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Acclimation to hypothermic incubation in developing chicken embryos (*Gallus domesticus*)

II. Hematology and blood O_2 transport

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Summary

Hypothermic incubation reduces the ability of the late-stage chicken embryo to mount endothermic heat production. This study investigates whether blood O_2 transport is similarly limited by cooler incubation temperatures. Two populations of chicken embryos were incubated at 38°C and 35°C. At Hamilton–Hamburger (HH) developmental stage 41–42, hypothermic incubation had no significant effect on hematocrit (~ 26%) or [Hb] (7 g%). However, in the final stages of incubation (stage 43–44), hypothermic incubation reduced hematocrit from ~31% at 38°C to ~27.5% at 35°C. Hypothermic incubation similarly caused a reduced [Hb] from 8.5 g% (38°C) to 6.25 g% (35°C), indicating a reduction in blood O_2-carrying capacity in embryos. Incubation temperature had a strong effect on blood–O_2 affinity in late development (stage 43–44), with P_{50} at 38°C falling significantly from ~6 kPa in 38°C embyros to ~4 kPa in 35°C embryos. P_{O_2} values in chorioallantoic arterial blood at HH 41–42 were 4.3 kPa at pH 7.46 (38°C) and 3.4 kPa at pH 7.39 (35°C). In chorioallantoic venous blood these values were 9.1 kPa at pH 7.34 (38°C) and 8.1 kPa at pH 7.42 (35°C). With further development to HH 43–44, chorioallantoic arterial blood oxygenation fell to 2.4 kPa at pH 7.54 (38°C) and 2.8 kPa at pH 7.52 (35°C). Similarly, P_{O_2} in chorioallantoic venous blood fell slightly to 7.7 kPa at pH 7.42 (38°C) and 7.4 kPa at pH 7.48 (35°C). Collectively, these data reveal that beyond HH 41–42, 35°C embryos experience retarded hematological development, and the findings that support the observed delayed metabolic response to acute temperature changes.

Key words: chicken embryo, *Gallus domesticus*, thermoregulation, hypothermic incubation, hemoglobin–oxygen affinity, heterokairy.

Introduction

The avian embryo undergoes a thermoregulatory transition from being a poikilotherm in its egg to being a homeotherm at or shortly after hatching. Thermoregulation in hatchling chicks relies on non-shivering endogenous heat production to control body temperature (T_b) (Whittow and Tazawa, 1991; Roberts, 1996; Tazawa et al., 2001), but the physiological adjustments and the timing of their onset as they occur during the transition to homeothermy have not been well described. In a companion study (Black and Burggren, 2004), we showed that late-stage chicken embryos (HH 41–44; Hamburger and Hamilton, 1951) maintained their rate of O_2 consumption (and presumably the attendant heat production) during acute reductions in body temperature, suggesting the onset of at least some of the physiological control mechanisms necessary for thermoregulation. Black and Burggren (2004) also investigated the influence of chronic hypothermic incubation in chicken embryos. Not surprisingly, fundamental developmental parameters such as growth and the time of hatching events (internal and external pipping) were slowed by chronic hypothermic incubation (35°C). These changes, however, were all relative – i.e. when expressed as a percentage of total incubation period, embryonic growth and hatching events occurred at predictable points in development in hypothermic chicks. These data suggested that temperature alone did not induce heterokairic changes (heterokairy is the change in the relative timing of onset of physiological processes and their control; Spicer and Burggren, 2003). However, late-stage embryos incubated under hypothermic conditions were not as proficient at maintaining metabolism during a gradual decline in ambient temperature (T_a) as were populations incubated at the normal 38°C, suggesting that chronically low incubation temperature interfered with the normal onset of maturity of their thermoregulatory system, and in particular the ability to elevate oxygen consumption and heat production.

Maturity of both the respiratory and cardiovascular systems is crucial to ensure the efficient delivery of O_2 to support metabolic heat production and regulate body temperature. In this study we examine the effects of hypothermic incubation on the metabolic and thermoregulatory physiology of chicken embryos. We examine hematology, blood O_2 transport

characteristics, and *in vivo* arterial and venous blood P_{O_2} and pH of late-stage chicken embryos incubated in either normothermal conditions (38°C) or hypothermal conditions (35°C). Our goal was to determine if differences in blood parameters might underlie our finding that lower incubation temperature negatively affects the ability of embryos to respond metabolically to decreases in ambient temperature. Specifically, we hypothesized that chronic incubation at low ambient temperature (35°C) would modify both blood O_2-carrying capacity and hemoglobin–O_2 binding affinity in the late-stage chicken embryo, and that these changes might result in a modified thermoregulatory phenotype.

Materials and methods

Source and incubation of eggs

Fertilized White Leghorn eggs (*Gallus domesticus* L.) were obtained from Texas A&M University (College Station, Texas, USA) and shipped to the University of North Texas (Denton, TX, USA) where they were incubated in commercial incubators. All experimental procedures were approved by The University of North Texas' Institutional Animal Care and Use Committee.

Samples were incubated at 38.0°C (control), or 35.0°C (hypothermic) and a relative humidity of 60%. To determine the gross effects of incubation temperature on development, nine or more embryos incubated at each temperature were staged for developmental maturity on days (D) 13–14, 15–16, 17–18 and 19–20 (Hamburger and Hamilton, 1951). Hypothermic embryos have a slower rate of development than embryos incubated in control conditions, so staging was completed to determine the length of incubation required for the 35°C embryos to reach developmental stages equivalent to those of the 38°C embryos.

All subsequent experiments were conducted on embryos at the following stages: HH 41–42, reached on days 15–16 for 38°C and days 17–18 for 35°C; HH 43–44, reached on days 17–18 for 38°C and days 19–20 for 35°C.

