.003; Fig. 2D) but the ATP1β1 mRNA levels remained constant ($F_{2,15}$=0.06, $P$>0.9; Fig. 2F). The $Na^+/K^+$-ATPase catalytic activity increased significantly after hatching ($F_{2,15}$=10.99, $P$<0.002; Fig. 3A).

In skeletal muscle, ATP1α1 gene expression was elevated 8 days after hatching compared with prenatal stages of development ($F_{2,15}$=5.06, $P$<0.003; Fig. 2C), and ATP1β1 mRNA levels were significantly decreased after hatching ($F_{2,15}$=9.26, $P$<0.003; Fig. 2E). Similar to liver, $Na^+/K^+$-ATPase catalytic activity increased significantly after hatching ($F_{2,15}$=12.27, $P$<0.002; Fig. 3B).

### Control by thyroid hormone

Hypothyroid treatment had no effect on the mitochondrial oxidative capacity in liver (state 3 respiration rates: $t_{10.599}$=–2.21, $P$>0.05; state 4+ respiration rates: $t_{15}$=0.011, $P$>0.9; data not shown) and skeletal muscle (state 3 respiration rates: $t_{13}$=0.629, $P$>0.5; state 4+ respiration rates: $t_{13}$=0.374, $P$>0.7; data not shown). However, PGC1α gene expression levels were significantly reduced in skeletal muscle of the hypothyroid animals compared with the control group ($t_{14}$=–2.78, $P$<0.02; Fig. 4A). In liver, PGC1α mRNA levels were lower than in muscle but reduced thyroid hormone levels had no affect on PGC1α gene expression ($t_{14}$=–1.59, $P$>0.1; Fig. 4A).

