

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

I. Developmental effects and chronic and acute metabolic adjustments

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Summary

Chronic exposure to a low incubation temperature clearly slows the development of poikilothermic chicken embryos (or any other poikilotherms), but little is known about the more subtle developmental effects of temperature, especially on physiological regulatory systems. Consequently, two populations of chicken embryos were incubated at 38°C and 35°C. When compared at the same development stage, incubation temperature had no significant impact on embryonic survival or growth. Moreover, the relative timing of major developmental landmarks (e.g. internal pipping), expressed as a percentage of development, was unaffected by temperature. The ability to maintain the rate of oxygen consumption (\dot{V}_{O_2}) during an acute drop in ambient temperature (T_a) improved from Hamburger–Hamilton (HH) stages 39–40 to 43–44 in the 38°C but not the 35°C

populations. Late stage (HH43–44) embryos incubated at 38°C could maintain \dot{V}_{O_2} (approximately 27–33 $\mu\text{l g}^{-1} \text{min}^{-1}$) during an acute drop in T_a to approximately 30°C. However, at the same stage 35°C embryos acutely measured at 38°C were unable to similarly maintain their \dot{V}_{O_2} , which fell as soon as T_a reached 36°C. Thus, while hypothermic incubation does not affect gross development (other than would be predicted from a simple effect of Q_{10}), there is a significant delay in the relative timing of the onset of thermoregulatory ability induced by hypothermic incubation.

Key words: chicken embryo, *Gallus domesticus*, thermoregulation, hypothermia, incubation, development, heterokairy.

Introduction

The developing chicken embryo is a poikilotherm, and consequently any decrease in ambient temperature precipitates a series of cascading events: embryo temperature follows the decline in ambient temperature (T_a), which in turn reduces metabolic rate and energy production, slowing embryonic growth and maturation (Tazawa et al., 1988; Pelster, 1997). The most obvious consequence of experimental hypothermic incubation is that it will take longer for a chronically hypothermic embryo to reach the required level of maturity for hatching. Not surprisingly, embryonic growth and the development of physiological systems in chicken embryos incubated at 35°C are retarded compared to embryos incubated at the optimum temperature of 38°C, with an additional 4 days required for hatching (Tazawa et al., 1988).

Less obvious than the simple lengthening of the embryonic period by chronic hypothermia, but potentially of considerable physiological importance, is the impact on both the absolute and relative timing of the onset of different physiological processes and their regulation. Indeed, temperature changes during development often have complex affects that go beyond simply accelerating or decelerating the development of the

embryo as a unit (Bull, 1980; Temple et al., 2001; Gutzke and Crews, 1988; Johnston et al., 1996; Spicer and Burggren, 2003). Susceptible to change in the chronically hypothermic embryo, then, are the absolute as well as the relative timings of onset of different physiological process and the systems that regulate them. This phenomenon has recently been termed ‘heterokairy’ to differentiate it from the rather broadly and loosely used ‘heterochrony’ (see Spicer and Burggren, 2003).

The shift from poikilotherm to homeotherm in bird embryos is an important physiological transition, beginning within the last 20% of incubation. This thermoregulatory transition requires heat-producing metabolism and supporting oxygen transport to mature as essential steps in the precise regulation of body temperature. Such developmental events may be particularly susceptible to temperature-induced qualitative adjustments. In this and the following study on the late stages of incubation of the chicken embryo (*Gallus domesticus*), we examine both acute and chronic temperature influences on metabolism, changes in blood-oxygen transport supporting metabolism, and thermoregulatory responses of chicken embryos. Our goal is to determine if chronic hypothermia

(35°C) alters standard developmental patterns, providing additional evidence for heterokairy. As a self-contained embryo that is very well characterized anatomically and reasonably well understood physiologically, the chicken embryo is an excellent model to examine how the changes in the thermal environment can quantitatively and qualitatively influence the developmental timeline.

In this first study, we test the hypothesis that chronic incubation in hypothermia (35°C) not only lengthens the embryonic period but also alters the relative timing of hatching events, the normal pattern of changes in metabolic activity, and the ability of the embryo to respond physiologically to acute decreases in T_a .

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.

Populations of eggs were incubated at 38.0°C (control), or 35.0°C (hypothermic), all at 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 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 through hatching to determine the length of incubation required for the 35°C embryos to reach developmental stages equivalent to the 38°C embryos.

All subsequent metabolic experiments were conducted on embryos at the following stages: stage 39–40, reached on days 13–14 for 38°C and days 15–16 for 35°C; stage 41–42, reached on days 15–16 for 38°C and days 17–18 for 35°C; stage 43–44, reached on days 17–18 for 38°C and days 19–20 for 35°C.

Rates of survival and timing of hatching

Fertilized eggs ($N=40$ for 38°C and $N=32$ for 35°C) were incubated as described above. Eggs were candled every 2 days (D) from D4 to D18 of incubation, to determine survival. From D19 to D25 of incubation eggs were candled daily to determine survival at the pre-pip stage, internally pipped stage, externally pipped stage and hatching success. Survival rates were calculated as number of eggs alive compared to total number of eggs incubated at that temperature. Counts of eggs on each day were converted to relative frequencies and plotted for each day of incubation. Egg counts also allowed the calculation of percentage survival and the timing of pipping and hatching events.

The effect of temperature on length of incubation to internal

pping, external pipping and hatching was expressed by the temperature quotient (Q_{10}) calculated using the van't Hoff equation:

$$Q_{10}=(k_2/k_1)^{10/(t_1-t_2)},$$

where k_1 and k_2 are the timing of events expressed as a percentage of incubation at temperatures $t_1=38^\circ\text{C}$ and $t_2=35^\circ\text{C}$, respectively.