Surgical protocol

Each egg was candled to locate a large chorioallantoic membrane (CAM) vessel. The overlying shell and membranes were removed without disturbing the underlying vasculature. The tip of a 30 gauge needle attached to a 1 ml syringe was inserted into a CAM artery and/or vein against the direction of blood flow. Dead space in the needle (approximately 10 µl) was filled with heparinized saline (53 mg 100 ml^{-1}). A minimum of 200 µl of blood was drawn for determination of P_{50}, hemoglobin concentraction and hematocrit, while 100 µl was drawn for measurement of arterial and venous P_{O_2} and pH or from separate embryos for ATP and 2,3-BPG determinations. Caution was taken to avoid contamination by the accidental uptake of fluid.

Hemoglobin and hematocrit determination

A 50 µl blood sub-sample was injected into an OSM2 Hemoximeter (Radiometer, Copenhagen) for determination of the hemoglobin content of the blood (g%). Hematocrit was measured by drawing 10 µl of blood into a heparinized capillary tube, sealing one end, and centrifuging for 5 min in a micro-hematocrit centrifuge. The hematocrit was determined as the volume of packed red blood cells to the total volume of blood.

P_{50} *determination*

A 50 µl sub-sample of freshly drawn arterial blood was added to a cuvette containing 4 ml of Hemox solution, 20 µl of Additive A, and 10 µl of Anti-Foaming agent, all solutions from TSC Scientific (New Hope, PA, USA). The sample was placed in the measurement cuvette of a HEMOX™ Analyzer (TSC Scientific), which continuously records blood O_2 saturation while changing blood P_{O_2}, plotting the results as a continuous rather than punctuated O_2 equilibirum curve. Blood placed in the measurement cuvette was allowed to thermally equilibrate at either 35°C or 38°C, depending on the experimental protocol. Measurements of percentage saturation and P_{O_2} were simultaneously recorded on a computer using Chart software (ADInstruments, Colorado Springs, CO, USA). Humidified air was gently bubbled through the sample until 100% O_2 saturation was obtained (approximately 7 min). The sample was then bubbled with humidified nitrogen for 7 min, until O_2 saturation of the blood reached zero. The sample was then reoxygenated through equilibration with air for an additional 7 min. Recorded saturations and P_{O_2} values were plotted to obtain a sigmoidal O_2 equilibrium curve. Two P_{50} measurements were determined on each sample, with the replicates averaged to generate the P_{50} for that sample.

Hill plots and 'n' coefficient determination

The percentage saturation (S) of the blood corresponding to P_{O_2} values of 1.6 kPa, 2.5 kPa, 4.1 kPa, 6.6 kPa, 10.5 kPa and 16.6 kPa was determined for each O_2 equilibrium curve to characterize the shape of the O_2-equilibrium curve for creation of a Hill plot. Values were converted as follows: $x=\log P_{O_2}$, $y=\log[S/(100-S)]$, where S is saturation. A linear Hill plot with $\log P_{O_2}$ as the independent variable and $\log[S/(100-S)]$ as the dependant variable was fitted with a regression line (Sigmaplot 2001). The 'n' coefficient, the slope of the regression line, summarizes the shape of the curve.

Arterial and venous P_{O_2} *and pH*

A 100 µl sample of CAM arterial or venous blood was injected into thermostatted P_{O_2} and pH electrodes (Microelectrodes Inc., Bedford, NH, USA) connected to a blood gas analyzer (Blood Gas Meter BMG 200, Cameron Instrument Company, Port Aransas, TX, USA) for simultaneous measurements of P_{O_2} and pH.

*Physiological (*in vivo*)* O_2*-equilibrium curves*

The O_2-binding properties of embryonic chick hemoglobin are highly influenced by pH (Bohr shift). To allow direct

comparisons of blood properties between incubation temperatures without the confounding effects of differences in acid–base status, most previously published O_2 equilibrium curves have been generated at 7.47, a pH characteristic of late-stage embryos incubated at 38°C (Tazawa, 1980). We elected to measure the P_{50} of each blood sample at two pH values, 7.47, corresponding to previous measurements of arterial pH for embryos incubated at 38°C, and at 7.57, the arterial pH for embryos incubated at 35°C (Tazawa, 1973, 1980). Bohr shifts were calculated for each developmental stage and incubation temperature. Then, using the calculated Bohr shifts, a series of stage- and treatment-specific O_2-equilibrium curves determined *in vitro* at pH values of 7.27–7.87 (in 0.1 pH unit increments), were constructed. Each *in vivo* blood P_{O_2} value collected from a sample was then plotted on the O_2-equilibrium curve most closely corresponding to that sample's pH. The *in vivo* O_2-equilibrium curves used for placement were then removed to reveal the *in vivo* O_2-equilibrium curves at the mean arterial and venous pH values.

ATP and 2,3-bisphosphoglycerate concentrations

A 100 µl sample of blood was added to an equal volume of trichloroacetic acid (TCA) for ATP measurement and to 300 µl of TCA for 2,3-bisphosphoglycerate (2,3-BPG) measurement. Samples designated for ATP measurement were stored on ice for a maximum of 2 h before measurement. Samples for 2,3-BPG measurement were stored at –70°C for 4 weeks before measurement, conditions that maintain stable amounts of organic compounds. Concentrations of organic phosphates were determined with an end-point spectrophotometry technique at a wavelength of 345 nm (Sigma–Aldrich measurement kit; St Louis, MO, USA).

