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Development of endothermic metabolic response in embryos and hatchlings of the emu (*Dromaius novaehollandiae*)

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Abstract

During hatching, there is a maturation of the mechanisms controlling the respiratory physiology involved in endotherm in precocial avian species. Here we examined the timing of the development of an endothermic response of oxygen uptake ($\dot{M}_{O_2}$) to an alteration of ambient temperature (T_a) in a model precocial species, the preterm and hatching emu (*Dromaius novaehollandiae*). Late stage pre-pipped and pipped embryos and hatchlings were measured for responses of $\dot{M}_{O_2}$ and shell or skin temperature (T_s) to altered T_a (ΔT_a). $\dot{M}_{O_2}$ remained unchanged in pre-pipped and internally pipped (IP) embryos at the end of 1.5 h exposure to ΔT_a of $\pm 10\,^{\circ}C$. Externally pipped (EP) embryos responded to a cooling and a warming exposure with marked increase and decrease in $\dot{M}_{O_2}$, as hatchlings responded to ΔT_a with an endothermic change in $\dot{M}_{O_2}$. The demonstration of the endothermic inverse metabolic response first appearing in EP embryos suggests that pre-EP embryos may also possess the ability to produce the endothermic inverse metabolic response, but they are restricted by the eggshell gas conductance. Late pre-pipped and IP embryos were measured again for responses of $\dot{M}_{O_2}$ to ΔT_a in air and then in a 40% O_2 environment. The metabolic response of pre-pipped embryos at 90% of incubation was partially altered by switching from air to hyperoxia. IP embryos responded to ΔT_a in 40% O_2 with apparent inverse changes in $\dot{M}_{O_2}$. The late stage emu embryo possesses the ability to produce an endothermic metabolic response at an earlier stage of development than in chickens, but this response is limited by the eggshell gas conductance.

Keywords: Emu; Embryo; Hatchling; Thermoregulation; Cooling and warming bout; Thermo-conformity; Endothermic response; Oxygen uptake; Eggshell gas conductance

1. Introduction

One of the key physiological transitions during the development of the avian embryo is the switch from ectothermy to endothermy. Late stage pre-pipped and externally pipped (EP) chicken embryos (*Gallus gallus domesticus*) show preliminary endothermic metabolic responses to cooling; that is, in response to small or gradual decreases in ambient temperature (T_a), oxygen uptake ($\dot{M}_{O_2}$) acutely remains unchanged or temporarily increases (Freeman, 1964, 1971; Tazawa et al., 1988, 1989a,b; Nichelmann et al., 2001; Black and Burggren, 2004). The endothermic metabolic response to cooling is augmented during hatching and any drop of the hatchling's body temperature (T_b) during exposure to lowered T_a is substantially mitigated (Freeman, 1967; Wekstein and Zolman, 1969; Tazawa and Rahn, 1987; Nichelmann and Tzshentke, 2002; Tazawa et al., 2004). Thus, the initial, feeble thermoregulatory competence in late pre-pipped and pipped chicken embryos matures markedly during hatching to provide the animal with the ability to maintain endothermy after hatching.

This switch from ectothermy to endothermy occurs because of the development of physiological regulatory mechanisms governing metabolism. Development of these regulatory pathways should involve changes in gene and protein expression and cellular physiology. One of the central questions in developmental physiology is whether the timing of the development of physiological regulatory pathways is fixed or can be modified by environment (Adolph, 1968; Spicer and Burggren, 2003; Burggren and Warburton, 2005). The development of endothermy provides an excellent paradigm to compare the timing of the regulatory processes within precocial avian species. Presumably, precocial birds pass through similar stages of endothermic development before gaining full thermoregulatory competence. This assumes that embryos pass through a stage where the

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endothermic metabolic response is restricted by eggshell gas conductance (i.e., O_2 conductance-limited stage) and a stage during which insufficient endothermic competence further improves with development (power-limited stage) (Tazawa et al., 1988; Whittow and Tazawa, 1991). However, the developmental pattern and time course of the maturation of physiological regulation, including the expression of metabolic genes and proteins, cellular level changes, and whole animal physiology is likely to differ between avian species, warranting comparative studies.