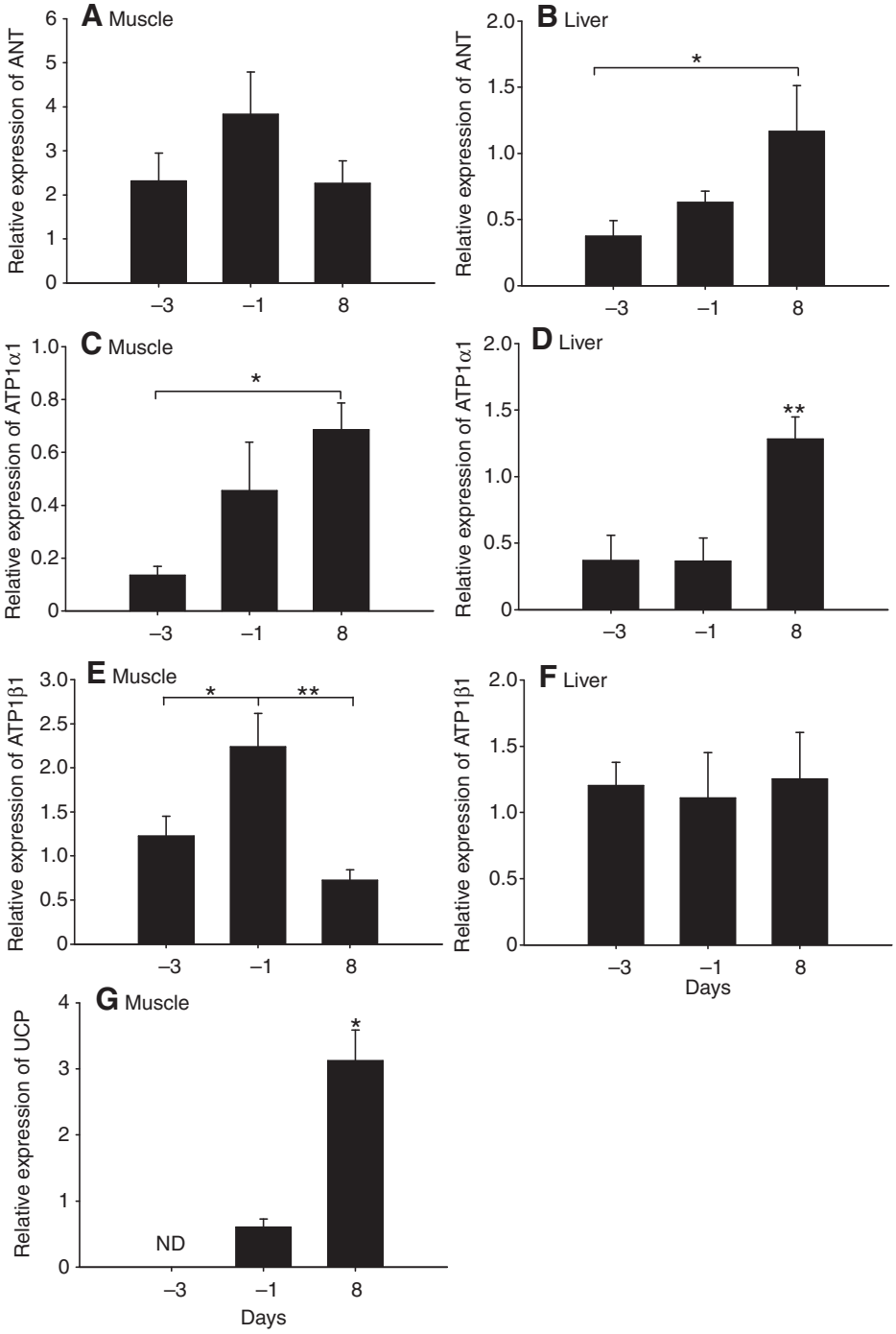


Fig. 2. Gene expression in skeletal muscle and liver at different stages of development All data are expressed as relative to adult mRNA levels (means ± s.e.m.). (A,B) Relative expression of ANT mRNA remained stable during development in skeletal muscle (A) but in liver (B) levels were significantly increased after hatching. (C,D) Relative expression of the $Na^+/K^+$-ATPase subunit ATP1α1 mRNA significantly increased 8 days after hatching in muscle (C) and liver (D). (E,F) mRNA levels of ATP1β1 were significantly elevated before hatching in muscle (E) but remained stable during development in liver (F). (G) Gene expression of UCP (uncoupling protein) significantly increased during development in muscle and was not detectable (ND) 3 days before hatching.

Uncoupling of mitochondrial respiration remained unchanged after treatment to induce hypothyroid in liver (palmitate stimulated uncoupling: $t_{15}$=0.628, $P$>0.5; uncoupling fraction mediated *via* ANT: $t_{15}$=–1.432, $P$>0.1; data not shown) and skeletal muscle (palmitate stimulated uncoupling: $t_{13}$=–2.84, $P$>0.7; uncoupling fraction mediated *via* ANT: $t_{13}$=–0.944, $P$>0.3; data not shown). ANT gene expression was significantly reduced by low thyroid hormone levels in liver ($t_{14}$=–2.91, $P$<0.02; Fig. 4B) but not in muscle ($t_{14}$=–1.5, $P$>0.1; Fig. 4B). Interestingly, low thyroid hormone levels significantly reduced UCP gene expression compared with the control group in muscle ($t_{14}$=–3.25, $P$<0.006; Fig. 4C).

In liver, gene expression of ATP1α1 and ATP1β1 did not differ between the low thyroid treatment and control groups (ATP1α1: $t_{10}$=1.06, $P$>0.3; ATP1β1 $t_{10}$=1.75, $P$>0.1; Fig. 4D,E), but the $Na^+/K^+$-ATPase activity was significantly higher in the hypothyroid treatment group compared with the control group ($t_{10}$=2.43, $P$<0.036; Fig. 3C). In skeletal muscle, mRNA levels of both of the $Na^+/K^+$-ATPase subunits as well as their catalytic activity remained unchanged after reduction of thyroid hormone levels (ATP1α1: $t_{7.15}$=–0.87, $P$>0.4; ATP1β1: $t_{10}$=–1.13, $P$>0.2; $Na^+/K^+$-ATPase activity: $t_{10}$=0.789, $P$>0.4; Fig. 4D,E, Fig. 3D).