Static \dot{V}_{O_2} measurements at 35°C and 38°C

Six eggs from each incubation temperature, at each stage, were implanted with thermocouples. Thermocouple wires were inserted immediately beneath the shell through a 0.5 mm hole, and held in place with a 1 cm² piece of tape. Preliminary experiments revealed no detectable thermal gradient from inside the embryo's body to the shell exterior in incubating eggs. Consequently, 'surface temperature' measured immediately under the shell is assumed to be embryo temperature. The eggs were placed in an air-tight, water-tight respirometer (240 ml) through which air was pumped continuously at 70–75 ml min⁻¹. Water and carbon dioxide were removed from the outflow air by passing it through Drierite™ and soda lime, respectively. Analysis of O₂ content of the air and calculation of oxygen consumption was carried out using an oxygen analyzer (model FC-1B, Sable Systems Inc., Henderson, NV, USA). The ventilated chamber was partially submerged in a programmable water bath (ISOTEMP 1028P, Fisher Scientific, Hampton, NH, USA) and allowed to equilibrate to incubation temperature for a minimum of 30 min before measurements were started. Measurements of oxygen consumption were recorded simultaneously with egg temperature and ambient temperature (Chart software and Powerlab data acquisition system, ADInstruments, Colorado Springs, CO, USA). All values of \dot{V}_{O_2} ($\mu\text{l O}_2 \text{ g}^{-1} \text{ min}^{-1}$) were expressed on an embryo mass-specific rather than egg mass-specific basis, unless otherwise indicated.

Basal \dot{V}_{O_2} measurements made at constant T_a were designated as 'static' \dot{V}_{O_2} measurements (in contrast to measurements during gradual cooling or warming, described below). Static measurements were always recorded first at the chronic incubation temperature of that particular egg. After this initial measurement (for approximately 30 min), the water bath temperature was changed at a rate of 3°C h⁻¹ to expose the egg to the other treatment incubation temperature (e.g. 35°C if the incubation temperature was 38°C) for a minimum of 2 h. \dot{V}_{O_2} was then determined for the same egg at the other treatment temperature as described above.

\dot{V}_{O_2} measurements during gradual cooling

\dot{V}_{O_2} was also measured during gradual cooling, because the T_a at which basal \dot{V}_{O_2} (and the accompanying heat production) can no longer be maintained during cooling – the T_{crit} – is an indication of the maturity of thermoregulatory ability. Hence, eggs ($N \geq 6$ for each incubation temperature) with implanted thermocouple wires were placed in metabolic chambers. Chamber and egg surface temperature were simultaneously

recorded as described above. Static basal \dot{V}_{O_2} measurements were determined at the egg's chronic incubation temperature before the start of the gradual cooling protocol. For those eggs incubated at 38°C, measurement of \dot{V}_{O_2} at 38°C was followed by continuous \dot{V}_{O_2} measurements during gradual cooling (3°C h^{-1}) of the water bath and metabolic chamber to a final egg temperature of 30°C. For those eggs incubated in hypothermia (35°C), a static \dot{V}_{O_2} measurement was determined at 35°C and the temperature of the water bath was then increased to 38°C. Eggs were allowed a minimum of 2 h at 38°C before entering the same gradual cooling protocol described for the control eggs incubated at 38°C. Preliminary measurements indicated that the temperature in the deep interior of the egg was always identical to surface temperatures at the cooling rates used in these experiments.

Stage and mass determination

Upon completion of \dot{V}_{O_2} measurements, embryos were killed by placing eggs in a container containing halothane vapor. Each embryo was then removed from the egg, blotted dry with towels and its wet mass determined using a microbalance (Denver Instrument Company, Denver, CO, USA). The ventricle was dissected from the embryo, blotted dry and weighed. The embryo was staged to confirm that it was at the expected developmental stage, weighed, and then dried in a 40°C oven for 2 days for subsequent determination of dry mass.

Statistical analysis of \dot{V}_{O_2} and mass data

All data were tested for normality of distributions (Shapiro–Wilks normality test) and equality of variances. SAS (Version 8.0) was used to conduct all statistical analyses. All statistical decisions were made with a 0.05 level of significance and all values are presented as means \pm S.E.M.

Significance of differences between static oxygen consumption measurements at 35°C and 38°C for embryos incubated at the same temperature was determined using a paired *t*-test. The same oxygen consumption data were tested for significance of differences between incubation temperatures at a particular measurement temperature using independent *t*-tests.

The rate of oxygen consumption during gradual cooling at each stage and each incubation temperature were tested with repeated-measures ANOVA (block design) to determine T_{crit} , the temperature at which a significant decrease in rate of oxygen consumption from control values occurred during the cooling protocol. Comparisons of oxygen consumption rates during gradual cooling between stages and incubation temperatures were performed using a one-way ANOVA. Student–Newman–Keuls (SNK) multiple range tests were performed following each ANOVA for *post-hoc* detection of specific differences between means.

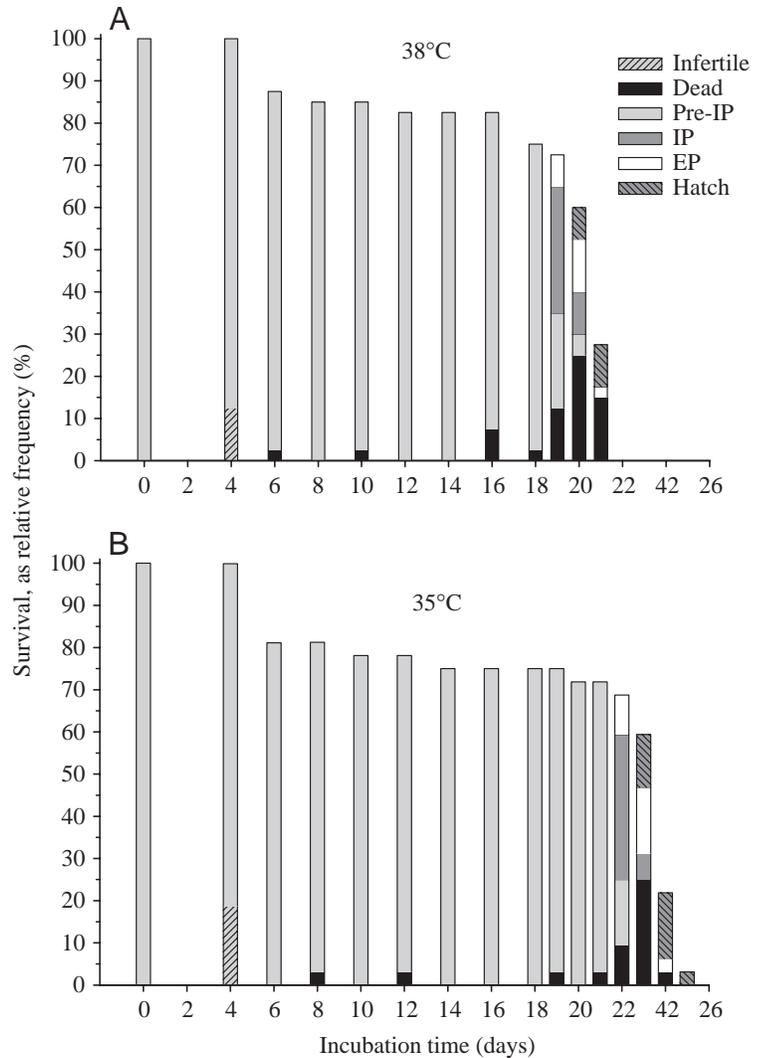


Fig. 1. The length of incubation, survival through incubation, and timing of hatching events (IP, internal pipping; EP, externally pipping) for embryos chronically incubated at 38°C (A) and 35°C (B). Sample sizes are indicated in Table 1.