The ratites such as ostrich, emu and rhea comprise an important model for the comparative study of the development of thermoregulatory pathways. The $\dot{M}_{O_2}$ of emu and rhea reaches a maximum and then declines after about 75% of development (Vleck et al., 1980). During this period, it has been suggested that the animal's physiology has matured to a point at which it could hatch at any subsequent time, thus allowing these species to exhibit synchronized hatching (Vleck et al., 1980; Buttemer et al., 1988). Consequently, we predict that the physiological regulatory mechanisms governing endothermy should begin to function earlier in the ratites than in the chickens.

Two studies measured heart rate (f_H) responses to altered T_a (ΔT_a) in hatchlings and embryos of the emu (Tamura et al., 2003; Fukuoka et al., in press). Emu hatchling f_H exhibited the typical endothermic response to ΔT_a on the first day of post-hatch life (Tamura et al., 2003). Similarly, EP embryos increased f_H in response to cooling, exhibiting endothermic f_H response earlier than in chickens (Fukuoka et al., in press). Because f_H and metabolic rate are highly coupled, our previous results suggest that the regulatory mechanisms controlling the endothermic metabolic response develop earlier in the emu compared with the chicken. Thus, the emu makes a very useful comparative model for examining the physiological, cellular and genetic mechanisms behind the development of endothermy.

Here we present our initial findings on the development of the physiological regulation of the endothermic response in developing emus to changes in T_a. We hypothesize that regulatory mechanisms controlling endothermy develop prior to hatching in the emu and that the physiological machinery is in place prior to hatching but the endothermic response of the embryo is restricted by eggshell O_2 conductance. In the present report, two experiments are conducted. We first examine the development of the endothermic metabolic response in emu embryos and hatchlings. We then elucidate whether changes in $\dot{M}_{O_2}$ in response to changes in T_a during the O_2 conductance-limited stage are a muted endothermic metabolic response.

2. Material and methods

2.1. Protocol for development of endothermic metabolic response

Fertile eggs of the emu were collected in breeding yards of the Cross Timbers Emu Ranch, Flower Mound, Texas and incubated at a temperature of 36 °C and relative humidity of 30% in a Hatchrite incubator at the University of North Texas in Denton. Eggs were turned automatically every 4 h until Day 47 of incubation when they were transferred to a hatching chamber maintained at 36 °C and a relative humidity of 40%. Embryos pipped the chorioallantoic and inner shell membranes (internal pipping, IP) on Days 48–49 of a 50-day incubation. IP was identified by auscultation of embryo vocalization. IP was followed by subsequent external penetration of the eggshell (external pipping, EP) with hatching occurring a bit more than a half-day after EP.

Experiments with embryos began on Day 47 of incubation. The day of pipping and hatching events differed between individual embryos and the eggs were grouped into IP and EP embryos irrespective of chronological age. Pre-IP embryos were divided into two groups according to the days remaining until hatching, which was identified after the experiment. Embryos tested 3 days before hatching were termed −3D pre-IP embryos ($n = 5$). Two of these five −3D pre-IP embryos were tested again 2 days before hatching (referred to as −2D pre-IP embryos; $n = 7$) and one embryo was examined 2 days later as an IP embryo. Among the seven −2D pre-IP embryos, two embryos were examined again on the following day as IP-embryos ($n = 6$). Accordingly, three IP embryos were subjected to the second test during IP and the remaining three embryos were examined first during IP. EP embryos ($n = 4$) were examined once.

