Transition from ectothermy to endothermy: the development of metabolic capacity in a bird (*Gallus gallus*)

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The evolution of endothermy is one of the most significant events in vertebrate evolution. Adult mammals and birds are delineated from their early ontogenetic stages, as well as from other vertebrates, by high resting metabolic rates and consequent internal heat production. We used the embryonic development of a bird (*Gallus gallus*) as a model to investigate the metabolic transition between ectothermy and endothermy. Increases in aerobic capacity occur at two functional levels that are regulated independently from each other: (i) upregulation of gene expression; and (ii) significant increases in the catalytic activity of the main oxidative control enzymes. Anaerobic capacity, measured as lactate dehydrogenase activity, is extremely high during early development, but diminishes at the same time as aerobic capacity increases. Changes in lactate dehydrogenase activity are independent from its gene expression. The regulatory mechanisms that lead to endothermic metabolic capacity are similar to those of ectotherms in their response to environmental change. We suggest that the phylogenetic occurrence of endothermy is restricted by its limited selective advantages rather than by evolutionary innovation.

Keywords: cytochrome *c* oxidase; aerobic capacity; lactate dehydrogenase; anaerobic metabolism; RT-PCR; mitochondria

1. INTRODUCTION

Understanding the processes that lead to high basal metabolic rates and the consequent heat production typical of endothermy are key to understanding vertebrate evolution (Hulbert & Else 2000). The energy requirements necessary to sustain endothermic metabolism dominate the ecology and behaviour of mammals and birds and counterbalance benefits such as increased levels of activity, more effective parental care and thermoregulation (Ruben 1995; Farmer 2000; Koteja 2000).

Basal aerobic metabolic rates are 5–8 times greater in mammals and birds compared to ectothermic vertebrates. Interestingly, however, metabolic pathways are highly conserved among vertebrates (Smith & Morowitz 2004). In all animals, increases in metabolic capacity may result from increased activity of regulatory enzymes such as citrate synthase (CS) and cytochrome *c* oxidase (COX) in aerobic pathways and lactate dehydrogenase (LDH) in anaerobic pathways (Choi *et al.* 1993; Crawford *et al.* 1999). Mechanisms that may regulate metabolic enzyme activity include changes in protein structure, modification of membranes and regulation of gene expression (Yang & Somero 1996; Hardewig *et al.* 1999; Hulbert & Else 2000).

Many ectothermic vertebrates up- or downregulate enzyme activities in fluctuating thermal environments, thereby maintaining constant metabolic capacity (Seebacher *et al.* 2003; Guderley 2004). Similarly, changes in the activity of enzymes in oxidative metabolic pathways also play a significant role in establishing endothermic metabolic capacity during the ontogeny of

birds (Grav *et al.* 1988; Choi *et al.* 1993). Necessary correlates for endothermic metabolism is increased capacities of the respiratory and cardiovascular systems, increased efficiency of the gastro-intestinal tract, and maybe neural and endocrine modifications (Chiba *et al.* 2004; Rose & Kuswanti 2004). Sufficient capacity in the latter systems may exist already in ectotherms, for example four chambered hearts and pulmonary–systemic pressure separation in crocodilians (Franklin & Axelsson 2000; Seymour *et al.* 2004), or it may have coevolved alongside selection for increased metabolic capacity.

The evolutionary conservatism of metabolic pathways means that ectotherms and endotherms are qualitatively similar, and it is intriguing to consider the molecular mechanisms that facilitate the transition from ectothermic to endothermic metabolic capacity. Of particular interest is the fact that most pre- and neonatal birds and mammals are essentially ectothermic and develop endothermic capacity during their ontogeny (Whittow & Tazawa 1991; Visser 1998). We used the early ontogeny of a precocious bird (*Gallus gallus*) as a model to determine molecular mechanisms that underlie the development of metabolic capacity during the transition from ectothermy to endothermy.

2. MATERIAL AND METHODS

We targeted COX and CS to investigate the development of aerobic capacity. COX catalyses the final reaction of the mitochondrial electron transport chain (complex IV) and its activity determines oxidative capacity (St Pierre *et al.* 1998). A principal function of the tricarboxylic acid (TCA) cycle is the synthesis of FADH₂ and NADH which act as electron donors for the electron transport chain, and CS controls flux

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through the TCA cycle. We evaluated oxidative capacity at three levels of regulation: CS and COX enzyme activities, COX gene expression and mitochondrial respiration. Anaerobic activity was characterized by the gene expression and enzyme activity of LDH which catalyses the reduction of pyruvate to lactate in the major anaerobic ATP producing pathway. Additionally, activity of phosphofructokinase (PFK) was measured to evaluate glucose metabolism.