Differences in embryo wet mass, dry mass, and heart mass were determined using ANOVA and SNK multiple range test.

Results

Survival and timing of hatching events

The length of incubation, survival, and timing of hatching events for embryos incubated in normothermal conditions (38°C) and hypothermal conditions (35°C) are summarized in Figs 1 and 2. In the early stages of incubation there was little difference in survival rates between the two incubation temperatures. At both temperatures there was an initial loss due to the presence of infertile eggs between D0 and D4 (12.5% at 38°C and 18.7% at 35°C, respectively). Embryos incubated at 38°C had a slightly higher rate of survival (75.0%) between D4 and internal pipping than 35°C embryos (68.8%), and a

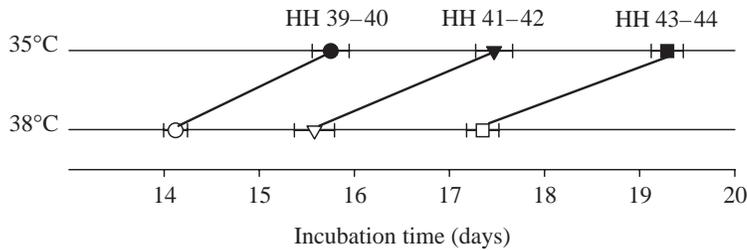


Fig. 2. Developmental stage and corresponding day of incubation (mean \pm 1 S.E.M.) for embryos incubated at 35°C and 38°C. Sample sizes are indicated in Table 1.

higher overall survival (48% and 31.3% for 38°C and 35°C, respectively).

Hypothermic embryos required an incubation time of 23.7 days, an average of 3.1 days longer than 38°C embryos (Table 1). Internal pipping in 35°C embryos occurred an average of 2.9 days later than in normothermal conditions, where it occurred at 22.2 days. Similarly, external pipping occurred at 22.8 days, 3.0 days later than in warmer embryos. The effect of hypothermic incubation on the relative timing of each hatching event was determined by converting the length of each event interval to a percentage of total incubation length, and then determining Q_{10} (Table 1). Incubation in hypothermic conditions had no effect on the relative timing of each hatching event, as indicated by Q_{10} values of 1.00 for each event interval. Thus, internal pipping occurred at 93% of development and external pipping at 96% of development, regardless of incubation temperature.

Growth and developmental progress

Embryos incubated at 35°C reached HH 39–40 on D15–D16 of incubation (Fig. 2), and by D17–D18 they were at HH 41–42. After 19–20 days 35°C embryos were at HH 43–44. Developmental progress of hypothermic embryos in late stages of incubation generally lagged behind 38°C embryos by 2–3 days.

Growth of embryos at each incubation temperature was determined by measuring both wet and dry embryo mass (Fig. 3). At the earliest stage (39–40) there was no significant

difference ($P>0.05$) in wet mass or dry mass between embryos incubated at 35°C (7.60 \pm 1.03 g and 0.79 \pm 0.13 g, respectively) and 38°C (5.54 \pm 0.29 g and 0.51 \pm 0.03 g, respectively). All embryos experienced a significant increase in both wet and dry mass from stage 39–40 to stage 41–42 (15.71 \pm 0.81 g and 2.38 \pm 0.17 g for 35°C embryos and 13.75 \pm 0.40 g and 2.20 \pm 0.11 g for 38°C embryos, $F=62.29$, $P<0.01$), but there was still no differences attributable to incubation temperature. By stage 43–44 the 35°C embryos had a significantly higher wet mass (22.26 \pm 0.98 g) than 38°C embryos (19.42 \pm 0.51 g) ($F=62.29$, $P<0.01$), but there was no significant difference in dry embryo mass (4.16 \pm 0.25 g and 4.62 \pm 0.36 g, respectively).

No apparent differences in embryo body mass were detected between incubation temperatures at stage 41–42; nonetheless, the wet ventricle mass of 35°C embryos (0.22 \pm 0.04 g) was significantly larger than that of the 38°C embryos at stage 41–42 (0.10 \pm 0.01 g) ($F=5.72$, $P=0.0016$), and the ratio of ventricle mass to embryo mass was significantly larger in 35°C embryos at all stages examined ($F=30.66$, $P<0.0001$) (Fig. 4). Between stages 41–42 and 43–44 there was no significant change in ventricle mass in embryos at either incubation temperature. Because embryonic wet mass significantly increased as development progressed, the ratio of ventricle mass to embryo mass decreased significantly in all embryos ($F=30.66$, $P<0.0001$).

Static \dot{V}_{O_2} at 35°C and 38°C

There was a general decrease in mass-specific \dot{V}_{O_2} with increasing stage (Fig. 5), with stage 43–44 embryos from both incubation temperatures having a significantly lower \dot{V}_{O_2} than at stage 39–40. Mean mass-specific \dot{V}_{O_2} for 35°C embryos ranged from 25 \pm 1.7 $\mu\text{l g}^{-1} \text{min}^{-1}$ at stage 39–40 to 13 \pm 0.35 $\mu\text{l g}^{-1} \text{min}^{-1}$ at stage 43–44 (Table 2). The mass-specific \dot{V}_{O_2} of 38°C embryos ranged from 33 \pm 0.82 $\mu\text{l g}^{-1} \text{min}^{-1}$ at stage 39–40 to 27 \pm 2.2 $\mu\text{l g}^{-1} \text{min}^{-1}$ at stage 43–44 (Table 2). Mass-specific \dot{V}_{O_2} of 35°C and 38°C embryos at the same developmental stage were not significantly different.

