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METABOLIC RESPONSES TO GRADUAL COOLING IN CHICKEN EGGS TREATED WITH THIOUREA AND OXYGEN

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Abstract—1. Late prenatal chicken embryos in eggs injected with saline showed a feeble homeothermic metabolic response to gradual cooling. This response was absent in thiourea-treated eggs. This suggests that the incipient homeothermic metabolic response before paranatal life may be attributed to thyroid development.

2. The compensatory metabolic response disappeared in embryos exposed to a hypoxic environment, while it was augmented in eggs in pure O_2, decreasing as ambient temperature fell.

3. These results may indicate that the homeothermic metabolic response in late embryos is O_2-conductance-limited and power-limited as previously suggested.

INTRODUCTION

The homeotherm produces heat at the same rate as it is lost to the surroundings. Avian embryos on the other hand need heat from their surroundings to maintain adequate metabolism for development. After about the midpoint of incubation in chicken eggs, the heat produced by metabolism overtakes heat loss, raising the egg temperature above that of the surroundings (Romanoff, 1941; Romijn and Lokhorst, 1955, 1956, 1960; Tazawa and Nakagawa, 1985; Tazawa and Rahn, 1986). The thermal conductance of chicken eggs is about 72 mW/°C (Tazawa *et al.*, 1988), while the rise in egg temperature above the environmental temperature is, for instance, about 0.5 and 2°C for young (12 days) and late (18 days) embryos, respectively. The metabolic heat required to produce these rises is about 35 and 140 mW in young and late embryos, respectively. This implies that when chicken eggs incubated at 38°C are exposed to an ambient temperature which is lowered by only 2°C, the heat lost through the egg becomes greater than that produced by metabolism; therefore the prenatal embryos cannot be homeothermic. Nevertheless, this does not necessarily mean that prenatal chicken embryos are not provided with some homeothermic capability, *viz.* a compensatory metabolic response to egg cooling. If the cooling temperature is very low, a feeble compensatory ability may be overwhelmed by the very much larger losses of heat (Tazawa *et al.*, 1988).

Freeman (1964) reported the first indication of a hemoethermic metabolic response on the 19th day of incubation; if the reduction in the ambient temperature is small (about 5°C), there is a transient rise in metabolic rate. It has been reported that there is an increase in carbohydrate catabolism in the 19-day-old embryo during cooling (Freeman, 1967) and the respiratory quotient of the late embryos is increased in response to cold exposure (Romijn and Lokhorst, 1955). In neonatal chicks, the importance of the thyroid in thermoregulation was suggested (Freeman, 1970, 1971).

In a previous report (Tazawa *et al.*, 1988), developing chick embryos were examined for metabolic responses to a gradual cooling procedure which kept the imbalance between heat loss and heat production as small as possible. The results suggested that thermoregulatory mechanisms are "switched on" several days prior to external pipping. This coincides with thyroid development during the late stages of prenatal life (Thommes and Hylka, 1977; Decuypere *et al.*, 1979; Ockleford *et al.*, 1983), which has also been reported for Japanese quail embryos (McNabb, 1987). In addition, the previous report (Tazawa *et al.*, 1988) suggested that the diffusive conductance of the eggshell to O_2 is a constraint on thermoregulatory mechanisms, i.e. "O_2-conductance limited". The present study was therefore designed to investigate the effects of thiourea, which antagonized the metabolic effect of thyroid hormones, and of altered ambient O_2, on the metabolic response of late chick embryos to gradual cooling.

MATERIALS AND METHODS

Fertile eggs of the domestic fowl (*Gallus domesticus*) were incubated at 38°C in a forced-draft incubator and turned automatically every 3 hr.

The eggs were treated with thiourea; the quantity administered was similar to that reported by Wittmann *et al.* (1984). Eggs were removed from the incubator at 17 days of incubation and the eggshell was pierced with a sharp syringe needle. Fifty mg of thiourea was dissolved in 5 ml saline and 0.25 ml of this solution (32.8 μmol) was injected into the egg through the hole. The hole was then covered with epoxy glue. The eggs were replaced in the incubator until the

gradual cooling test was conducted some day after the following day to 24 days of incubation. In order to examine the possible adverse effect of saline injection on the metabolic response of embryos to cooling, other eggs (i.e. sham eggs) were subjected to injection of the same volume of thiourea-free saline at 17 days and to the gradual cooling test some day after the following day to 20 days.