Statistical analyses

All data was tested for normality of distributions (Shapiro–Wilks normality test) and equality of variances before specific statistical analyses were performed. Data from within and between stages and at the two incubation temperatures were tested for statistical significance with an ANOVA. Significance between groups was determined with a Student–Newman–Keuls (SNK) multiple range *post hoc* test. All statistical tests were conducted using SAS software and decisions were made with a 0.05 level of significance. Values are means ± 1 S.E.M.

Results

Blood oxygen transport

Incubation temperature had no significant effect ($P>0.05$) on hematocrit or [Hb] in HH 41–42 embryos (Fig. 1). Further development to HH 43–44 in 38°C embryos resulted in a significant increase in both hematocrit ($F=6.17$, $P=0.001$) and [Hb] ($F=6.28$, $P=0.001$). However, the stage-dependent increases in 38°C incubated embryos did not develop in 35°C embryos, where no significant increase occurred in either hematocrit or [Hb].

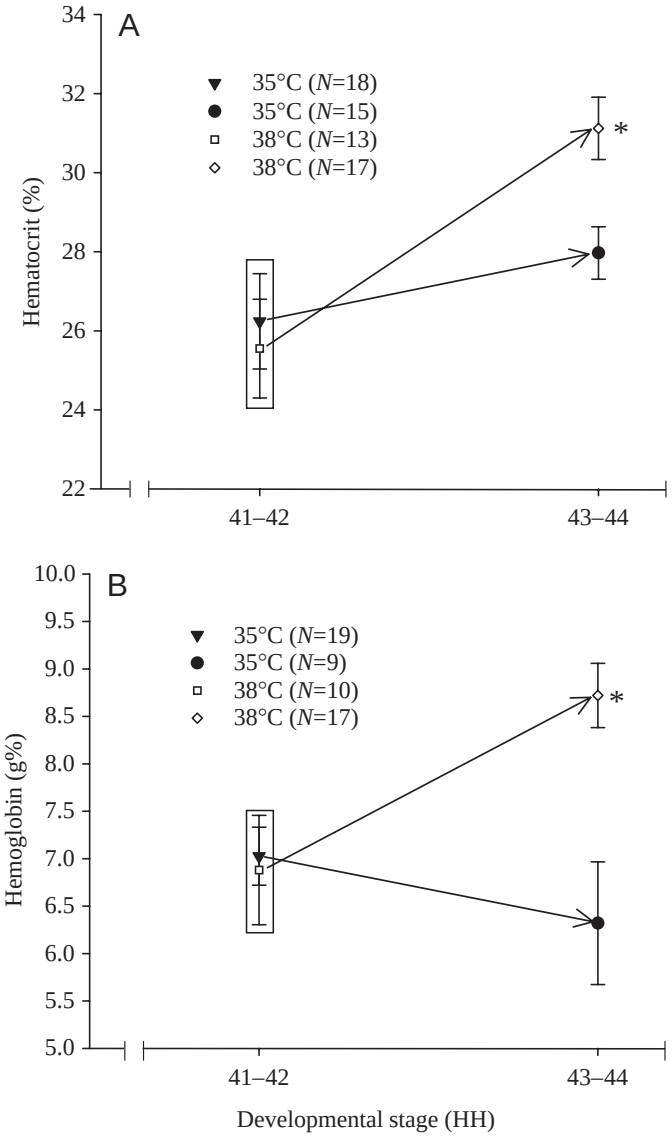

Fig. 1. Hemoglobin concentration and hematocrit for chicken embryos incubated at 38°C and 35°C at HH 41–42 and 43–44. Mean values within a box are not significantly different from each other ($P>0.05$). An asterisk indicates a significant difference between stages, at the same incubation temperature ($P<0.05$). Values are means ± 1 S.E.M.

At HH 41–42, blood of both 35°C and 38°C embryos had relatively high P_{50} value, low O_2 affinity, and was insensitive to changes in measurement temperature (Fig. 2). By HH 43–44, the blood–O_2 affinity of the 38°C embryos at 38°C had not changed significantly, but was now temperature sensitive, with P_{50} dropping from 6.0 kPa±0.4 at 38°C to 4.4 kPa±0.4 at 35°C (Fig. 2B, $F=4.55$, $P<0.0001$). In considerable contrast to the 38°C embryos, the blood of the 35°C embryos remained temperature insensitive at HH 43–44, showing no change in O_2 affinity measured at 38°C (Fig. 2B, SNK, $P>0.1$).

Temperature- and stage-induced O_2 affinity changes in 35°C and 38°C embryos were not accompanied by any significant change in the shape of the O_2 equilibrium curve, summarized