(a) Study animals

Fertilized chicken eggs (*G. gallus*) were obtained commercially and incubated at 38–39 °C. Birds ($n=6$ /sampling time) were sampled on the following days of incubation and post-incubation: 10, 15, 18/19, 20/21, 22, 25/26, 280 (adults). Internal pipping occurred on day 20/21 and hatching on day 22. Animals were euthanased by cervical dislocation, and liver and pectoral muscle tissues were collected and frozen immediately in liquid nitrogen.

(b) Enzyme assays

Assays for COX, CS, LDH and PFK were conducted at 30, 35 and 40 °C. All enzymes were assayed in all samples taken from day 15 onwards. Liver tissue was homogenized in 50 mM imidazole/HCl, 2 mM MgCl₂, 5 mM ethylene diamine tetra acetic acid (EDTA), 1 mM reduced glutathione and 1% Triton X-100, and assays were conducted spectrophotometrically (Seebacher *et al.* 2003). Assay media contained 100 mM K₂HPO₄/KH₂PO₄ buffer (pH 7.0), 0.16 mM NADH and 0.4 mM pyruvate for LDH; 100 mM Tris/HCl (pH 8.0), 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic) acid, 0.1 mM acetyl CoA and 0.15 mM oxaloacetate for CS; 100 mM K₂HPO₄/KH₂PO₄ (pH 7.5) and 0.05 mM cytochrome *c* reduced with Na₂S₂O₄ for COX, and assays were conducted against a reference of 0.05 mM cytochrome *c* oxidized with 50 µM K₂F(CN)₆; 75 mM Tris/HCl (pH 8.0), 7 mM MgCl₂, 200 mM KCl, 1 mM KCN, 2 mM AMP, 0.15 mM NADH, 1 mM ATP, 4 mM fructose-6-phosphate, 2 IU ml⁻¹ aldolase, 2 IU ml⁻¹ glycerol-3-phosphate dehydrogenase and 10 IU ml⁻¹ triose phosphate isomerase for PFK. All enzyme activities are expressed as units g⁻¹ tissue wet weight, where 1 unit = 1 mM of substrate converted per second. Enzyme activity data were analysed by analysis of variance followed by Tukey *post hoc* tests.

Thermal sensitivity of enzyme activity was expressed as $Q_{10} = (k_2/k_1)^{10/t_2-t_1}$, where k_1 is the reaction rate at temperature t_1 and k_2 is the reaction rate at temperature t_2 .

(c) Mitochondrial O₂ consumption

O₂ consumption of isolated mitochondria was measured on days 19 ($n=5$) and 25 ($n=6$) of development. Mitochondria were isolated by homogenizing pectoral muscle and liver in ice-cold 140 mM KCl, 10 mM EDTA, 5 mM MgCl₂, 20 mM HEPES, 0.5% bovine serum albumin at pH 7.3. The homogenate was centrifuged (at 4 °C) at 1400g for 5 min, and the supernatant was centrifuged at 9000g for 7 min. The pellet containing the mitochondria was resuspended in assay medium (200 µl/100 mg tissue), containing 140 mM KCl, 20 mM HEPES, 5 mM Na₂HPO₄, 0.5% BSA (pH 7.3). Fifty microlitres of the mitochondrial solution was further diluted in 500 µl of assay medium to give a final protein concentration of 547.7 µg ml⁻¹ (± 20.4 s.e.) for pectoral muscle and 817.6 µg ml⁻¹ (± 52.3 s.e.) for liver (Bicinchoninic Acid (BCA) protein assay, Pierce, USA). Oxygen consumption of the mitochondrial solution was

measured at 38–39 °C with a microelectrode (model 1302, Strathkelvin Instruments, Scotland) connected to an oxygen meter (model 782, Strathkelvin Instruments) in a temperature-controlled respiration chamber (Mitocell MT200, Strathkelvin Instruments). State III respiration rate was measured as the rate of the linear decrease in O₂ concentration after adding ADP as substrate (5 mM final solution; Johnston *et al.* 1994); state III respiration rate is reported as mM of O₂ consumed min⁻¹ mg⁻¹ of mitochondrial protein. The coupling of electron transport to oxidative phosphorylation (ATP production) was expressed as the respiratory control ratio (RCR); RCR is the ratio between state III O₂ consumption rate and the rate when ADP has been exhausted (state IV; Johnston *et al.* 1994). Data were analysed by analysis of variance followed by Tukey *post hoc* tests.