Each group of embryos, regardless of incubation temperature or stage, experienced a significant change in

Table 1. Effects of chronic incubation at 35°C and 38°C on the length of incubation and the relative amount of development each day

Event interval Start \rightarrow End	Sample size		Survival to event end (%)		Days to end event		Time to event (% of total)		Temperature effect (Q_{10})
	35°C	38°C	35°C	38°C	35°C	38°C	35°C	38°C	
0 \rightarrow IP	13	16	40.6	40.0	22.2	19.3	93.5	93.6	1.0
0 \rightarrow EP	9	9	28.1	22.5	22.8	19.8	96.1	96.2	1.0
0 \rightarrow H	9	9	28.1	21.8	23.7	20.6	100	100	1.0
IP \rightarrow EP	9	9	69.0	56.2	0.6	0.5	2.6	2.6	1.0
EP \rightarrow H	9	9	100.0	77.7	0.9	0.8	3.8	3.8	1.0

Incubation is divided into intervals based on hatching events from day 0 (0), internal pipping (IP), external pipping (EP) and hatching (H).

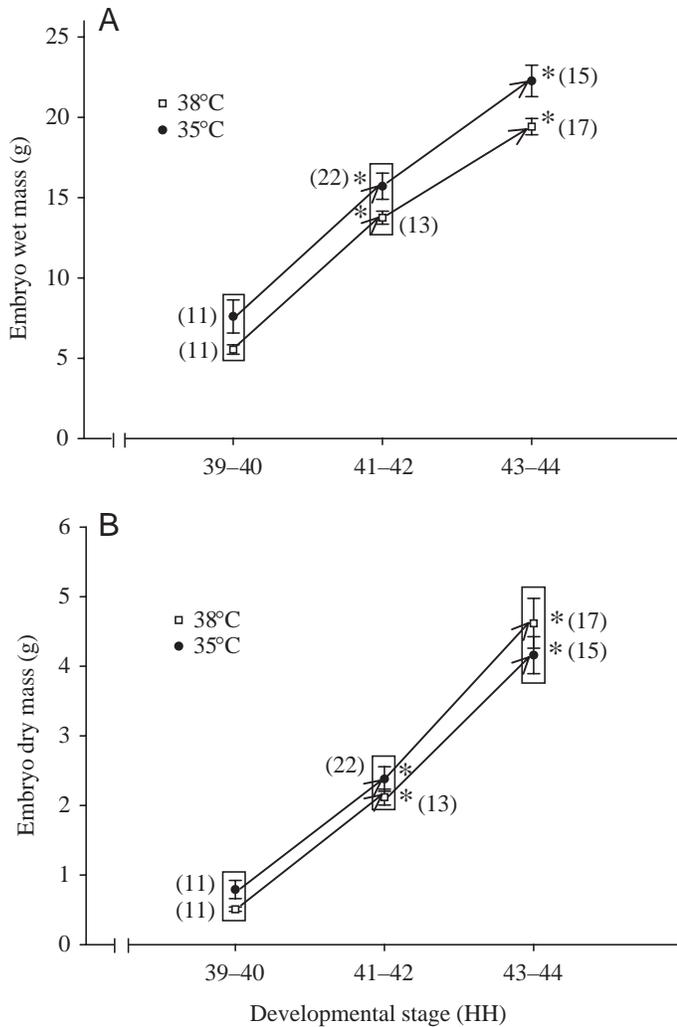


Fig. 3. Wet (A) and dry (B) mass of embryos incubated at 35°C and 38°C. A box surrounds points that are not significantly different from each other ($P>0.05$). Asterisks indicate a significant increase in mass from the previous stage, at the same incubation temperature ($P<0.05$). Values are means \pm 1 S.E.M. N values are in parentheses.

oxygen consumption after 2 h at the opposite incubation temperature (Matched-pairs t -tests with the probability for each group; $P<0.018$, Fig. 5).

\dot{V}_{O_2} during acute cooling

Some embryos from each stage and each incubation temperature were able to increase \dot{V}_{O_2} briefly during the earliest stages of the cooling protocol. However, all embryos eventually experienced a significant decline in \dot{V}_{O_2} at some point during the gradual 8°C drop in T_a (Fig. 6). The youngest embryos of each incubation temperature had significantly higher \dot{V}_{O_2} values than the older embryos (Fig. 7). This was evident for the entire duration of the cooling protocol in the 35°C embryos (Fig. 7A), and until T_a fell to 36°C in the 38°C-incubated embryos (Fig. 7B). There were no significant differences in \dot{V}_{O_2} during gradual cooling between stages

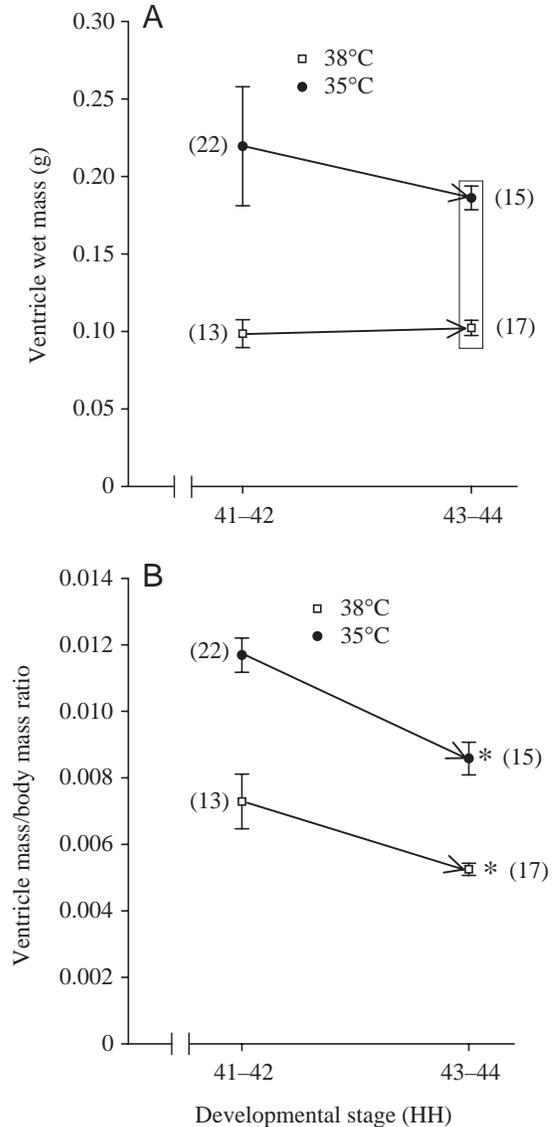


Fig. 4. Wet ventricle mass (A) and ratio of ventricle mass to embryo mass (B) for embryos incubated in 35°C and 38°C. Plotting convention as described for Fig. 3.