For experiments with hatchlings, a separate group of eggs, not previously subjected to cooling, was allowed to hatch in a hatching chamber. Hatchlings were kept in a walk-in incubator maintained at 25 °C. The day of hatching was designated as Day 0. Four hatchlings were individually marked and examined daily during the 4 days period from Day 0 to 3 of post-hatch. Water was provided ad libitum and food was not given until the end of experiment on Day 3.

The responses of $\dot{M}_{O_2}$ and shell or skin temperature of the egg and hatchling (T_s) to ΔT_a were determined over a 4.5 h period. Different sequences of exposure to ΔT_a were used for embryos and hatchlings, dictated by the temperature of their living environments. In embryos incubated at 36 °C, the sequence involved cooling from 35 to 25 °C and then warming back to 35 °C, while hatchlings brooded at 25 °C were warmed from 20 to 35 °C and then cooled back to 20 °C. Specifically, in measurements with embryos, the respirometer containing the egg was initially placed in a water bath maintained at 35 °C and $\dot{M}_{O_2}$ and T_s were measured for 1.5 h period. After the 1.5 h equilibrium and measurement period, the water in the bath was replaced quickly by the water of 25 °C and the measurement continued for another 1.5 h period. Following this measurement, the water was changed again with warm water of 35 °C and the measurements of $\dot{M}_{O_2}$ and T_s were continued for additional 1.5 h. In these cooling and warming procedures, T_a changed to an equilibrium level in about 30 min. Changes in $\dot{M}_{O_2}$ and T_s became small, but did not reach a plateau level during the 1.5 h period. Thus, the averaged values of $\dot{M}_{O_2}$ and T_s during the last 10 min period of each 1.5 h recording were used as the value of individual parameters at the altered T_a.

2.2. Protocol for metabolic response measurement in hyperoxia

Forty-six eggs were collected at the Pearson Emu Ranch, Pilot Point, Texas for this experiment and incubated under the same incubation conditions as in the first experimental series. Experiments were made on two ages of pre-IP embryos and IP embryos. The first pre-IP embryos were Day 40 embryos at 80% of incubation requiring 10 more days of development prior to hatching. The other group was embryos on Day 45 of incubation, which corresponded to 90% of incubation and were expected to pip the chorioallantoic and inner shell membranes a few days later. IP embryos were identified by auscultation of vocalization on Day 49 of incubation. The number of embryos examined in each group was 10, 14 and 12 for groups of Day 40, Day 45 and Day 49 (IP), respectively. Each embryo was examined once.

In the experiments examining potential gas exchange limitations to the endothermic response, the responses of $\dot{M}_{O_2}$ and T_s were determined first in air for a 4 h period and then in 40% O_2 environment for an additional 4 h period in individual embryos. Because the eggs were incubated at 36 °C, the exposure sequence was first cooling and then warming. After a 1 h equilibrium and measurement period at 35 °C in air, the eggs were exposed to 25 °C environment for a 1.5 h period with subsequent exposure to 35 °C environment for another 1.5 h period. The $\dot{M}_{O_2}$ and T_s were determined from the last 10 min recordings in individual air environments at 35, 25 and 35 °C. Then, the inflow gas to the respirometer was switched from air to a 40% O_2/N_2 gas mixture at 35 °C. After 1 h at 35 °C, the eggs were exposed to 25 °C for a 1.5 h period followed by a subsequent exposure to 35 °C environment for another 1.5 h period in 40% O_2/N_2 gas mixture. The $\dot{M}_{O_2}$ and T_s were determined from the last 10 min recordings at 35, 25 and 35 °C environments.

2.3. Measurements of shell or skin temperature and oxygen uptake

T_s and T_a were continuously measured using fine gauge Cu–Co thermocouples and recorded with a Powerlab 8SP data acquisition system and Chart 5.0 (ADInstruments). For measurement of egg T_s, a hole 2 mm wide was drilled in the eggshell, taking care not to injure the underlying shell membranes. The tip of the thermocouple wire was inserted into the space between the eggshell and the outer shell membrane, and the thermocouple was fixed in place with adhesive tape 2 cm^2. For hatchlings, the tip of the thermocouple was fastened with adhesive tape to the surface of the skin under the wing. The thermocouple wire was then led to the hatchling's back where the wire was held by the adhesive tape.