(d) Gene expression

RNA was isolated from approximately 50 mg of liver tissue using TRIreagent (Molecular Research Center, USA), following the manufacturer's instructions. One microgram of total RNA was treated with Dnase I (Sigma) and subsequently reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Australia) using hexamer primers.

Primers and dual-labelled probes were designed from chicken sequences obtained from GenBank for the mitochondrial encoded cytochrome *c* oxidase subunit 1 (*COX1*; Tsukihara *et al.* 1996) and the nuclear encoded lactate dehydrogenase isozyme A (*LDH-A*). Additionally, the nuclear encoded 'housekeeping gene' RNA polymerase II polypeptide E (*POLR2E*) served as a multiplexed endogenous control. The sequences were as follows:

COX1:

COX1-F 5'-CAAAGACATTGGCACYCTTT-3';

COX1-R 5'-CCGGGCTGTCctagtct-3';

COX1 probe 5'-(HEX)TGCTGTGCCGGCTATGCCC BHQ1).

LDH-A:

LDH-A-F 5'-GCATGCACGGAATAAAAGATG-3';

LDH-A-R 5'-TCTCCTCTTCATCAGGTTTTAGG-3';

LDH-A probe 5'-(HEX)TGCCACTGCTGCCAGTACA CA(TAMRA)-3'.

POLR2E:

RP2-F 5'-CATGCAGGAGGAGAACATCA-3';

RP2-R 5'-TGCAGAACTGCTCCAGGAT-3';

POLR2E probe 5'-(FAM)TGCTGCACCACAATCAGCG CACG(BHQ1)-3'.

Gene expression data were collected ($n=6$ chickens/sampling time) on days 10, 15, 18/19, 22, 25/26, 280. For every individual, each target gene (either *COX1* or *LDH-A*) was multiplexed with the endogenous control (*POLR2E*) in either triplicate (dilution series) or duplicate (test samples) 20 µl reactions. Each reaction contained 1 × ImmoMix (Bioline, USA: final concentrations of 1.5 U Immolase DNA Polymerase, 16 mM (NH₄)₂SO₄, 62.5 mM Tris-HCl (pH 8.3), 0.01% Tween 20, 1 mM dNTPs, 1.5 mM MgCl₂), additional MgCl₂ was added for a final concentration of 4.5 mM, 200 nM of each dual-labelled probe (Sigma-Genosys, Australia), 600 nM *POLR2E*-F primer, 200 nM *POLR2E*-R primer (Invitrogen, Australia), either 900 nM *COX1*-F

primer and 300 nM *COX1*-R primer or 200 nM of each *LDH-A-F* and *LDH-A-R* and 1 µl (ca 32 ng) of cDNA. Reactions were run on a Rotor-Gene 3000 Real-time PCR thermal-cycler (Corbett Research, USA) with the following conditions: 95 °C for 7 min, 40 cycles of 95 °C for 20 s, 68 °C for 5 s, 56 °C for 12 s, 60 °C for 45 s during which data was captured on the FAM/Sybr and JOE channels. For each gene cDNA, dilutions series from a pool of cDNA were run to calculate PCR efficiency (E) based on the equation $E = 10^{[-1/\text{slope}]}$ (Bustin 2000; Pfaffl 2001). Gene expression data were analysed with the pairwise fixed reallocation randomization test (2000 randomizations) in the Relative Expression Software Tool (Pfaffl *et al.* 2002). Expression of *COX1* and *LDH-A* at each stage of development was normalized by *POLR2E* expression and set relative to the adult expression (adults=1). For comparison gene expression data non-normalized by *POL2E* are also reported.

3. RESULTS

(a) Oxidative capacity

COX activity was significantly greater on day 22 (hatching) and day 25/26 ($F_{5,33} = 16.59$, $p < 0.001$) compared to earlier developmental stages and compared to adults (figure 1a). Up to day 18, COX activity decreased with increasing temperature between 35 and 40 °C ($Q_{10} < 1$; figure 1b). After day 18, Q_{10} values increased significantly ($F_{5,24} = 9.19$, $p < 0.0001$) with age between 35 and 40 °C, but remained stable throughout development between 30 and 35 °C ($F_{5,20} = 0.785$, $p = 0.57$; figure 1b).