41-42 and 43-44 for either incubation temperature, except for the initial static measurement at 35°C for the 35°C embryos.

Although there are no significant differences in gradual cooling \dot{V}_{O_2} between the 35°C and 38°C embryos compared at each stage, there are important differences in the temperature (T_{crit}) at which they first experienced a significant decline in \dot{V}_{O_2} from the baseline rate at 38°C (Fig. 7). Fig. 7A shows that the youngest embryos (stage 39-40) experienced a significant decrease in \dot{V}_{O_2} when T_a reached 34°C. By stage 41-42 (Fig. 7B) the 38°C embryos had a T_{crit} of 34°C, while 35°C embryos had a higher T_{crit} of 36°C. Just prior to hatching at stage 43-44 (Fig. 7C), 38°C embryos can endure a drop in T_a of 8°C (T_{crit} of 30°C) before suffering a significant decrease in \dot{V}_{O_2} , but the stage 43-44 35°C embryos were not as efficient, showing a significant decline at a T_{crit} of just 36°C – 1/4 the

Table 2. Rate of oxygen consumption by chicken embryos as a function of incubation and measurement temperatures and developmental age

Temperature incubation/ measurement (°C)	Day of incubation	\dot{V}_{O_2} ($\mu\text{l min}^{-1} \text{egg}^{-1}$)	Reference
38/38	16	382	Tazawa, 1973
38/38	16	417	Tazawa et al., 1992
38/38	16	350	Pearson et al., 1996
38/38	16	333	Howe et al., 1996
38/38	16	333±7 (9)*	Present study
38/38	18	400	Tazawa, 1973
38/38	18	433	Tazawa et al., 1992
38/38	18	383	Pearson et al., 1996
38/38	18	420	Dzialowski et al., 2002
38/38	18	450±38 (5)*	Present study
35/35	16	417±30 (6)*	Present study
35/35	18	383±20 (10)*	Present study
35/35	20	317±7 (12)*	Present study

*Values are means ± 1 S.E.M. (*N* = number of eggs).

temperature decrease at which the 38°C began to show a \dot{V}_{O_2} decline. \dot{V}_{O_2} values for each of these groups at 38°C and at T_{crit} are summarized in Table 3.

Discussion

Survival, timing of hatchling events and extension of developmental timeline

Bird embryos are poikilothermic throughout incubation, making them susceptible to fluctuations in body temperature dictated by the incubation environment (Tazawa et al., 1988, 1989; Whittow and Tazawa, 1991). One of the primary and most obvious impacts of chronic hypothermia on poikilotherms is a slowing of physiological rates and processes, including metabolism and development. Incubation at 38°C has long been considered the optimum temperature for rearing chicken embryos (Whittow, 2000), clearly based on imitating the incubation environment created by the hen. Yet, there is little evidence to suggest that incubation at a temperature other than 38°C has consequences for survival or fitness of the embryo. The results of this study confirm that, as expected, chicken embryos incubated at 35°C developed more slowly than those embryos incubated at 38°C, spending an average of 3.1 extra days in the egg before hatching. Similar studies reported that 35°C incubation is near the lower end of the viable incubation temperatures, and results in an incubation period of up to 25 days in the chicken embryo (Tazawa, 1973).

Importantly, we hypothesized that incubation at 35°C would

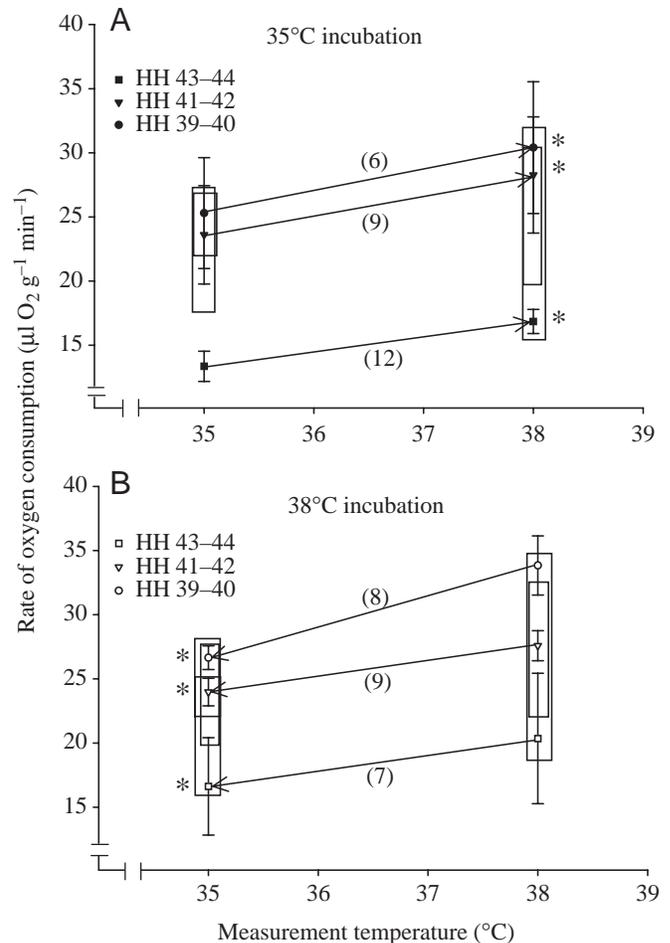


Fig. 5. Mass-specific static rate of oxygen consumption \dot{V}_{O_2} for embryos incubated at 35°C (A) and 38°C (B). The arrow direction indicates that the \dot{V}_{O_2} for each embryo was first determined at that individual's incubation temperature, and then again after 2 h at the other incubation temperature. Plotting convention as described for Fig. 3. *N* values for each 35/38°C pair are indicated in parentheses.

not only lengthen the developmental timeline, but would also change development qualitatively. Yet our data show that incubation temperature had no effect on the relative timing of internal and external pipping in the chicken embryo, two critical landmarks in avian development. There was a slight difference in the overall rates of survival between embryos incubated at 35°C and 38°C (31% and 48%, respectively) but, overall, temperature had little effect on survival of the chicken embryos throughout incubation. Moreover, small numbers of embryos at each temperature died at approximately the same points in development (Fig. 1A,B). These identical mortality rates during early incubation suggest that the embryos experienced a common failure of an organ or a system at a key point in development.