Two series of experiments were designed to investigate the effect of ambient O_2 on the metabolic response to cooling. One was to expose the eggs to a small reduction of ambient O_2, and the other to pure O_2, before starting egg cooling. The metabolic chamber employed for the measurement of $\dot{M}_{O_2}$ opened to air through a 3-way stopcock. In the hypoxic experiment, the stopcock was closed for about 5 min to reduce the O_2 concentration in the chamber due to consumption by the egg. For the pure O_2 environment, on the other hand, the metabolic chamber with the egg was vented by pure O_2 for 1 hr and then the $\dot{M}_{O_2}$ was determined.

The determination of $\dot{M}_{O_2}$ was made employing a modified volumetric microrespirometer (Scholander, 1942; Scholander and Edwards, 1942; Ackerman *et al.*, 1980; Tazawa and Rahn, 1986). The respirometer consisted of two equal-size Plexiglas cylindrical chambers (volume of *ca* 300 ml) connected by a U-type glass manometer filled with water. The experimental egg was placed in one chamber (metabolic chamber) containing KOH solution and the other (compensating chamber) contained an infertile egg. Both chambers, immersed in a thermostatted water bath, were opened to the atmosphere through stopcocks and the metabolic chamber was vented with air or pure O_2 through conduits installed in the chamber. When the $\dot{M}_{O2}$ was measured, all the air channels were closed and a glass syringe containing pure O_2 was connected to the metabolic chamber through the stopcock. As the egg consumed O_2 and the concomitantly produced CO_2 was absorbed by KOH, the pure O_2 in the syringe was infused to correct the displacement of water level in the manometer. The correction was made every 2 min for 8 min and the cumulative volume of O_2 infused was read. The slope of the regression line for the relationship between infused O_2 and time was corrected to the $\dot{M}_{O_2}$ of the egg at standard temperature and pressure. The $\dot{M}_{O_2}$ of individual eggs was measured first at 38°C for 1 hr, providing six determinations which were averaged for the control $\dot{M}_{O_2}$. Then, the water bath thermostat was switched off and the bath containing the egg chamber was left to cool. The temperature inside the metabolic chamber was recorded with a thermistor probe sensitive to changes to 0.1°C. The cooling time constant was about 5.6 hr (Tazawa *et al.*, 1988).

For eggs exposed to pure O_2, the $\dot{M}_{O_2}$ at 38°C was measured first in air and then over a period of 1 hr after 1 hr of O_2 exposure. The eggs were then cooled.

RESULTS

The $\dot{M}_{O_2}$s in air at 38°C determined for intact control eggs, saline-injected sham eggs, thiourea-treated eggs, ambient O_2-deficient eggs, and that of eggs before and after exposure to pure O_2 are summarized in Table 1. The $\dot{M}_{O_2}$s of individual experimental groups (B–E) measured in air are not significantly different from the O_2 consumption of intact control eggs (A) ($P > 0.1$ by Student's unpaired t-test). The increased availability of O_2 due to exposure to pure O_2 raised the O_2 uptake by about 18% on average (Table 1), which is intermediate between the previously reported values (Høiby *et al.*, 1983; Stock *et al.*, 1985).

The metabolic responses to gradual cooling in air were first determined for seven intact eggs 18–20 days old (group A in Table 1) to confirm the previous

Table 1. Oxygen uptake ($\dot{M}_{O_2}$ in ml/day) at 38°C, in air, for intact control eggs (A), sham eggs (B), thiourea-treated eggs (C), ambient O_2-deficient eggs (D) and eggs subsequently exposed to pure O_2 (E)

	A	B	C	D	E
No.	7	7	12	8	13
Days	18–20	18–20	18–24	18–20	18–20
$\dot{M}_{O_2}$	620 ± 66	634 ± 34	603 ± 81	606 ± 67	628 ± 83
					744 ± 94*

Values are mean ± SD. *$\dot{M}_{O_2}$ in pure O_2.

results (Tazawa *et al.*, 1988). There was a "plateau phase" in metabolism at the beginning of cooling as previously reported for prenatal embryos aged 18 days onward (Fig. 2b of Tazawa *et al.*, 1988). In this phase, metabolism remained essentially constant (i.e. within 95% of 38°C control $\dot{M}_{O_2}$) until ambient temperature declined to about 35°C. The sham eggs injected with saline (group B in Table 2) presented metabolic responses identical to those of the control eggs (Fig. 1). The saline was given at 17 days of incubation and the metabolic responses to cooling were determined in 3, 3 and 1 embryos for 18, 19 and 20 days of incubation, respectively.

Two out of 12 embryos administered thiourea at 17 days pipped externally, but did not hatch, and the remainder neither pipped the egg, nor escaped from the eggshell up to the time when the embryo died or our measurements ceased at 24 days of incubation.