## DISCUSSION

The transition from ectothermy to endothermy is accompanied by an increased cellular demand for ATP resulting from increased $Na^+/K^+$-ATPase activity. This increase in demand is paralleled by

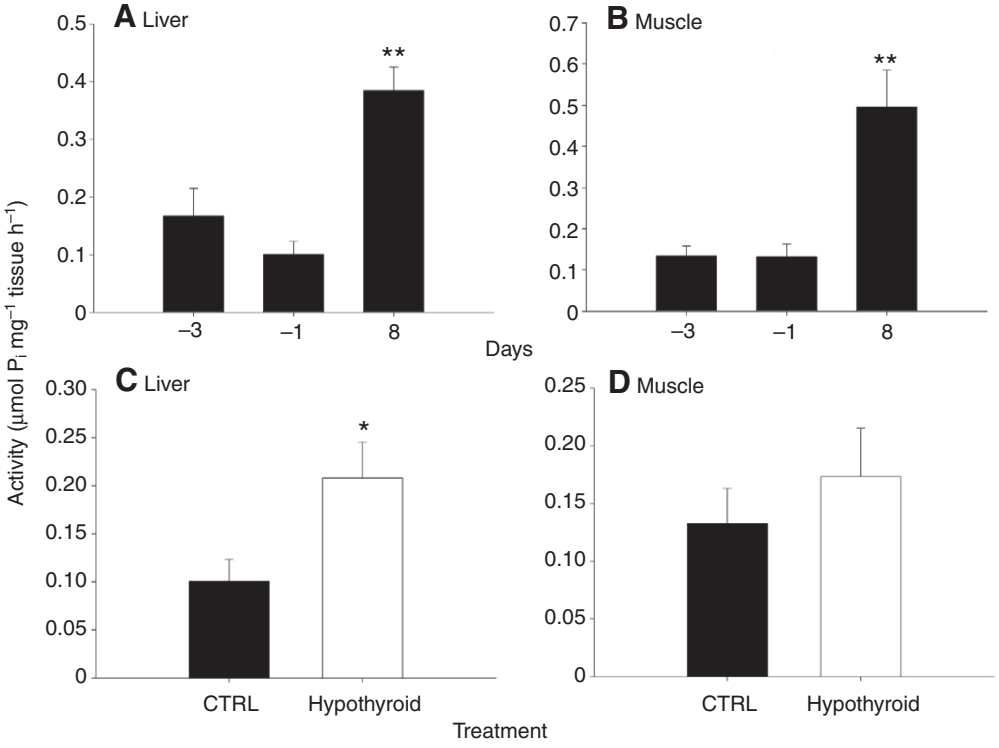


Fig. 3. The catalytic activity of $Na^+/K^+$-ATPase (μmol $P_i$ $mg^{-1}$ tissue $h^{-1}$; means ± s.e.m.) increased significantly after hatching in (A) liver and (B) muscle. Induced hypothyroidism significantly increased the enzyme activity of the treatment group compared with the control group in (C) liver but not in (D) muscle were $Na^+/K^+$-ATPase activity did not change with treatments.

an increase in tissue metabolic capacity resulting from an increase in state 3 respiration, and an increase in citrate synthase and cytochrome *c* oxidase activities (Seebacher et al., 2006); the latter most probably reflects a quantitative increase in mitochondrial density. This increase in total tissue metabolic flux during development will also increase heat production even if proton leak rate remained constant. At the same time, however, skeletal muscle and liver mitochondria become more uncoupled during development, and have greater state 4 respiration rates, thereby augmenting heat production. Similar to mammals, increases in oxidative capacity of muscle occurs as a result of the induction of PGC1α gene expression by thyroid hormone. Endothermy in birds is fundamentally different from mammals, however, because one of the principal mechanisms that facilitates heat production in mammals, uncoupling proteins, does not operate in birds.