The $\dot{M}_{O_2}$ of embryos and hatchlings was measured using flow-through respirometry previously described for chicken eggs (Dzialowski et al., 2002). Eggs or hatchlings were placed into individual metabolic chambers (approximately volume 1.5 *l*) in a thermostatted water bath. Two thermocouple wires passed through a small hole in the respirometer and the wires were sealed in the lid. One thermocouple measured T_a in the respirometer and the other measured T_s of the egg or hatchling, as described above.

The inflow gas mixture (20.9% O_2/N_2 or 40% O_2/N_2) to the respirometer was produced by mixing pure O_2 and N_2 with a Cameron Instruments gas mixer (model GF-3). This gas mixture ran through a copper coil in the water bath to achieve the correct temperature prior to entering the respirometer. The flow through each chamber was regulated with a fine needle valve (MF-8 Respirometer Manifold, Sable Systems) and measured by either timing the flow of a bubble through a calibrated pipette or a calibrated flow meter (Sable Systems Gas Analyzer Subsampler v.2). The outflow gas from the respirometer was passed through soda lime and Drierite to remove CO_2 and water vapor prior to measurement of O_2 concentration. The percent O_2 in the inflow and outflow gas was measured using Qubit O_2 analyzers in the first experiment and a Sable System Fox O_2 analyzer in the second experiment. The $\dot{M}_{O_2}$ was calculated using the equation of Hill (1972) and expressed as ml O_2/h per egg at standard temperature, pressure and dry (STPD).

2.4. Statistical analysis

For the first experiment, the differences in $\dot{M}_{O_2}$ (or T_s) between three exposures to T_a of 35–25–35 °C in embryos and T_a of 20–35–20 °C in hatchlings were examined by one-way repeated measures ANOVA for significance at $P < 0.05$ at each individual developmental stage or age. At an individual T_a, the significance of differences in $\dot{M}_{O_2}$ and T_s between developmental stages of embryos were examined by one-way factorial ANOVA at $P < 0.05$. The significance of differences in $\dot{M}_{O_2}$ and T_s between ages of hatchlings were examined by one-way repeated measures ANOVA. Differences in $\dot{M}_{O_2}$ (or T_s) for embryos exposed to the warming and cooling bouts at the two different levels of O_2 were compared within each age group using two-way repeated measures ANOVA. When ANOVA revealed significant differences between treatments, we carried out Tukey post hoc test to detect the differences between group means. Mean values ±1S.E. are presented.

3. Results

3.1. Metabolic responses in embryos and hatchlings

Because the exposure sequence to ΔT_a was different between the embryos and the hatchlings, changes in T_s differed between them (Fig. 1). T_s during the initial exposure to T_a of 35 °C was not significantly different between the developmental stages of embryos. Also T_s after the final exposure to T_a of 35 °C was not significantly different between the different ages of embryos. However, significant differences in T_s were detected between the initial and final T_a's within the pre-IP and IP embryos ($P < 0.0001$), but it was not significantly different in EP embryos. T_s at T_a of 25 °C was not significantly different between any developmental stages. In hatchlings, T_s during warming to T_a of 35 °C was not significantly different between any ages. There were also no differences between T_s's during the initial and final exposure to T_a of 20 °C within or between any ages.