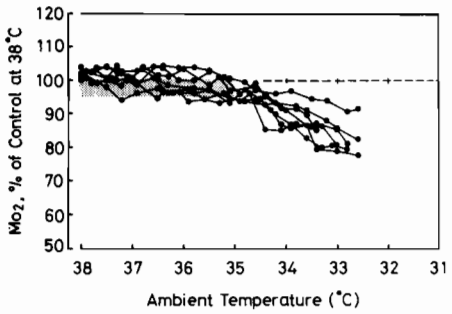

Fig. 1. Oxygen uptake of prenatal embryos, aged 18–20 days, during gradual cooling, shown as a percentage of the 38°C control $\dot{M}_{O_2}$. The eggs were injected with 0.25 ml saline at 17 days of incubation. Dotted area indicates the $\dot{M}_{O_2}$ maintained within 95% of the control value despite a 3°C fall in ambient temperature.

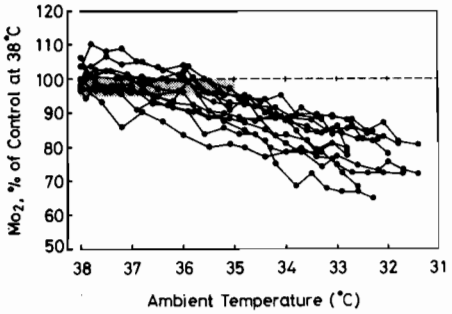

Fig. 2. Oxygen uptake of embryos, aged 18–24 days, during gradual cooling, shown as a percentage of 38°C control $\dot{M}_{O_2}$. The eggs were injected with 38.2 μmol thiourea in 0.25 ml saline at 17 days of incubation. Dotted area is the same as for Fig. 1.

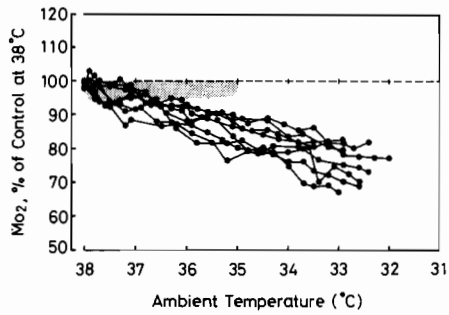

Fig. 3. Oxygen uptake of prenatal embryos, aged 18–20 days, during gradual cooling, shown as a percentage of the 38°C control $\dot{M}_{O_2}$. The embryos were exposed to O_2-deficient air. Dotted area is the same as for Fig. 1.

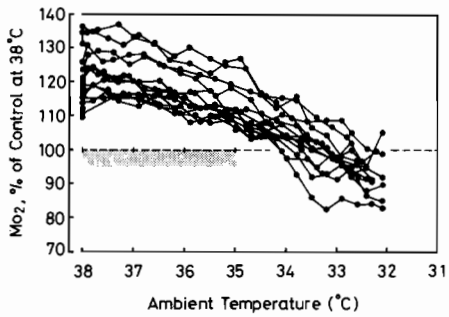

Fig. 4. Oxygen uptake of prenatal embryos aged 18–20 days, during gradual cooling, shown as a percentage of the 38°C control $\dot{M}_{O_2}$. The embryos were exposed to pure O_2. Dotted area is the same as for Fig. 1.

Figure 2 represents 12 determinations of the metabolic responses of embryos aged from 18 to 24 days (group C in Table 1; one embryo each for 18 and 19 days old and two embryos for each day from 20 to 24 days). Two embryos pipped the egg externally at 20 days, and one was measured for its metabolic response the following day, and another at 23 days.

The O_2 deficiency in the environment was due to consumption by the egg. The metabolic response to cooling was measured in eight embryos (group D in Table 1; 5, 1 and 2 embryos for days 18, 19 and 20, respectively). While the O_2 uptake at 38°C was essentially the same as that of control eggs in air, the $\dot{M}_{O_2}$ of all eggs decreased as the ambient temperature fell, failing to reach a plateau phase (Fig. 3).

The metabolic response to cooling in a pure O_2 environment was determined in 13 eggs (group E in Table 1; 5, 6 and 2 eggs for 18, 19 and 20 days of incubation, respectively). The $\dot{M}_{O_2}$ decreased to the control level in air when the ambient temperature was lowered below 34–32°C (Fig. 4).