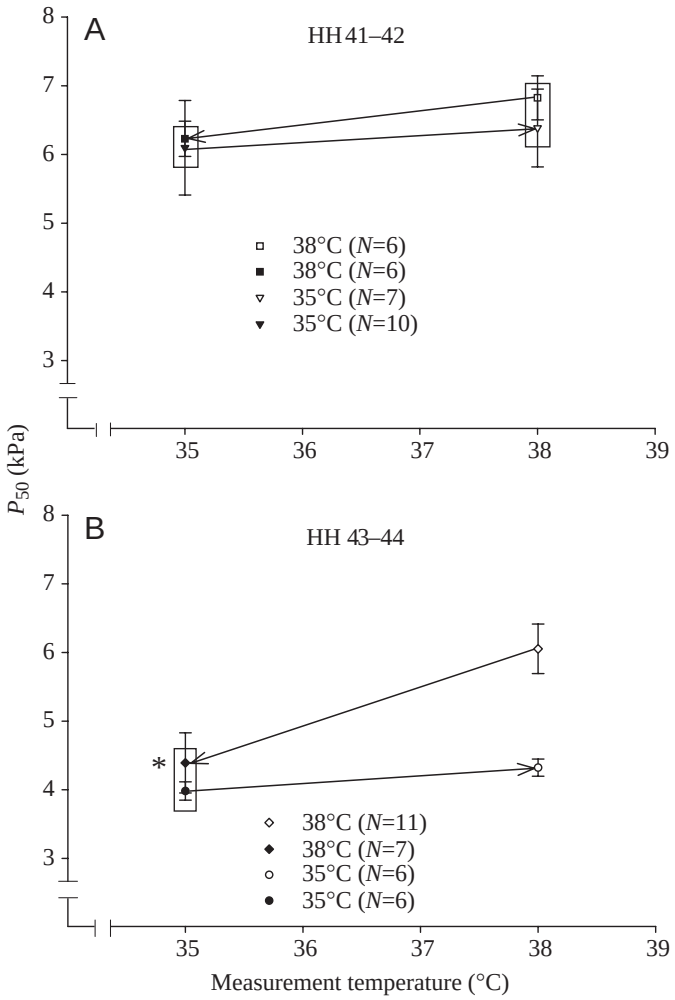

Fig. 2. P_{50} of embryonic blood at pH 7.47 from chickens incubated at 38°C and 35°C at HH 41–42 (A) and HH 43–44 (B). Plotting convention as described in title of Fig. 1. Values are means ± 1 S.E.M.

by the Hill coefficients (*n*). Values of *n* ranged from 2.42±0.056 to 2.64±0.059 for the HH 41–42 embryos (Fig. 3A inset). Similarly, at HH 43–44 there were no significant changes in the shape of the curves for either the 35°C or 38°C embryos, with Hill coefficients ranging from 2.10±0.19 to 3.10±0.11 (Fig. 3B inset).

In vivo *respiratory properties of arterial and venous CAM blood*

Blood from the CAM artery, carrying relatively deoxygenated blood from the tissue of the embryos to the CAM gas exchanger underlying the shell, had low P_{O_2} values, in the order of 3.7–4.2 kPa (Table 1). 35°C embryos at HH 41–42 had a significantly lower arterial P_{O_2} than the 38°C embryos (SNK, $P<0.05$), but between HH 41–42 and 43–44 the arterial P_{O_2} of the 35°C embryos remained unchanged, while that of the 38°C embryos dropped significantly (SNK, $P<0.001$).

Venous CAM blood (draining the CAM gas exchanger) of HH 43–44 embryos had similar P_{O_2} values regardless of incubation temperature (between 8.1±0.3 kPa and 9.1±0.4 kPa for 35°C and 38°C, respectively). Only the 38°C embryos experienced a significant drop in venous P_{O_2} as development continued (SNK, $P=0.024$). Within each incubation temperature and stage the venous P_{O_2} was always significantly higher than that of the arterial blood (SNK, $P<0.001$).

There were no significant differences in the pH of arterial or venous blood between incubation temperatures or stages, and the pH of the arterial blood was not significantly different from the venous blood for any of the treatment groups (Table 1).

When plotted on O_2-equilibrium curves representing *in vivo* arterial and venous pH conditions, the *in vivo* physiological range of P_{O_2} and saturation experienced by the embryo can be determined for each group (Fig. 4). HH 41–42 embryos maintained a 60–80% O_2 saturation in 'arterialized' blood in both incubation temperature groups. With further development to HH 43–44, oxygen saturation climbed slightly to 65–90% in both groups. There was more variation in CAM arterial blood saturations. Minimum saturation values of about 20% were evident in 35°C embryos at HH 43–44 compared with less than 10% in 38°C embryos at HH 43–44.

Organic phosphate modifiers of hemoglobin–oxygen affinity

Blood [ATP] ranged from approximately 80–120 μmol dl^{-1} (Fig. 5A), while blood [2-3-BPG] ranged from approximately

Table 1. *Physiological range of the partial pressure of oxygen (P_{O_2}) and pH of arterial and venous CAM blood from embryos incubated in 35°C and 38°C*

Temperature (°C)	Stage (HH)	Chorioallantoic arterial blood		Chorioallantoic venous blood	
		P_{O_2} (kPa)	pH	P_{O_2} (kPa)	pH
35	41–42	3.4±0.3 (7)[1]	7.39±0.05 (6)	8.1±0.3 (8)	7.42±0.04 (7)
35	43–44	2.8±0.3 (7)	7.52±0.03 (7)	7.4±0.3 (7)	7.48±0.05 (7)
38	41–42	4.3±0.3 (8)[1,†]	7.46±0.07 (9)	9.1±0.4 (11)*	7.34±0.04 (10)
38	43–44	2.4±0.2 (10)[†]	7.54±0.05 (10)	7.7±0.4 (10)*	7.42±0.04 (10)

Values are means ± 1 S.E.M., with sample size in parentheses.
[1]A significant difference between incubation temperatures ($P<0.05$).
*A significant difference between stages for 38°C embryos ($P<0.05$).
[†]A significant difference between stages for 38°C embryos ($P<0.001$).

0.1–0.6 μmol dl^{-1} (Fig. 5B). The 35°C embryos had significantly lower concentrations of ATP at HH 41–42 (F=4.20, P=0.012), but by HH 43–44 there was no significant difference in [ATP] between incubation temperatures. Concentrations of 2,3-BPG were not significantly different between incubation temperatures at HH 41–42. By HH 43–44 the 38°C embryos experienced a significant decline in blood 2,3-BPG, giving the 35°C embryos a significantly higher [2,3-BPG] at the latest stages (F=6.99, P<0.001).

In general, 35°C embryos maintained constant [ATP] and [2,3-BPG] between HH 41–42 and 43–44, while the concentrations of both organic phosphates tended to decrease between these same stages in the 38°C embryos (Fig. 5).