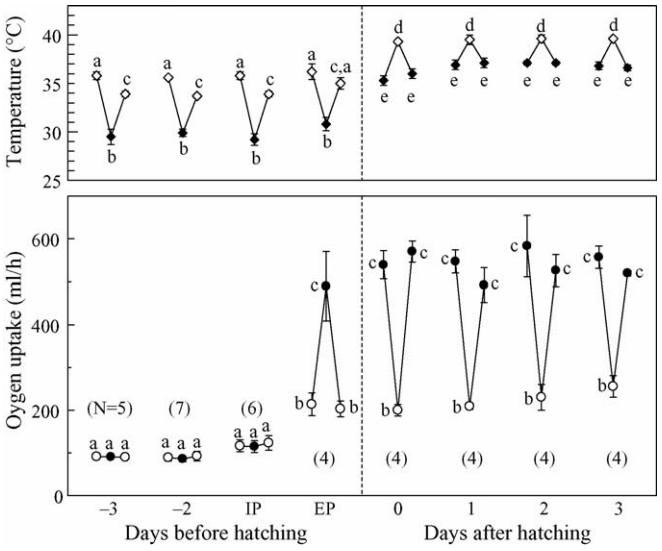

Fig. 1. Responses of shell or skin temperature (top panel, diamonds) and $\dot{M}_{O_2}$ (bottom panel, circles) to altered ambient temperature, measured in embryos and hatchlings in the emu. The number in the parentheses indicates the sample size for each measurement. Open and closed symbols indicate the measurements in warm and cool environments, respectively. Significant differences between group means are shown with different letters.

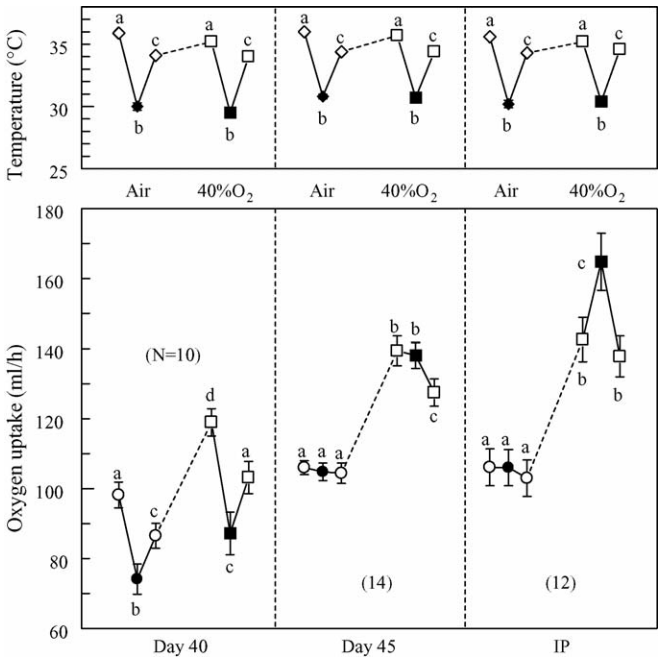

Fig. 2. Responses of shell temperature (top panel) and $\dot{M}_{O_2}$ (bottom panel) to altered ambient temperature in air (diamonds and circles) and 40% O_2/N_2 environment (squares), measured in pre-pipped emu embryos on Days 40 and 45 and IP embryos on Day 49 of incubation. Significant differences between group means are shown with different letters.

The effect of T_a on the $\dot{M}_{O_2}$ was dependent on developmental stage of the embryos (Fig. 1). At 35 °C, there was no significant difference in $\dot{M}_{O_2}$ (90–120 ml/h) between pre-IP and IP embryos. However, the $\dot{M}_{O_2}$ of EP embryos (210 ml/h) was significantly greater compared with that of pre-IP and IP embryos ($P < 0.0001$). When embryos were exposed to a decrease in T_a of 10 °C, the $\dot{M}_{O_2}$ of pre-IP and IP embryos remained unchanged, but EP embryos significantly increased $\dot{M}_{O_2}$ to 490 ml/h in response to cooling. The $\dot{M}_{O_2}$ returned to control levels during re-warming in the EP embryos.