DISCUSSION

In a previous report (Tazawa *et al.*, 1988), developing chick embryos aged from 12 to 20 days and externally pipped embryos were measured for metabolic responses to gradual cooling in order to investigate if prenatal embryos are provided with a compensatory metabolic response to cooling. The responses of young (12 days to about 17 days old) and late embryos (about 18 days onward) were different (Fig. 2 of Tazawa *et al.*, 1988). While the $\dot{M}_{O_2}$ of young embryos decreased with decreasing ambient temperature, late embryos maintained their $\dot{M}_{O_2}$ within 95% of the 38°C control until the ambient temperature decreased below 35°C; the metabolic rate was "uncoupled" from ambient temperature, forming a plateau in $\dot{M}_{O_2}$ response. The externally pipped embryos responded to gradual cooling by increasing the $\dot{M}_{O_2}$ above the control value. The results suggested that a feeble homeothermic metabolic response to egg cooling appears around 18 days of incubation and the response is strengthened after external pipping. The emergence of a compensatory metabolic response to cooling in late prenatal embryos was investigated in another way (Tazawa *et al.*, 1989), where the embryo was measured for $\dot{M}_{O_2}$ at 38°C and after reaching a quasi-equilibrium state (5–9 hr) at lowered ambient temperature. While the Q_{10} for $\dot{M}_{O_2}$ was about 2 in young embryos (12 and 16 days old), the near-term embryos (18 days old) presented a Q_{10} of about 1.5 at moderately lowered temperature (32°C).

The previous report (Tazawa *et al.*, 1988) further suggested that incipient endothermic homeothermy which precocial hatchlings *in ovo* may exhibit, may be prevented by a low gas conductance of the eggshell, effectively "throttling" the embryo's heat production capacity. Therefore, in the present experiment, in addition to the gradual cooling test made in thiourea-treated eggs, the effect of the eggshell was examined indirectly by lowering or raising O_2 concentration in the surroundings.

The sham eggs which were injected with thiourea-free saline presented a plateau phase of $\dot{M}_{O_2}$ in response to gradual cooling similar to that of the intact eggs, indicating that the saline injection had little effect on the metabolic response (Fig. 1). When the eggs were then treated with thiourea, most embryos failed to pip the egg externally and none of the embryos hatched, as indicated previously (Wittmann *et al.*, 1984). The $\dot{M}_{O_2}$ at control temperatures [603 ± 81 (SD) ml/day, Table 1] was insignificantly lower than that of intact control eggs (620 ± 66 ml/day), and there was no trend to change with age. The thiourea, antagonizing the metabolic effects of thyroid hormones, likely induced a hypothyroid state (Wittmann *et al.*, 1984). The 20- and 23-day-old embryos (one embryo for each day) initially responded to cooling with increasing $\dot{M}_{O_2}$, but thereafter failed to maintain it. The embryos which would have hatched in the absence of thiourea; i.e. those aged 21–24 days, also reduced their $\dot{M}_{O_2}$ as the ambient temperature fell, indicating that the homeothermic metabolic response to cooling was evidently blocked by the thiourea.

The feeble compensatory response was also impeded by a lack of O_2 in the environment (Fig. 3), while it was augmented by increasing ambient O_2 (Fig. 4). The diffusive conductance of the eggshell to O_2 is fixed (Paganelli *et al.*, 1978), and hypoxia is the price the embryo must pay to increase its heat production. This would impose a limit on the amount of heat that an embryo in an enclosed egg can produce, which was previously defined as "O_2-conductance limited" (Tazawa *et al.*, 1988). The

deficiency in ambient O_2 produced in the present experiment was small enough not to reduce significantly the $\dot{M}_{O_2}$ at 38°C (606 ± 67 ml/day, $N = 8$) compared with control values for eggs in air (620 ± 66 ml/day, $N = 7$) (Table 1). The O_2 concentration in the chamber was not measured, but judging from the O_2 consumption of the egg and the chamber volume, it was probably not less than 19.5%. The hypoxia, even if it is small, therefore prevents a feeble compensatory metabolic response, indicating indirectly that the eggshell O_2 conductance limits the thermoregulatory function of late prenatal embryos.

While the compensatory metabolic response emerging in chicken embryos during the last stages of prenatal development disappears in O_2-deficient air, the metabolic response to cooling is augmented in O_2-rich air (Fig. 4). The O_2 supply to the egg was improved by increasing the O_2 tension gradient in the face of a fixed shell conductance. The $\dot{M}_{O_2}$ in air at 38°C (628 ± 83 ml/day, $N = 13$) increased by about 18% in pure O_2 at 38°C (744 ± 94 ml/day) (Table 1). After exposure to gradual cooling, the increase in $\dot{M}_{O_2}$ above the 38°C air control value did not fall until the ambient temperature was reduced below 34–32°C. While the metabolic response to cooling was strengthened by increasing the O_2 supply, it could not be maintained as the ambient temperature fell further. This may be due to an O_2 supply for the embryo limited by the blood O_2 carrying capacity and physiological shunt in the allantoic circulation (Piiper *et al.*, 1980). Simultaneously, the thermoregulatory capacity of the embryo is "power-limited" as previously suggested for newly hatched neonates (Tazawa *et al.*, 1988).

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