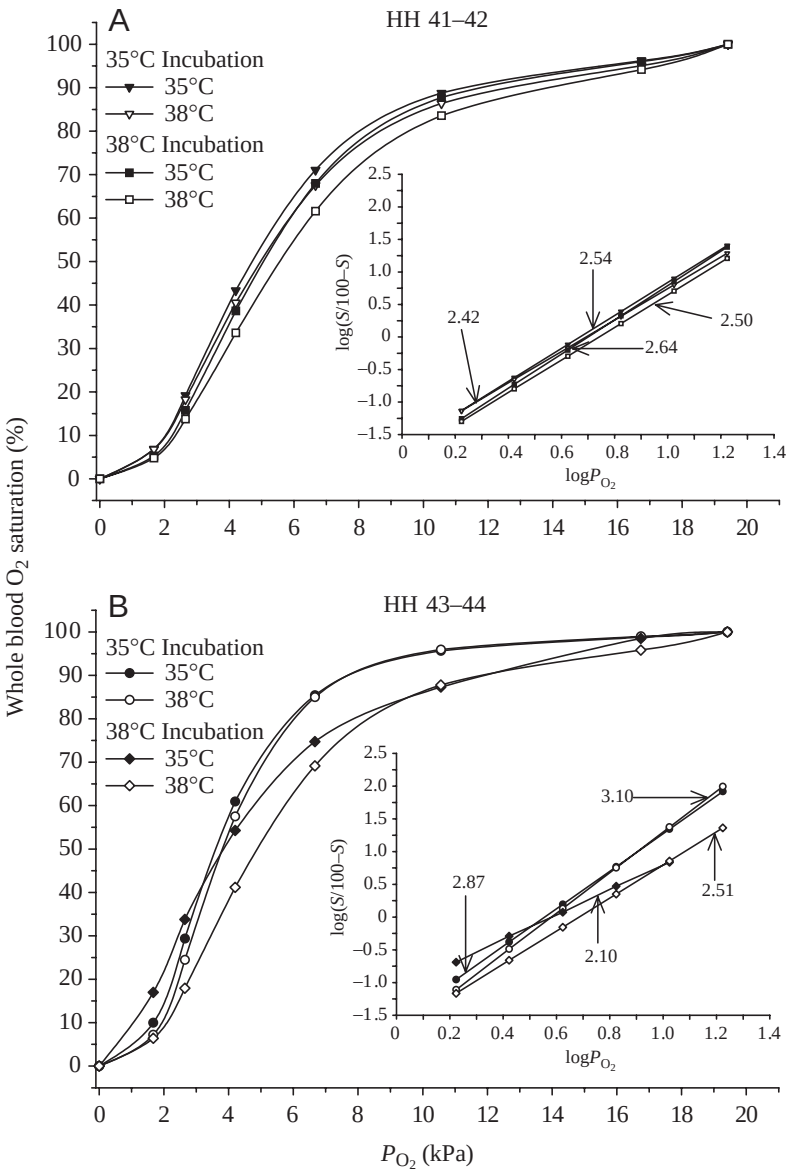

Fig. 3. Whole blood O_2 equilibrium curves for embryos incubated at 35°C and 38°C and measured at 35°C or 38°C at stages HH 41–42 (A) and HH 43–44 (B). Insets are Hill plots showing corresponding Hill coefficients (n). The P_{50} values of these curves correspond to the P_{50} values plotted in Fig. 2.

Discussion

Incubation temperature and blood O_2-carrying capacity

Hematocrit and hemoglobin content for 38°C late-stage chicken embryos agree with values reported in the literature (Tazawa et al., 1971; Tazawa, 1972; Baumann et al., 1983; Dzialowski et al., 2002). The 38°C embryos showed a significant increase in both hematological parameters between HH 41–42 and 43–44, again in line with previous reports (Tazawa et al., 1971).

Hypoxic incubation can increase both hematocrit and [Hb] of embryonic chicken blood (Dusseau and Hutchins, 1988; Dzialowski et al., 2002), suggesting that increasing the total O_2-carrying capacity of the blood may be an important acclimation response ensuring adequate O_2 delivery to tissues prior to hatching. It is interesting to note that, in contrast to the 38°C embryos, there was no similar significant increase in hematocrit and [Hb] values in 35°C embryos with development from HH 41–42 to HH 43–44 (Fig. 1). Perhaps hypothermic incubation precludes important acclimation responses that operate at 38°C.

The lower O_2-carrying capacity of the 35°C embryos corresponds to the significant increase in Hb–O_2 affinity between HH 41–42 and 43–44. The concurrence of these events suggests that the 35°C embryos may be maximizing the loading of O_2 at the respiratory surface to compensate for restriction of total O_2-carrying capacity. Blood gas data supports this notion, with the 35°C HH 43–44 embryos creating CAM venous (‘arterialized’) O_2 saturations of close to 90% (Fig. 4). A left-shifted curve should allow the embryo to saturate blood more completely at P_{O_2} values *in vivo* similar to those experienced by the 38°C embryos. HH 41–42 embryos incubated at 35°C and all 38°C embryos maintained CAM venous saturations of less than 80%, corresponding to the findings of other studies (Misson and Freeman, 1972; Tazawa and Mochizuki, 1978; Tazawa, 1980).