Uncoupling is an essential component of endothermy (Brand et al., 1991), and accounts for up to 20% of basal energy metabolism (Rolfe and Brand, 1997). Furthermore in mammals mitochondrial uncoupling, facilitated by UCP1, plays an important role in adaptive heat production; that is, in adjusting metabolic heat production to maintain stable body temperatures in variable environments (Cannon and Nedergaard, 2004). In chickens, ANT is an important mechanism of uncoupling oxidative phosphorylation, in a process similar to that in mammals in which its concentration determines basal proton leak (Brand et al., 2005). By contrast, avUCP has no detectable effect on mitochondrial uncoupling, and it is therefore unlikely to be important in adaptive heat production after hatching. The function of avUCP has been controversial since its discovery (Raimbault et al., 2001). AvUCP is most closely related to crocodilian UCP3, and both crocodilian UCP3 and avUCP form a distinct clade with UCP3 of mammals (Schwartz et al., 2008). Considering the absence of UCP1 in birds, it has been suggested that avian UCP has assumed a similar function in endogenous heat production (Schwartz et al., 2008; Toyomizu et al., 2002). We can now say that this does not appear to be the case, at least for *Gallus gallus*. However, ultimate verification of the role of avUCP would require a knockdown model to confirm that avUCP is not involved in mild uncoupling of mitochondrial respiration from oxidative phosphorylation. Other possible functions of avUCP are defence against free radical damage (Criscuolo et al., 2005), and a role in fatty acid metabolism (Brand and Esteves, 2005). In addition, UCP3 may function as a transporter of pyruvate out of mitochondria at high membrane potentials, which would have a negative feedback effect on the Krebs cycle, and would lead to preferential use of pyruvate by lactate dehydrogenase (Criscuolo et al., 2006; Mozo et al., 2006). It is possible that avUCP functions as a pyruvate carrier because it is exclusively expressed in skeletal muscles which consist, to a large extent, of glycolytic fibres. Additionally, the remarkable increase in UCP mRNA levels, 8 days after hatching, points towards a role of UCP in skeletal muscle as locomotory activity increases. In liver, ANT-mediated uncoupling remained constant throughout development although mitochondrial uncoupling increased after hatching which indicates that there are other uncoupling agents present. Other anion carriers such as the aspartate–glutamate antiporter (Samartsev et al., 1997) or dicarboxylate carrier (Wieckowski and Wojtczak, 1997) can mediate uncoupling in liver (Skulachev, 1999), but their potential role in endothermic heat production needs further investigations.

The increased $Na^+/K^+$-ATPase activity in endothermic hatchlings compared with earlier developmental stages is likely to be a response to increasing membrane permeability to $K^+$ and $Na^+$ ions in endotherms (Hulbert and Else, 1990). Increases in membrane polyunsaturated fatty acids during development (Noble and Cocchi, 1990) may explain their increased leakiness and the resulting increase in $Na^+/K^+$-ATPase activity (Wu et al., 2004). In chickens, mRNA levels of the catalytic $\alpha_1$ subunit correlate with the activity of the enzyme, so that during the transition from ecothermy to endothermy $Na^+/K^+$-ATPase activity is transcriptionally controlled by the catalytic $\alpha_1$ subunit but not by the glycosylated $\beta_1$ subunit.