Incubation temperature and embryonic growth

Embryonic wet mass for 38°C embryos at HH 39–40 and HH 41–42 closely resembled previously reported values

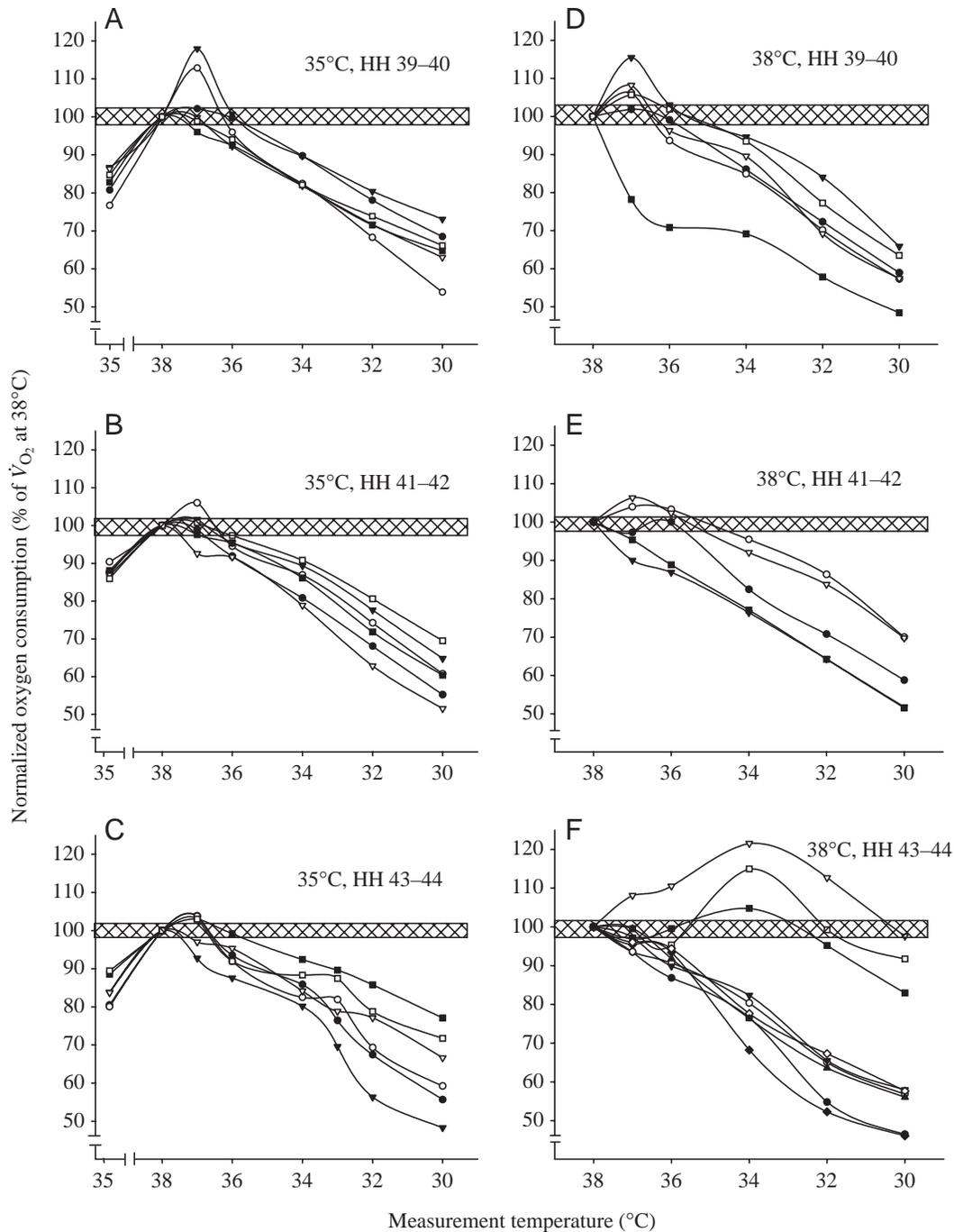
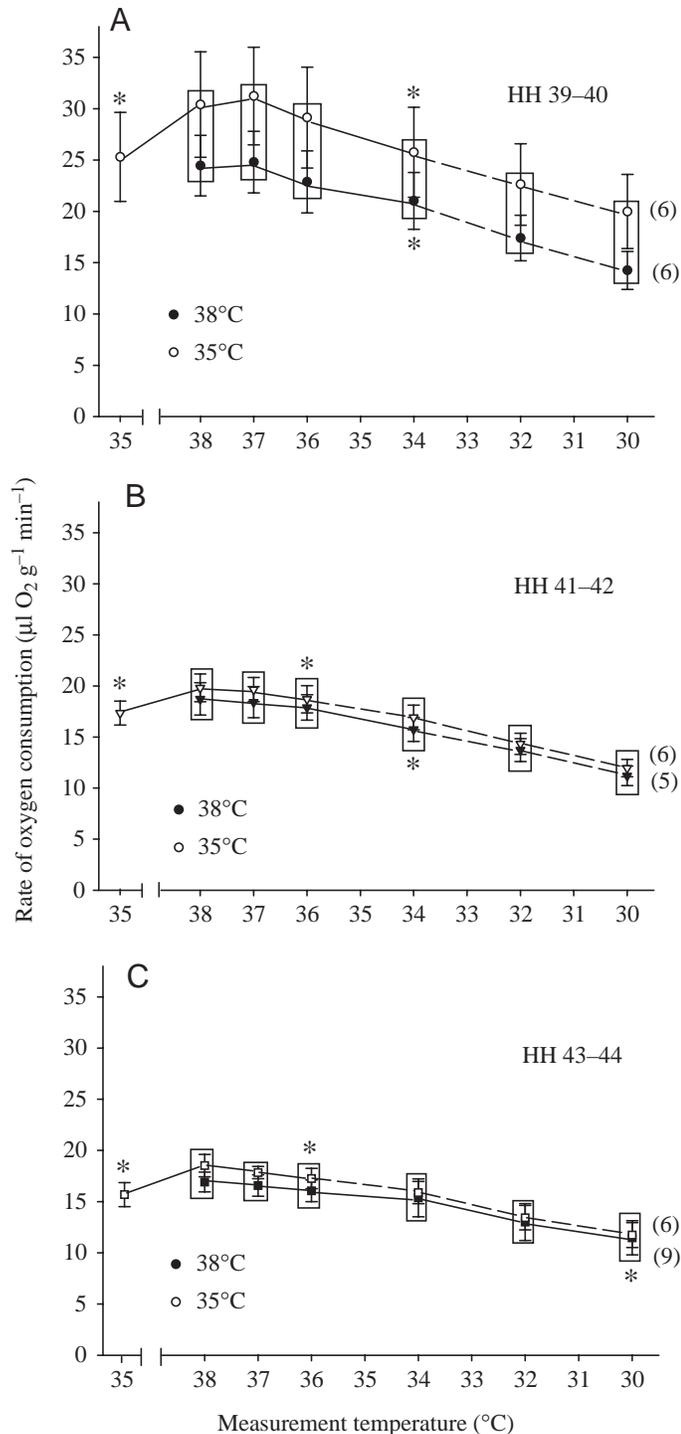