Incubation temperature and Hb–O_2 affinity

Hemoglobin–O_2 binding affinity represents a compromise between the conflicting needs of efficient loading of O_2 at the respiratory surface and adequate delivery of O_2 to the metabolically active tissues (Reeves, 1984). Balancing these requirements becomes more complicated in the late stages of incubation in the chicken embryo. An egg incubating in normal temperature and O_2 conditions becomes increasingly hypoxic *in ovo* as the restrictions of diffusive gas exchange across the egg shell fail to meet the growing O_2 demands of the rapidly

developing embryos (Wagensteen et al., 1970; Rahn et al., 1974; Ar et al., 1980; Tazawa, 1980; Reeves, 1984). As a consequence, air cell P_{O_2} declines from 18.6 kPa in early incubation to approximately 13.3 kPa just prior to pipping (Reeves, 1984; Burggren et al., 2000). Venous P_{O_2} follows that same pattern, decreasing from 10.6 kPa at D12 of incubation to 8.1 kPa by D17 (Tazawa et al., 1971). Following an inverted pattern, blood P_{CO_2} increases during development, resulting in a progressive decrease in blood pH (Tazawa, 1973).

Maintaining adequate O_2 loading in the face of these developmental respiratory transitions is assisted by Hb–O_2 affinity transitions in the chicken embryo. Development is characterized by a progressive left-shift in the O_2 equilibrium curve, from a P_{50} of 7.0 kPa at D8 to P_{50} values at D18 down to 4.2–5.8 kPa (Tazawa, 1980) or even as low as 4.1 kPa (Reeves, 1984). In the present study, chicken embryos incubated normothermically at 38°C generally followed the expected pattern, with a decrease in P_{50} from 6.8 kPa at HH 41–42 down to 6.0 kPa at HH 43–44 (days 17 and 18 of incubation), with P_{50} values at these later stages slightly higher than values reported in the literature. Embryos incubated at 35°C followed the expected pattern more closely, reaching a P_{50} of about 4.0 kPa by HH 43–44.

Why hemoglobin(s) of HH 41–42 embryos remain insensitive to a 3°C change in measurement temperature is unclear. The sensitivity of whole blood to changes in temperature depends on its hemoglobin types (Baumann and Meuer, 1992). By D8 of incubation the blood of the chicken embryo contains three types of hemoglobins: a hatching hemoglobin (HbH), which plays only a minor role in O_2 transport, and two adult hemoglobins (HbA and HbD). In adult chickens the ratio of HbA to HbD is approximately 3:1, but HbD plays a larger role in the chicken embryo, existing at a ratio of 1:1.05 at D9 of incubation (Baumann and Meuer,