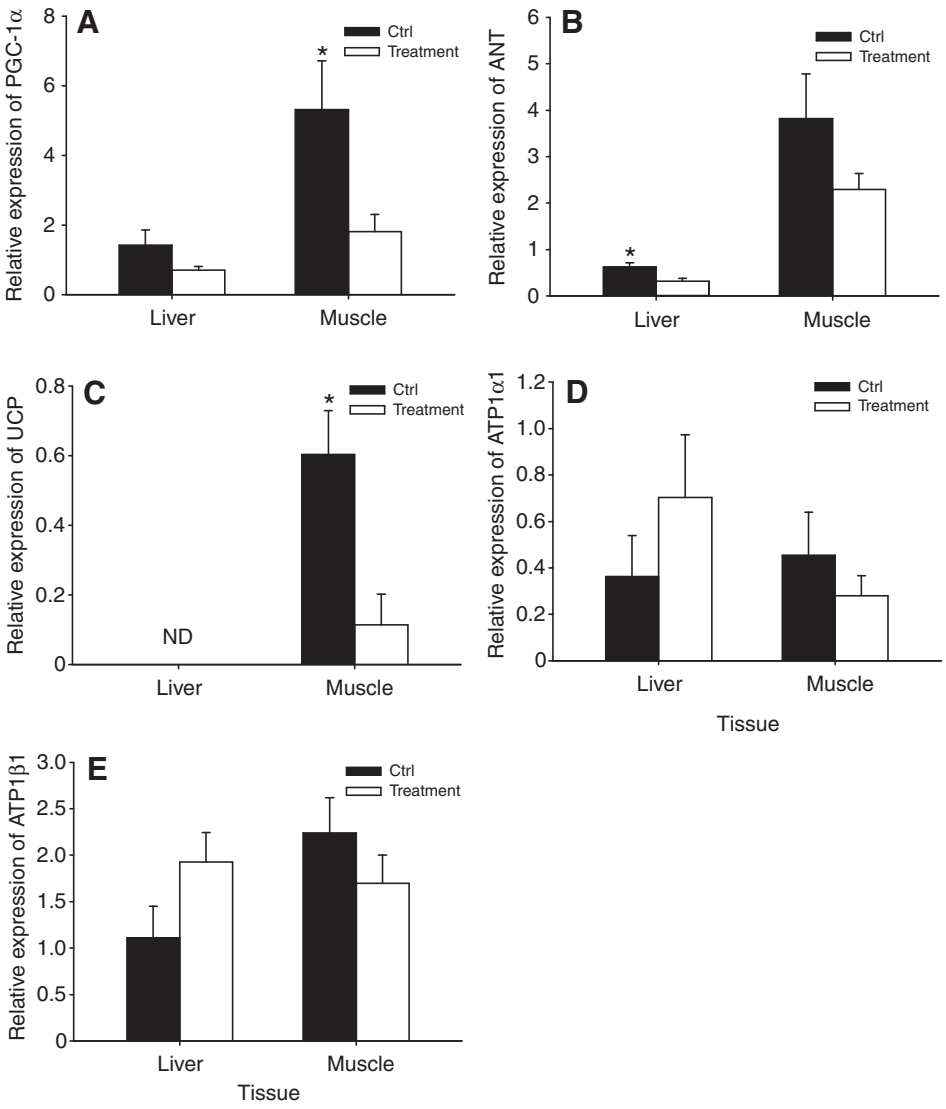


Fig. 4. Effect of induced hypothyroidism on gene expression in liver and skeletal muscle. Data are expressed as relative to adult mRNA levels (means ± s.e.m.); white columns treatment group (induced hypothyroidism); black bars, control group. (A) Treatment significantly reduced PGC-1α gene expression in skeletal muscle but had no effect on PGC-1α expression in the liver. (B) ANT gene expression was significantly reduced in the treatment group in liver but not in skeletal muscle. (C) UCP gene expression was not detectable (ND) in liver but was significantly reduced in the hypothyroid group compared with a control group in skeletal muscle. (D,E) Relative mRNA expression of the $Na^+/K^+$-ATPase ATP1 α1 subunit (D) and the β1 subunit (E) did not change with treatment in liver or skeletal muscle.