Fig. 6. $\dot{V}O_2$ during gradual cooling for individual embryos incubated at 35°C and 38°C to stages 39–40 (A and D, respectively), stages 41–42 (B and E, respectively), and stages 43–44 (C and F, respectively). Data are normalized to the measured $\dot{V}O_2$ value at 38°C; cross hatched bars represent 5% variation from the measurement at 38°C.

(Tazawa et al., 1971; Dzialowski et al., 2002). The difference in wet body mass at HH 43–44 between incubation temperatures in the present study is accounted for by the higher total body water content in the 35°C embryos. Calculations of % total body water from the wet and dry mass data confirmed that at stage 43–44 the 35°C the wet mass of the embryo consisted of 81% water, compared to only 76% in the 38°C embryos. Dzialowski et al. (2002) reported similar values of

total body water in D18 (HH 44) embryos at 81.8%. Their study also determined that D12 embryos (HH 38) contained 91.7% body water, a value comparable to that in our study for HH 39–40 embryos incubated at both 35°C (89.6%) and 38°C (90.8%).

Overall, these data reveal that embryo growth is proportional to developmental stage, irrespective of how long it takes to reach that stage. This emphasizes the importance of making



comparisons at equivalent stages of development, rather than solely at arbitrary periods of development defined chronologically. Because the development of embryos and organ systems is non-linear, absolute time is not a good descriptor when comparing embryos incubated at different temperatures. The concept of 'degree days' (days of development \times the temperature of development) has been used to normalize the effect of temperature on development and thus address the discrepancies between absolute time and

Fig. 7. Gradual cooling mass-specific rate of oxygen consumption for embryos at stages 39-40 (A), 41-42 (B) and 43-44 (C). A box surrounding points indicates no significant difference between stages ($P > 0.05$). The asterisks indicate the temperature at which the oxygen consumption first became significantly lower than the initial oxygen consumption at 38°C. The subsequent transition to broken lines after the asterisk indicates that all subsequent oxygen consumptions remain significantly different during further cooling. Numbers of embryos used in each cooling run are indicated in parentheses. Values are means \pm 1 S.E.M.

developmental time (see Pritchard et al., 1996; Weltzien et al., 1999). However, as Spicer and Burggren (2003) and Burggren and Crossley (2002) emphasize, temperature effects on development are complex, selective and not easily unraveled.

In contrast to whole embryo mass, ventricular mass showed evidence of complex temperature effects. The ventricle mass of stage 43-44 embryos incubated at 38°C ($0.10 \text{g} \pm 0.01$) closely resembled the heart mass data for D18 chicken embryos ($0.12 \text{g} \pm 0.01$) obtained by Dzialowski et al. (2002). Our study revealed that both ventricle mass and the size of the ventricle relative to embryo mass were significantly higher in the 35°C embryos (Fig. 4) at stage 41-42. Previous studies examining the effects of hypoxic stress on the development of the chicken embryo saw increases in heart mass of D12 embryos that were exposed to hypoxia between days 6 and 12 of incubation (Dzialowski et al., 2002). That study, however, was unable to show a similar response in D18 embryos or in hatchlings. Future experiments to examine the differential effects of incubation temperature on heart mass will help to unravel why hypothermic incubation leads to cardiac hypertrophy.

Temperature and \dot{V}_{O_2}

\dot{V}_{O_2} measurements obtained in the present study agree with previous measurements for D12, 14, 16 and 18 chicken embryos incubated at 38°C (Table 2). However, this is the first study to complete measurements of \dot{V}_{O_2} in embryos chronically incubated at 35°C. The mass-specific \dot{V}_{O_2} of stage 43-44 embryos incubated at 35°C appears low in comparison with measurements from 38°C embryos at the same stage in this and previous studies. The significantly larger embryo wet mass of the 35°C embryos contributes to this difference, but this group also demonstrated a large amount of variation in \dot{V}_{O_2} . Observations made during incubation indicated that the chorioallantoic membrane (CAM) in the 35°C embryos failed to line the entire inner surface of the shell. Although CAM surface area data was not collected in these experiments, a smaller surface area for gas exchange might have an impact on the embryos with the largest metabolic demand, i.e. HH 43-44 embryos. The importance of CAM surface area is debated. Okuda and Tazawa (1988) covered up to 50% of the chicken egg with epoxy, effectively reducing the surface area of the CAM able to exchange gases with the environment. They found that the reduction in gas conductance reduced \dot{V}_{O_2} . In contrast, Wagner-Amos and Seymour (2002) reported that metabolic

Table 3. Rate of oxygen consumption during gradual cooling from 38°C

Incubation temperature (°C)	Stage (HH)	N	Baseline \dot{V}_{O_2} ($\mu\text{l min}^{-1} \text{g}^{-1}$)	T_{crit} (°C)	\dot{V}_{O_2} at T_{crit} ($\mu\text{l min}^{-1} \text{g}^{-1}$)	Probability (SNK)
38	39–40	6	24.3±2.9*	34	21.1±2.7	0.002
38	41–42	5	19.0±1.7	34	16.4±1.2	<0.05
38	43–44	9	16.8±1.0	30	11.3±1.7	0.017
35	39–40	6	30.0±5.1	34	26.1±4.4	<0.001
35	41–42	6	20.0±1.4	36	17.0±1.3	0.037
35	43–44	6	17.8±0.9	36	16.8±1.0	0.013

Baseline temperature = 38°C; T_{crit} = temperature at which basal \dot{V}_{O_2} can no longer be maintained.