The $\dot{M}_{O_2}$ at 35 °C in hatchlings (~200–250 ml/h) was not significantly different between any measured post-hatch days, although mean $\dot{M}_{O_2}$ tended to increase from 200 ml/h on Day 0 to 256 ml/h on Day 3. At the lower T_a of 20 °C, $\dot{M}_{O_2}$ was significantly larger than that at 35 °C at any age ($P < 0.0001$) and was not different between hatchling ages.

3.2. Metabolic responses of embryos in hyperoxia

Responses of T_s and $\dot{M}_{O_2}$ to cooling and warming in air and 40% O_2/N_2 differed as an animal developed from a pre-IP embryo at 80% of incubation (Day 40) to an IP embryo (Fig. 2). On all 3 days of the experiment, T_s's during the first and last exposure to T_a of 35 °C were significantly different both in air and in a 40% O_2 environment ($P < 0.0001$). On individual days, T_s during the first T_a exposure of 35 °C was not different between air and 40% O_2 environment. T_s during exposure to T_a of 25 °C was significantly lower compared with T_s during T_a of 35 °C in the three groups ($P < 0.0001$).

As incubation progressed, there was an apparent development of the endothermic metabolic response to cooling. On Day 40 of incubation, $\dot{M}_{O_2}$ was significantly different between all three T_a exposures in both air and 40% O_2 environment ($P < 0.0001$). Exposure to 40% O_2 resulted in a significant increase in $\dot{M}_{O_2}$ compared with the normoxic values. During both gas exposures, the $\dot{M}_{O_2}$ decreased significantly during the cooling bouts. On Day 45, there was no significant difference in $\dot{M}_{O_2}$ during the exposures to the three T_a's in air. When exposed to 40% O_2, there was a significant increase in $\dot{M}_{O_2}$ compared with exposure to air ($P < 0.0001$), but $\dot{M}_{O_2}$ did not increase further upon cooling. However, $\dot{M}_{O_2}$ decreased significantly during re-warming ($P < 0.01$). In IP embryos, the $\dot{M}_{O_2}$ in air was not different during exposure to the three T_a's as observed in the initial experiment. However, in the 40% O_2 environment $\dot{M}_{O_2}$ increased significantly during the exposure to T_a of 35 °C, followed by a further significant increase when exposed to T_a of 25 °C ($P < 0.0001$). The $\dot{M}_{O_2}$ during exposure to the initial T_a of 35 °C and the final T_a of 35 °C was not significantly different in the IP embryos during 40% O_2 exposure.

4. Discussion

4.1. Maturation of the endothermic metabolic response

The embryos of precocial birds are substantially ectothermic during most of their incubation period. For chicken embryos, the $\dot{M}_{O_2}$ at a T_a of 24 °C was about 40% of that at 37 °C when measured up to hatching (Tazawa and Rahn, 1987). This reduction in $\dot{M}_{O_2}$ in response to a decrease in T_a of 13 °C corresponded to a temperature coefficient (Q_{10}) of 2 and conformed to the Arrhenius limitation of temperature on chemical kinetics; i.e., Arrhenius-limited stage. A similar ectothermic response to cooling was observed in Day 40 emu embryos which had

completed only 80% of incubation (Fig. 2). Large decreases in ΔT_a are thought to overwhelm or swamp any feeble compensatory endothermic competence that the chicken embryo may possess (Tazawa et al., 1988, 1989a). Gradual cooling of eggs to minimize this swamping effect revealed a very weak endothermic metabolic response in chicken embryos prior to pipping with subsequent augmentation of this response during hatching (Tazawa et al., 1988). Prolonged cooling tests with graded ΔT_a, complementary to the gradual cooling tests, also revealed a feeble endothermic metabolic response in pre-pipped chicken and duck embryos (Tazawa et al., 1989a; Kuroda et al., 1990).