PGC1α, the major metabolic regulator in mammals, and its target PPARγ are significantly elevated in pectoral muscle and liver during embryonic development of chickens compared with adults (Walter and Seebacher, 2007). Here we show that muscle PGC-1α gene expression, and thereby mitochondrial function and capacity, is regulated by thyroid hormone in birds. In mammals, PGC1α can be regulated directly by T3 *via* an upstream thyroid hormone response element (Wulf et al., 2008). Additionally, it is possible that T3-mediated increase in PGC1α gene expression is regulated indirectly *via* other transcription factors that bind to CREB or LXXLL binding motives at the N-terminal region of PGC1α (Puigserver and Spiegelman, 2003). Unlike in muscle, however, reduced thyroid levels did not influence PGC1α gene expression in liver, where it may be induced instead by cAMP and glucocorticoids to controls gluconeogenesis (Yoon et al., 2001). In addition to stimulating PGC1α expression and thereby mitochondrial capacity, thyroid hormones can also control the activity of ANT and UCP (Collin et al., 2003). The downregulation of ANT in liver after induced hypothyroidism indicates a regulatory effect of thyroid hormones on ANT. However, thyroid hormone did not affect ANT- or UCP-mediated uncoupling or $Na^+/K^+$-ATPase activity in muscle, so that despite its importance in regulating metabolic capacity it does not act as an overall controller of capacity, uncoupling, and demand during the development of endothermy.

Of the several theories attempting to explain selection pressures that have led to endothermy: thermoregulation (McNab, 1978), improved parental care (Farmer, 2000), and increased aerobic activity (Bennett and Ruben, 1979), the latter is the most widely accepted. It posits that greater capacity for oxidative ATP production led to selective advantages by allowing more sustained levels of activity. Heat production would have been a corollary of increased metabolic capacity without being directly selected for. In chickens however, the increases in oxidative capacity (state 3 respiration rate) are accompanied by increased uncoupling of oxidative phosphorylation and increased cellular ATP demand, both of which reduce the ATP available for activity. It is unlikely, therefore, that the advantages of increased activity or any other single initial selection pressure led to modern endothermic phenotypes (Kemp, 2006). Complex organismal functions such as endothermy evolve by the gradual integration of genetic changes that affect suborganismal traits, without the need for evolutionary transitions of individual traits to contribute directly to the more complex function (Lenski et al., 2003). Accordingly, it is expected that there are pronounced variations in individual traits (e.g. mitochondrial function, enzyme activities, uncoupling, etc.) among species