*Values are means \pm 1 S.E.M.

SNK, Student–Newman–Keuls test.

activity was not significantly correlated with reductions in gas conductance, accomplished by applying wax to the shell.

Incubation temperature did not profoundly affect basal \dot{V}_{O_2} of chicken embryos in the final stages of development. The very similar \dot{V}_{O_2} exhibited by 35°C- and 38°C-incubated embryos at HH 43–44 and the general trend of a decrease in mass-specific oxygen consumption with development may both be explained by the internal O_2 levels late in development. In such late stages, \dot{V}_{O_2} of the embryo is constrained by O_2 diffusion rates across the shell. Reduced embryonic mass-specific \dot{V}_{O_2} results because the embryo continues to grow at the expense of establishing hypoxia within the egg (Romijn and Lokhorst, 1951; Wagensteen et al., 1970; Rahn et al., 1974; Ar et al., 1980; Tazawa, 1980). If late stage chicken embryos are provided with increased O_2 (hyperoxic environment), they increase their metabolic activity (Tazawa et al., 1992). An examination of the early stages of development would probably reveal lower \dot{V}_{O_2} in embryos incubated at 35°C, supporting the slower rate of growth and development and the increased length of the developmental timeline.

Temperature and thermoregulatory activity

A homeotherm must be able to regulate precisely endogenous heat production as well as heat loss in order to maintain a stable body temperature in the face of fluctuating ambient temperatures. Chicken embryos are certainly not homeotherms, but at some point in very late development the embryo makes the transition from poikilothermy to homeothermy, using elevated \dot{V}_{O_2} to produce heat used to maintain body temperature. Tazawa et al. (1988) slowly cooled late-stage embryos from 38°C to 30°C over a period of 8 h, minimizing the imbalance between heat loss and heat production. They noted that embryos as young as stage 43 were able to maintain a maximal oxygen consumption until the ambient temperature reached 34°C, and proposed that the embryo was an apparent poikilotherm, unable to maintain body temperature, only because of the thermal constraints of the egg environment, not because they lacked the mechanisms required for regulating metabolic activity. In support of the contentions of Tazawa et al. (1988), we propose two lines of evidence

suggesting the onset of thermoregulatory ability in late-stage embryos: (1) the ability of some individual embryos actually to increase briefly their \dot{V}_{O_2} by 5–10% upon a 1°C drop in T_a and (2) the ability of the embryo generally to maintain \dot{V}_{O_2} during an 8°C drop in T_a .

Incubation temperature appears to modify the developmental onset of the chicken embryo's ability to trigger endogenous heat production as part of developing thermoregulatory mechanisms. Specifically, hypothermic incubation delays the onset of the embryo's ability to maintain stable \dot{V}_{O_2} in the face of acutely declining temperature. The youngest embryos examined from both incubation temperatures significantly reduced \dot{V}_{O_2} at a T_{crit} of 34°C (Fig. 7A). By stages 41–42 and 43–44, the 35°C embryos experienced significant decreases in metabolic activity earlier, but only at 36°C. The ability of the 38°C embryos to maintain \dot{V}_{O_2} improved with continued development, and by stage 43–44 they experienced a significant decline in oxygen consumption only after a full 8°C decline in egg surface temperature, thus surpassing the performance reported for chicken embryos by Tazawa et al. (1988). While some of the statistically significant differences individually may be of limited biological significance, collectively these data show that over all incubation conditions, hypothermic incubation temperatures produce embryos that are less efficient in responding physiologically to acute T_a decreases.

Important differences exist between measurements of static \dot{V}_{O_2} made after longer periods of exposure (2 h) to altered ambient temperature compared with the 'gradual cooling' experiments. First, after longer exposure, the temperature effect on \dot{V}_{O_2} was the same for all stages and both incubation temperatures, with Q_{10} values ranging from 1.49±0.08 to 1.57±0.17. Although there is something inherently different about the 38°C embryos that allows them to better resist gradual decreases in ambient temperature, these data suggest this effort is short-lived, lasting for only a few hours. The constraints imposed by the egg environment include a complete lack of insulation (Whittow and Tazawa, 1991), high thermal conductance (Tazawa et al., 1988) and limited diffusion of respiratory gases (Wagensteen et al., 1970; Rahn et al., 1974).

Each of these factors is likely to contribute to an eventual overwhelming demand on metabolic heat production, such that after 2 h of exposure there is no difference between embryos incubated at 35°C and 38°C. Secondly, these Q_{10} values are also lower than those that describe the effect of temperature on \dot{V}_{O_2} during gradual cooling over the same temperature range. With a Q_{10} as high as 1.88, temperature has a greater effect on \dot{V}_{O_2} during the initial stages of cooling. The lower Q_{10} after a longer period of exposure implies that the exponential decrease in oxygen consumption initially overshoots the appropriate \dot{V}_{O_2} but compensates within the 2 h period of this experiment (Tazawa et al., 1989).

Incubation temperature and heterokairy

Chicken embryos incubated under hypothermic conditions at 35°C appear to follow the same relative developmental timeline as embryos incubated at 38°C. Embryo masses were the same, \dot{V}_{O_2} values were similar, and internal and external pipping occurred at the same relative points along the developmental timeline. These responses would suggest that temperature, in fact, does not induce heterokairy, at least as it relates to changes in the relative timing of the onset of key physiological processes and their control that specifically affect growth or the maturation of the respiratory system (judging from the timing of pipping events). Yet, the different responses of 35°C and 38°C embryos to gradual cooling reveal significant effects of chronic hypothermic incubation on the maturity of physiological components required for endogenous heat production for thermoregulation. These ontogenetic differences support the concept of heterokairy, but additional experiments will be required to determine which regulatory components are responsible for the differences between 35°C and 38°C embryos.

Temperature has been shown to have complex and selective effects on physiological and metabolic development in chicken embryos. In the companion paper we show how blood O_2 transport properties are similarly affected in complex ways by incubation temperature.

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