Eggshell O_2 conductance may be a limiting factor on $\dot{M}_{O_2}$ and the subsequent heat that embryos enclosed in the egg can produce to increase minute competence of thermoregulation, i.e., O_2 conductance-limited stage. After the onset of EP, the eggshell constraint on $\dot{M}_{O_2}$ partially lessens, because diffusive gas exchange through the chorioallantoic membrane becomes supplemented by pulmonary gas exchange (Ar et al., 1980; Menna and Mortola, 2002). However, the maximum $\dot{M}_{O_2}$ of the EP embryo still produces less heat than that needed to offset heat loss and the egg cools. Because of an insufficient ability to generate heat, endothermy evades the EP chicken embryo. This phase of development is characterized as power-limited endothermy. After chicken embryos hatch, Tb increases above the brooding T_a during the first few days and reaches a plateau (Tazawa and Rahn, 1987). The metabolic response to ΔT_a of 10 °C is an up-regulation of $\dot{M}_{O_2}$ during cooling and down-regulation of $\dot{M}_{O_2}$ during warming. The changes in $\dot{M}_{O_2}$ are inversely related to those of T_a, which first appears in chickens immediately after hatching (Tazawa et al., 2004).

$\dot{M}_{O_2}$ in pre-IP and IP emu embryos did not change in response to cooling and warming exposures (Fig. 1). The ΔT_a of 10 °C in this experiment was relatively large compared to smaller temperature changes in the previous cooling tests for chicken embryos (Tazawa et al., 1988, 1989a; Black and Burggren, 2004). Pre-pipped emu embryos even at 90% of incubation maintained a constant $\dot{M}_{O_2}$ in the face of relatively large ΔT_a for at least a 1.5 h exposure period (Fig. 2), indicating that they have a higher degree of endothermic competence than even EP embryos of the chicken.

During the EP period when $\dot{M}_{O_2}$ rapidly increases (Vleck et al., 1980), the emu embryos responded to the cooling and warming bouts by up-regulating and down-regulating $\dot{M}_{O_2}$, respectively, in an endothermic fashion (Fig. 1). As was hypothesized, the metabolic response to ΔT_a in EP emu embryos was a firm endothermic inverse pattern, indicating that thermoregulatory competence develops prior to hatching in emu, in contrast to chickens, despite the O_2 conductance limitation of the eggshell.

Although EP emu embryos were exposed to smaller ΔT_a than hatchlings, upon cooling they produced changes in $\Delta\dot{M}_{O_2}$ that were comparable to hatchlings (Fig. 1). However, the heat generated by the increased $\Delta\dot{M}_{O_2}$ seemed unable to defend body temperature of EP embryos against altered T_a, because their egg T_s during cooling decreased to the same level as pre-IP and IP embryos that had not increased $\dot{M}_{O_2}$. Unlike the changes in T_s of pre-IP and IP embryos, the egg T_s of EP embryos during the second re-warming returned to the pre-cooling, indicating that improvement of thermoregulatory competence occurs during the EP period. After hatching, $\Delta\dot{M}_{O_2}$ produced by hatchlings in response to ΔT_a was identical at all ages and concomitantly skin T_s at T_a of 20 °C was identical throughout the period examined, which was much higher than egg T_s during exposure to T_a of 25 °C and comparable with that at T_a exposure of 35 °C. From this we infer that the power-limited competence of thermoregulation matures quickly during the EP period in the emu to obtain homeothermic endothermy upon hatching.

4.2. Effect of eggshell gas conductance on the endothermic metabolic response

It has been suggested that during the late stages of embryonic development the $\dot{M}_{O_2}$ of embryos is limited by the eggshell O_2 conductance. If this is the case, an increase in the O_2 partial pressure difference across the eggshell should result in an increase in $\dot{M}_{O_2}$, because the rate of gas transport across the eggshell is a function of gas partial pressure difference and the diffusive conductance of the particular gas (Wangensteen and Rahn, 1970/71; Rahn et al., 1974; Ar et al., 1980). Here we increased the O_2 partial pressure difference across the eggshell by putting the emu eggs in a hyperoxic environment. In late stage chicken embryos, ambient hyperoxia increased $\dot{M}_{O_2}$ from 4 to 22% (Høiby et al., 1983; Stock et al., 1985; Tazawa et al., 1989b, 1992; Ar et al., 1991). During the EP stage, chicken embryos did not significantly change $\dot{M}_{O_2}$ in response to various increases in ambient O_2 concentrations (Tazawa et al., 1992). In the present experiment, the average increase of $\dot{M}_{O_2}$ in response to 40% O_2 was 21, 32 and 34% for pre-pipped emu embryos on Days 40 and 45 of incubation and IP-embryos on Day 49, respectively. These findings demonstrate that gas exchange is limited by the eggshell gas conductance in emu embryos as early as Day 40 of incubation.