COX gene expression was significantly elevated between days 10 and 26 compared to adult birds ($p < 0.01$; figure 1c), but gene expression did not change significantly during that period. Relative to adults, *POLR2E* expression was significantly lower than COX expression during development, but it was significantly elevated above adult levels during development except on day 18/19 ($p < 0.01$ for days 10, 15; $p < 0.05$ for days 22, 25/26). Nonetheless, COX gene expression remained significantly greater than in adults after correcting for changes in *POLR2E* expression during development.

Rates of mitochondrial O₂ consumption were similar in pectoral muscle and liver ($F_{1,16} = 1.25$, $p = 0.28$), but there was a significant decrease in mitochondrial O₂ consumption in both tissues (interaction $F_{1,16} = 2.76$, $p = 0.12$) between day 18/19 and day 25/26 ($F_{1,16} = 48.74$, $p < 0.001$; figure 2a). Despite similar mitochondrial O₂ consumption rates, tissues differed significantly in the extent to which electron transport was coupled to ATP production. The RCR was significantly lower in liver compared to muscle ($F_{1,17} = 46.47$, $p < 0.001$), and it did not change during development in liver.

CS activity, which controls flux through the TCA cycle, increased significantly after day 22 (hatching; $F_{5,34} = 6.13$, $p < 0.001$), and it remained elevated in adults compared to day 22 hatchlings (figure 3a).

(b) Anaerobic capacity

Activity of LDH was extremely high in early development (days 15–18), but it decreased significantly between day 18 and hatching (day 22; $F_{5,34} = 31.03$, $p < 0.001$; figure 4a). Unlike COX, the thermal sensitivity of LDH remained stable throughout development between 30 and

Figure 1. Development of oxidative capacity in liver tissue. (a) Cytochrome *c* oxidase enzyme activity increased significantly after day 18 of incubation and remained significantly higher 3 days after hatching (hatching = day 22) compared to adults. (b) The thermal sensitivity of COX enzyme activity did not change during development between 30 and 35 °C, but Q_{10} values increased significantly after day 18 between 35 and 40 °C. (c) COX gene expression remained stable during development, but it was significantly greater than in adults; for comparison, the expression of RNA polymerase (*POLR2E*) is also shown. Hatching occurred on day 22 (indicated by the arrow above the x-axis).

35 °C ($F_{5,34} = 2.43$, $p = 0.055$) and between 35 and 40 °C ($F_{5,34} = 1.74$, $p = 0.152$). Interestingly, gene expression did not follow the same pattern as enzyme activity. LDH gene expression remained constant and slightly lower than adult levels throughout development ($p > 0.05$; figure 4b).

PFK, an indicator of glycolytic activity, was significantly greater in adults compared to embryos in the egg (day 22 and before; $F_{5,33} = 7.09$, $p < 0.001$; figure 3b).

4. DISCUSSION

The requirements for embryonic growth during development would in themselves necessitate greater levels of

Figure 2. (a) Mitochondrial O₂ consumption per mg of mitochondrial protein decreased significantly from day 19 of incubation to 3 days after hatching (day 25) in both liver and pectoral muscle. (b) Aerobic metabolism was significantly more uncoupled from ATP production (respiratory control ratio) in liver compared to pectoral muscle.

protein synthesis than in adults, and increased *COX* gene expression is necessary to sustain mitochondrial proliferation during growth. The elevated expression of *POLR2E*, particularly during early development, reflects this increased protein requirement. The fact that relative to adults, *COX* gene expression is significantly greater than *POLR2E* expression, however, indicates that expression levels are beyond that necessary to maintain the early low metabolic status-quo in newly formed cells during growth. Patterns of *COX* expression, therefore, point towards mitochondrial proliferation to supply mitotically formed daughter cells, as well as to increase densities within cells. The decrease in mitochondrial oxygen consumption between days 19 and 25 indicates that increases in oxidative capacity during that period are achieved by increasing mitochondrial density within cells (Hulbert *et al.* 1991; St. Pierre *et al.* 1998). Nonetheless, an increase in cristae density just after hatching may contribute to the increase in oxidative capacity in iliofibularis muscle tissue (Eppley & Russell 1995). The significantly greater RCR in pectoral muscle is not unexpected, because the motor function of muscle cells would favour ATP production rather than the uncoupling of oxidative phosphorylation for heat production often observed in liver (Brand *et al.* 1994, 2004).