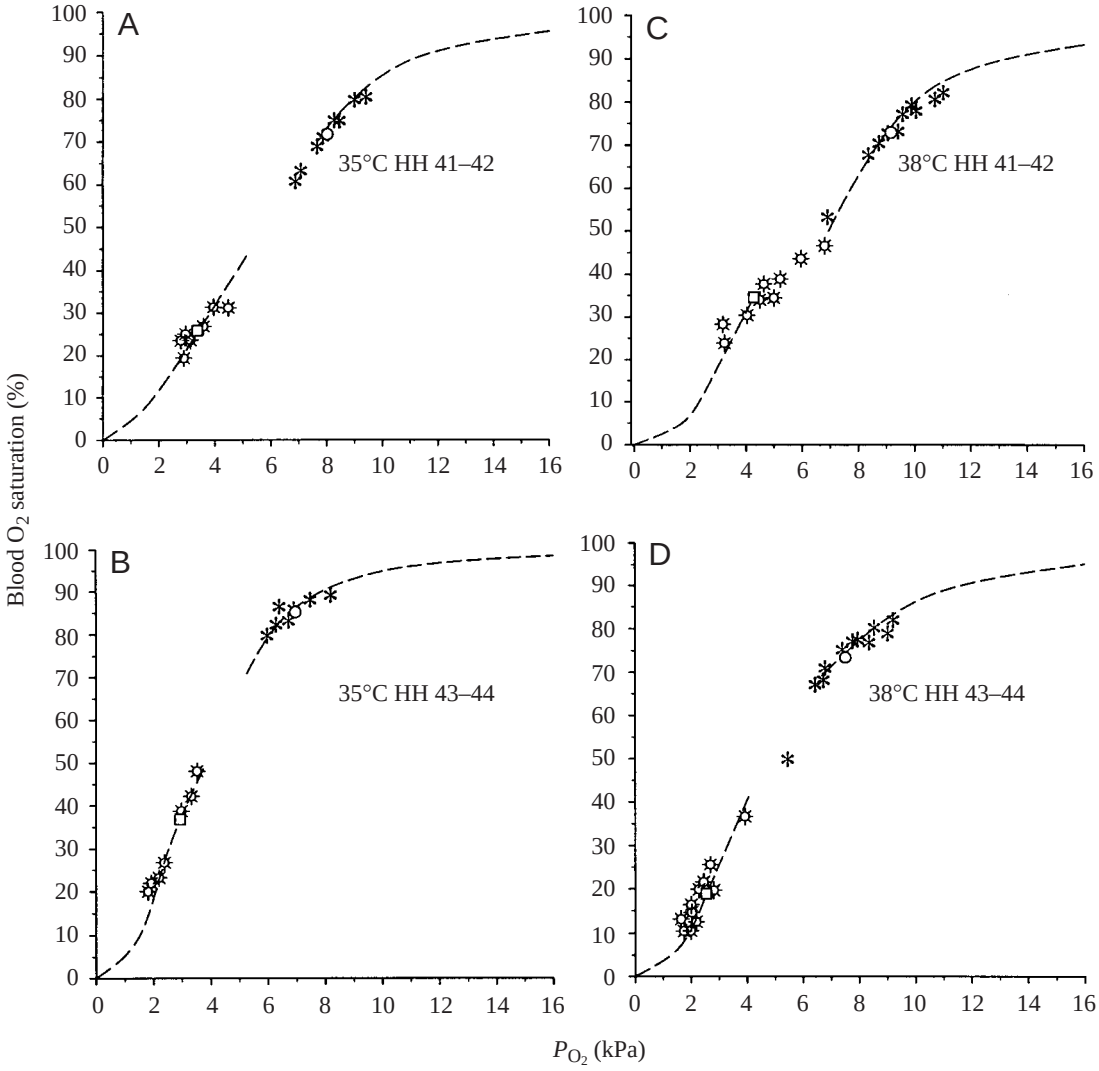

Fig. 4. Physiological O_2 equilibrium curves for embryos incubated in 35°C (A,B) and 38°C (C,D). Each point represents a single blood sample from less oxygenated blood in the CAM arteries (open asterisk) or more oxygenated blood in the CAM veins (black asterisk). The broken curves represent the O_2 equilibrium curves at the physiological pH. The open circle indicates the mean venous P_{O_2} and the open square indicates the mean arterial P_{O_2} (Table 1).

1992). The ratio of HbA to HbD continues to change in the late stages of incubation, and although the influence of each hemoglobin on the O_2 binding affinity of the blood is not yet fully understood, it is possible that the ratio of HbA to HbD may have an important impact on the temperature sensitivity and other characteristics of embryonic hemoglobin. Incubation temperature may impact the rate at which the ratio of HbA to HbD increases and in this fashion delay the onset of blood temperature sensitivity.

In addition to being altered by changes in embryo acid–base status, temperature and Hb type, O_2 affinity of embryonic chicken blood is, of course, also affected by organic phosphate concentrations (Baumann and Meuer, 1992). Up until approximately D12 of incubation in the chicken, ATP is the primary organic modifier of hemoglobin–O_2 affinity (Misson and Freeman, 1972; Bartlett and Borgese, 1976; Baumann and Meuer, 1992; Hochachka and Somero, 2002). The increase in hemoglobin–O_2 affinity between D8 and D18 of incubation corresponds to the period where metabolic rate reaches its maximum pre-pipping plateau, and hypoxia begins to develop within the egg. At this point the aerobic production of ATP is more difficult to achieve and there is a fall in blood [ATP] (Bartlett and Borgese, 1976; Nikinmaa, 1990). As [ATP] drops, anaerobic production of 2,3-bisphosphoglycerate (2,3-BPG) increases and this organic phosphate then acts as the primary allosteric modifier of hemoglobin until after hatching, when inositol polyphosphate (IPP) becomes the adult allosteric modifier (Bartlett and Borgese, 1976; Nikinmaa, 1990).

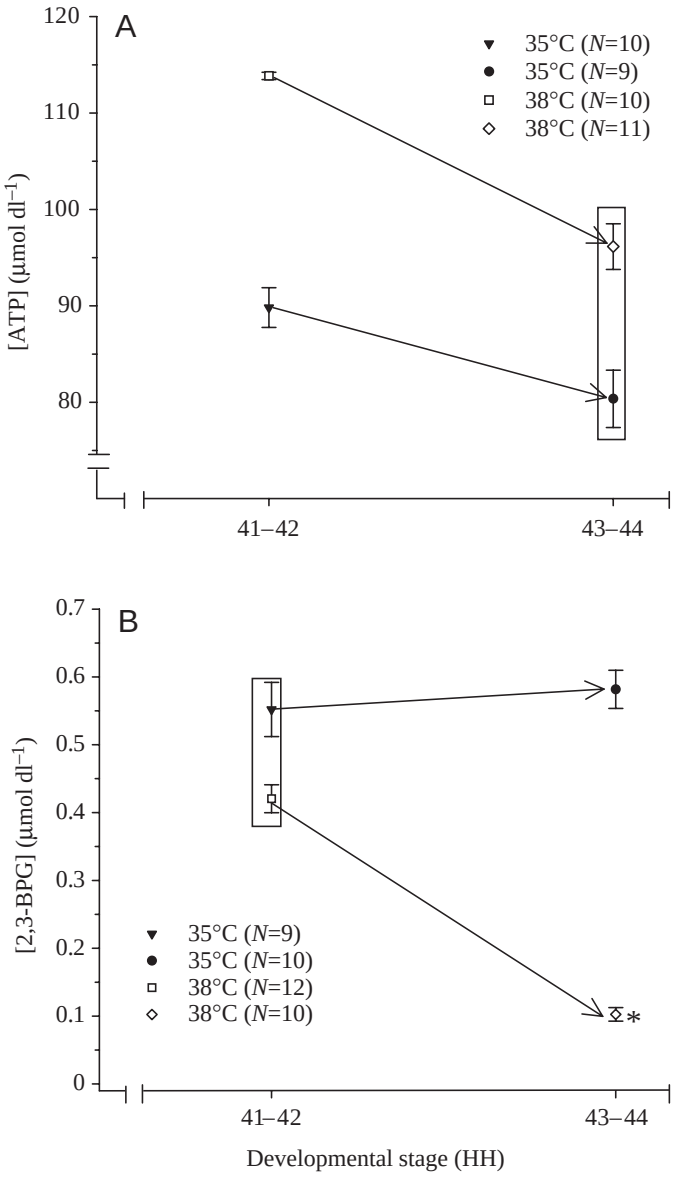

Fig. 5. Adenosine triphosphate (ATP) and 2,3-bisphosphoglycerate (2,3-BPG) concentrations in blood of chicken embryos incubated at 38°C and 35°C. Plotting conventions described in title of Fig. 1. Values are means ± 1 S.E.M.