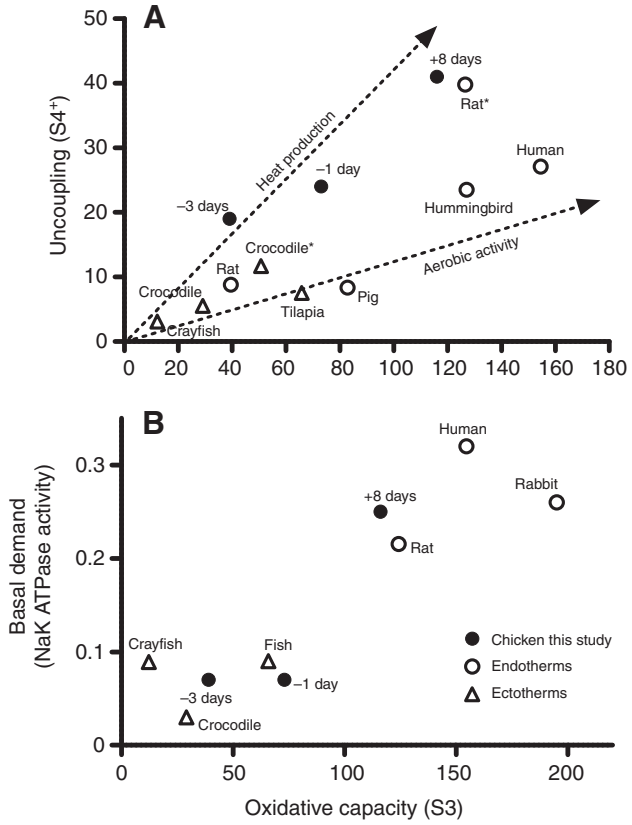


Fig. 5. Endothermy can be defined by the interactions between (A) metabolic capacity [here state 3 (S3) mitochondrial oxygen consumption (nmol $mg^{-1}$ protein $min^{-1}$)] and mitochondrial uncoupling [state 4 (S4+) mitochondrial oxygen consumption (nmol $mg^{-1}$ protein $min^{-1}$)] and (B) between metabolic capacity and cellular ATP demand [$Na^+/K^+$-ATPase activity (nmol $P_i$ $min^{-1}$ $g^{-1}$ tissue)]. Selection for increased aerobic activity would produce animals with high oxidative capacity and low uncoupling whereas selection for heat production would favour high oxidative capacity and high uncoupling (A). Mitochondrial oxygen consumption data are from Glanville and Seebacher (Glanville and Seebacher, 2006) for crocodile (crocodile=warm acclimated *Crocodylus porosus*; crocodile*=*C. porosus* cold acclimated), E. J. Glanville and F.S. (unpublished data) for rat (rat=summer caught *Rattus fuscipes*; rat*=winter caught *R. fuscipes*); Schnell and Seebacher (Schnell and Seebacher, 2008) for the fish, tilapia (*Oreochromis mossambicus*); Seebacher and Wilson (Seebacher and Wilson, 2006) for crayfish (*Cherax destructor*); Suarez et al. [(Suarez et al., 1986) for hummingbird (*Selasphorus rufus*); Porta et al. (Porta et al., 2006) for pig; Willis and Jackman (Willis and Jackman, 1994) for rabbit; Tonkonogi and Sahlin (Tonkonogi and Sahlin, 1997) for human]. $Na^+/K^+$ATPase data other than for chicken and humans (Fraser and McKenna, 1998) are from Else et al. (Else et al., 1996). Because of the paucity of tissue-specific data of $Na^+/K^+$ATPase activities we matched activities of *C. johnstoni* with mitochondrial oxygen consumption of *C. porosus* (crocodile in B), *Rattus norvegicus* with *Rattus fuscipes* (rat), and *Cyprinus carpio* with *Oreochromis mossambicus* (fish).

(Fig. 5A,B) because the evolution of endothermy does not follow a single pathway or selection pressure but results from the accumulation of changes in individual traits. If heat production was the principal selection pressure, uncoupling of mitochondria should be high relative to oxidative capacity (for example, the ratio of state 3 and state 4 mitochondrial respiration rates was 2), and this is the pattern observed in the development of chickens and also in wild rats in winter, which presumably gain advantage from increased heat production (Fig. 5A). The phenotypic plasticity in the ratio of state 3 and state 4 rates seen in cold- and warm-acclimatized rats is interesting because it emphasises that endothermy is not characterized by the evolution of a single metabolic state. However, if increased aerobic activity was the principal selection pressure, oxidative capacity should increase while uncoupling remains relatively constant (for example, state 3:state 4 =8; Fig. 5A). Most endotherms lie somewhere between these extremes but increases in oxidative capacity of ectotherms follow the 'increased activity' trajectory (Fig. 5A), except for cold-acclimated crocodiles which have similar values to summer-caught rats. In contrast to the continuous nature of the relationship between uncoupling and oxidative capacity, there is a clear break between ectotherms and endotherms in the cellular ATP demand ($Na^+/K^+$-ATPase activity; Fig. 5B); the higher oxidative capacity of endotherms (including day 8 chickens) is accompanied by high cellular ATP demand, which may represent a necessary causal link between these traits.

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