The increase in tissue-specific *COX* enzyme activity that coincides with changes in the thermal sensitivity of the enzyme toward the adult body temperature of around 40 °C is crucial, because it illustrates a mechanism that

Figure 3. (a) Activity of the principal control enzyme in the TCA cycle (citrate synthase) increased significantly after hatching (day 22). (b) PFK activity, an indicator of glycolytic activity, increased significantly after hatching (day 22), presumably reflecting a dietary shift from yolk to carbohydrate. Hatching occurred on day 22 (indicated by the arrow above the x-axis).

operates independently from transcriptional control. *COX* is situated in the mitochondrial membrane, and the thermal stability and activity of membrane proteins depend largely on membrane structure (Brand *et al.* 1994). Changes in the fatty acid and phospholipid composition of mitochondrial membranes may cause significant changes in the activity of membrane-bound enzymes that are independent from changes in enzyme concentration (St. Pierre *et al.* 1998; Hulbert & Else 2000). During the last week of development the levels of arachidonic and docosahexaenoic acids in the liver increase substantially (Noble & Cocchi 1990), and both fatty acids have been linked to increased membrane proton flux and endothermy (Brand *et al.* 1994; Wu *et al.* 2004).

Energy use (oxygen consumption) of precocious birds increases exponentially throughout development, paralleling growth. *Gallus gallus* embryos reach hatching mass as early as by 80% (day 18) of the developmental period in the egg, when growth ceases and oxygen consumption rates stabilize (Vleck & Vleck 1987). The upregulation of oxidative capacity after day 18, therefore, coincides with a decrease in growth-related energy demand, and upregulation of *COX* activity after day 18 represents the proximate physiological mechanism, and the onset of endothermic thermoregulation.

In the sequence of aerobic metabolic pathways, the electron transport chain is preceded by the TCA cycle, which functions in the synthesis of FADH₂ and NADH that act as electron donors for the electron transport chain. The timing of upregulation in CS activity lagged several days behind that of *COX* upregulation. Aerobic metabolic potential may, therefore, not be realized until CS activity is

Figure 4. (a) Anaerobic capacity, expressed as LDH enzyme activity, decreased significantly after day 18, (b) but *LDH* gene expression remained constant during development, and was not significantly different from adults; for comparison, the expression of RNA polymerase (*POLR2E*) is also shown. Hatching occurred on day 22 (indicated by the arrow above the x-axis).

sufficiently high to supply substrates for electron transport that is after hatching. In parallel with CS activity, PFK activity is significantly upregulated after hatching, and we suggest that this indicates a shift from a protein and fat-based diet (yolk) to a carbohydrate diet after hatching.

Unlike aerobic capacity, changes in anaerobic ATP producing capacity are decoupled from transcriptional control. The mismatch between the decrease in LDH activity between days 18 and 26, and the constancy of *LDH* gene expression during that period shows that anaerobic capacity is controlled post-transcriptionally. LDH is a tetrameric protein whose activity depends on stereospecific substrate binding. Catalytic rate constants of LDH change with conformational rigidity in response to temperature (Fields & Somero 1998), which, in ectothermic fish, results in pronounced changes of activity that are independent from levels of gene expression (Yang & Somero 1996). Similar conformational changes may determine LDH activity during bird development.

The independent evolution of endothermy in such distantly related organisms as synapsids, diapsids and lily flowers (Seymour 2001) may indicate that the predisposition for endothermy exists in the shared metabolic pathways and regulatory mechanisms, and does not require evolutionary innovation. Regulation of oxidative metabolism is controlled by the thyroid receptor transcription factor family and associated coactivators (Moyes 2003; Fan *et al.* 2004), and the origin of the metabolic difference between endotherms and ectotherms may be

found in differential expression of those genes. Endothermy carries a severe energetic cost that limits its selective advantage in high heat transfer situations, such as in aquatic environments or in very small organisms, in environments with unpredictable energy supply, or in extreme thermal environments (Seebacher 2003). Hence, selection may have favoured non-reversible upregulation of basal metabolic rate only rarely.

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