Contrary to our expectations, the significant increase in hemoglobin–O_2 affinity between HH 41–42 and 43–44 in the 35°C embryos is not associated with any change in [ATP] or [2,3-BPG]. This suggests that in hypothermic embryos organic phosphates played little role in modifying hemoglobin–O_2 affinity, and that incubation temperature and the nature of the hemoglobins present were probably the primary reasons for the significant drop in P_{50} observed by HH 43–44. The effect of temperature on organic phosphate concentration in the chicken embryo has never been examined but, in general, [ATP] and [2,3-BPG] from these experiments are similar to those of numerous other studies on chicken embryos (Isaacks and Harkness, 1975; Isaacks et al., 1976; Bartlett and Borgese, 1976; Baumann and Meuer, 1992) and other precocial embryos including turkeys, pheasants, guinea fowl and ducks (Isaacks et al., 1976; Bartlett and Borgese, 1976).

Although incubation temperature had no apparent affect on organic phosphate concentrations in late-stage chicken embryos, hypoxic incubation results in an earlier switch from ATP to 2,3-BPG. Exposure to hyperoxia allows chicken embryos to maintain high levels of ATP throughout incubation and delay the developmental switch to 2,3-BPG (Ingermann et al., 1983; Baumann et al., 1986). The arterial and venous pH and P_{O_2} values collected for embryos incubated at 35°C and 38°C were similar at each stage of development (Table 1), indicating that a reduction in incubation temperature does not increase the level of hypoxic stress beyond what is normally experienced by the late-stage embryo. From previous research, it is clear that O_2 regulates both the concentrations of organic phosphates and the timing of the switch from ATP to 2,3-BPG. It is not surprising then, that incubation temperature alone did not result in obvious changes in the patterns of organic phosphate concentration in the late-stage chicken embryo.

Oxygen transport and consequences for metabolism and thermoregulation

The adult chicken can increase pulmonary ventilation and cardiac output to elevate O_2 transport rates in support of increased $\dot{V}_{O_2}$. In contrast, $\dot{V}_{O_2}$ in the chicken embryo is limited by diffusive gas exchange across the shell, especially in later developmental stages when O_2 demands are increasing

(Tazawa et al., 1992). Essential changes occur in both [Hb] and blood Hb–O_2 affinity as the embryo reaches these final, most aerobically demanding stages. Interestingly, embryos incubated at 35°C and 38°C maintained surprisingly similar rates of O_2 consumption at equivalent stages of development – less than might be predicted with from typical temperature effects on animal metabolism (Black and Burggren, 2004). Yet, the mechanisms for achieving these similar O_2 demands appear quite different. Initially, HH 41–42 embryos present themselves very similarly at both incubation temperatures, with near identical basal metabolic rates, hematocrits, total hemoglobin contents and temperature-insensitive hemoglobins with low O_2 affinities. However, by HH 43–44 important differences emerge between 35°C and 38°C embryos. The 38°C embryos have developed significantly higher hematocrit and [Hb], and so presumably have a greater potential O_2-carrying capacity than the 35°C embryos. The 35°C embryos, on the other hand, have hemoglobins with higher O_2-binding affinities and are able to more completely saturate the blood at the respiratory gas exchanger surface. The similar metabolic rates of these two different incubation groups indicate that either set of compensatory changes can effectively support the O_2 demands of embryos.

Although basal metabolic rates of embryos incubated at 35°C and 38°C are not significantly different (Black and Burggren, 2004), 35°C embryos were less effective in responding metabolically to acute decreases in ambient temperature in late incubation. We speculate that at HH 43–44 the more temperature-sensitive hemoglobins of the 38°C embryos might provide an advantage over the temperature-insensitive hemoglobins of 35°C embryos at the same developmental stage. When exposed to decreases in ambient temperature, the hemoglobins of the 38°C embryos at HH 43–44 could have a higher O_2 affinity at the cooler respiratory surface and a lower O_2 affinity at the site of the warmer tissues, maximizing O_2 loading at the CAM. The presence of descending temperature gradients from the core of the embryo to the surface of the shell in the developing chicken egg is well established, making the temperature-sensitive nature of the hemoglobin molecule in the 38°C embryos an efficient strategy for O_2 transportation (Tazawa et al., 1988; Turner, 1990). The 35°C embryos at HH 43–44 maintain a high O_2-affinity regardless of temperature, which apparently still enables them to achieve efficient loading of O_2 but might inhibit efficient delivery of O_2 to the embryo. As one might expect, the 35°C embryos experienced a significant decline in $\dot{V}_{O_2}$ with a much smaller acute ambient temperature decrease than the 38°C embryos, suggesting a less developed or otherwise compromised capacity for thermoregulation through elevated aerobic metabolism.

Beyond HH 41–42, 35°C embryos experienced retarded hematological development and a delayed metabolic response to acute temperature changes compared to embryos incubated in control conditions of 38°C. These data providing the first supporting evidence in an endothermic species for ‘heterokairy’ – a within-individual change in the timing of the onset of regulatory mechanisms during development (Spicer and Burggren, 2003). Exposure to decreased ambient temperature represents an ecologically relevant threat to the chicken hatchlings (Whittow and Tazawa, 1991), especially the 35°C embryos with their retarded thermoregulatory abilities. A series of future experiments will examine how the hematological and metabolic differences revealed in these studies affect the ability of the hatchlings to cope with such thermal stress. Of course, physiological temperature-dependent differences induced by incubation temperature differences are not likely to arise in a naturally incubated chicken embryo receiving conductive heat from the incubating hen. However, even though the experimental induction of such changes is not directly relevant to the ecology of natural incubation, the present study has shown that using incubation temperature as a developmental variable is clearly a very useful tool for examining the capability of developing physiological and hematological systems to respond to environmental challenges *in ovo*.

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