If pre-pipped embryos have developed the regulatory mechanisms for endothermic thermoregulatory competence, but an eggshell gas conductance inhibits an endothermic metabolic response in air, then removal of the limitation of diffusive conductance to gas exchange by hyperoxia should allow for an up-regulation of $\dot{M}_{O_2}$ with a decrease in T_a. On Day 40 or at 80% of incubation, pre-pipped emu embryos responded to ΔT_a with a thermo-conformity change in $\dot{M}_{O_2}$ in air and this response persisted in 40% O_2 (Fig. 2). Pre-pipped embryos on Day 45 or at 90% of incubation did not exhibit this thermo-conformity, but rather showed a preliminary endothermic metabolic response in air, as obtained in pre-pipped embryos in the first experiment. Hyperoxia changed this preliminary endothermic response to the inverse response of $\dot{M}_{O_2}$ during re-warming. In IP embryos, the preliminary endothermic metabolic response in air was changed by hyperoxia to the apparent endothermic inverse response of $\dot{M}_{O_2}$. It is inferred that pre-IP embryos at 90% of incubation and IP embryos had a potential endothermic competence to regulate $\dot{M}_{O_2}$ in response to ΔT_a in air, but it was restricted by the eggshell O_2 conductance.

One of the determining factors for the maturation of an endothermic response may be the development of thyroid hormone secretion. Thyroid development reported during the late

stages of incubation in the chicken and the Japanese quail coincides with the initial development of endothermic competence under limited thermal stress in these species (Thommes and Hylka, 1977; Decuypere et al., 1979; Ockleford et al., 1983; McNabb, 1987). In the late pre-pipped and EP embryos of the chicken, minute compensatory metabolic response to cooling was blocked by thiourea, which blocks the metabolic effects of thyroid hormones (Tazawa et al., 1989b). It is possible that thyroid development is accelerated in the emu compared to the chicken, in a form of physiological heterochrony, contributing to the endothermic compensatory $\dot{M}_{O_2}$ response of the EP emu embryos. The timing and importance of thyroid development to the development of the endothermic response in the emu remains to be studied.

4.3. Conclusion

We have shown that the physiological capacity for endothermy of the emu develops prior to hatching and the endothermic metabolic response is limited by the eggshell gas conductance. The $\dot{M}_{O_2}$ of pre-pipped embryos at 80% of incubation exhibits an ectothermic response. As the pre-pipped embryos develop up to 90% of incubation, their response to ΔT_a becomes thermal independence. Once the embryo externally pips, the embryo can increase $\dot{M}_{O_2}$ during cooling in a similar fashion to the hatchlings. This is in marked contrast to chicken embryos, where the endothermic response does not develop until a day after hatching.

When the IP embryos were exposed to ΔT_a in hyperoxic environment, the thermal-independent response of $\dot{M}_{O_2}$ in air became inversely related to temperature. In addition, pre-pipped embryos at 90% of incubation responded to hyperoxia with a partial endothermic inverse response of $\dot{M}_{O_2}$. Accordingly, the physiological regulatory mechanisms governing the endothermic metabolic response are in place, but are restricted by the eggshell gas conductance in the emu. It appears that the metabolic machinery governing an endothermic response matures earlier in development in the emu compared with the chicken.

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