Hypoxic incubation blunts the development of thermogenesis in chicken embryos and hatchlings

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Azzam MA, Szdzuy K, Mortola JP. Hypoxic incubation blunts the development of thermogenesis in chicken embryos and hatchlings. Am J Physiol Regul Integr Comp Physiol 292: R2373–R2379, 2007. First published March 8, 2007; doi:10.1152/ajpregu.00885.2006.—We asked to what extent sustained hypoxia during embryonic growth might interfere with the normal development of thermogenesis. White Leghorn chicken eggs were incubated at 38°C either in normoxia (Nx, 21% O₂) or in hypoxia [Hx, 15% O₂, from embryonic day 5 (E5) until hatching]. The Hx embryos had lower body weight (W) throughout incubation, and hatching was delayed by about 10 h. For both groups, all measurements were conducted in normoxia. At embryonic day E11, the static temperature-oxygen consumption (ambient T-V˙O₂) curve was typically ectothermic (Q₁₀ = 1.92–1.94) and similar between Nx and Hx. Toward the end of incubation (E20), the Q₁₀ averaged 1.41 ± 0.06 in Nx and 1.79 ± 0.08 in Hx (P < 0.005), indicating that the onset of the thermogenic response in Hx lagged behind Nx. In the 1-day-old hatchlings (H1), body weight did not significantly differ between Nx and Hx. At H1, the T-V˙O₂ curves were endothermic-type, and more so in the older (>8 h old) than in the newly hatched (<8 h old) chicks, whether examined statically or dynamically as a function of time. In either case, the thermogenic responses of Hx were lower than those of Nx. In a 43–31°C thermocline, the preferred T of the Hx hatchlings was around 37.3°C, and similar to Nx, suggesting a similar setpoint for thermoregulation. We conclude that hypoxic incubation blunted the development of thermogenesis. This could be interpreted as an example of epigenetic regulation, in which an environmental perturbation during early development alters the phenotypic expression of a regulatory system.

embryonic development; epigenetic adaptation; hatching; hypometabolism; hypoxia; thermoregulation; development

MAMMALS AND BIRDS ARE HOMEOTHERMS, meaning that they regulate body temperature around a set value, over a wide range of ambient temperatures. This characteristic develops during the late prenatal period and begins to functional at birth and hatching, when it manifests itself with a combination of behavioral and autonomic mechanisms (4, 21, 43). One of these mechanisms is heat production, or thermogenesis, a metabolic response to cold that, in young mammals, is mostly contributed by the calorigenic properties of the brown adipose tissue and the activity of its uncoupling protein (31). Hypoxia influences all aspects of thermoregulation. In particular, in neonatal mammals, hypoxia depresses thermogenesis (27) and decreases the brown fat mass and the expression of its uncoupling protein (29, 30). In addition, as in the adults of many classes of animals, hypoxia lowers the preferred ambient temperature (6, 7, 15, 25, 44). The depression of heat production is one of the mechanisms to save on oxygen consumption (V˙O₂); hence, it represents an important strategy to survive hypoxia (22).

Similar to after birth, in the prenatal period, hypoxia also decreases metabolic rate, but the energy saving comes largely from the reduction in fetal growth (18, 21). In fact, during the embryonic development of a bird or a mammal, thermogenesis is absent and only begins to be operational in the perinatal period (e.g., 14, 28). Because of this late development, it is possible that prenatal hypoxia may have no effects on the formative process of thermogenesis. On the other hand, the development of thermogenic mechanisms may be sensitive to the prenatal metabolic history, as in the case of avian embryos incubated at below-normal temperatures. In these embryos, the sustained hypometabolism provoked by cold-incubation not only stunted body growth but also blunted the normal developmental process of thermogenesis (3, 23). The eventuality that prenatal hypoxia may interfere with the normal development of the mechanisms of heat production has received little attention, and the few data available are open to different interpretations.

In human infants born at high altitude, acute cold exposure caused a thermogenic response significantly lower than in infants at sea level (11). This result could mean that the hypoxic gestation of the high-altitude infants depressed the developmental processes of their thermogenic mechanisms. However, it could also reflect the fact that the measurements were conducted at high-altitude, and hence in a hypoxic condition that, as mentioned above, by itself reduces V˙O₂ and thermogenesis.

Two-day old rats born from dams exposed to hypobaric hypoxia since conception had lower body weight and V˙O₂ than controls; their brown fat was hypoplastic with decreased uncoupling protein, yet, when subjected to cold, their thermogenic response was almost as in normoxic-gestated animals (12, 30). These seemingly conflicting results were attributed to the stimulating effects on thermogenesis caused by stress-related mechanisms, possibly of maternal origin. In fact, during pregnancy, the maternal adaptation to hypoxia influences birth weight (19) and causes numerous hormonal responses, including thyroid and catecholamine release, known to have an impact on fetal development (9, 13, 41). After birth, maternal stress can diminish lactation and the care for the litter, causing complex effects on the pup’s metabolism and the biomolecular machinery involved in heat production (2, 20, 34, 36). Avian embryos, in which the chorioallantoic membrane provides gas exchange, offer an opportunity to study the effects of prenatal
hypoxia on the development of thermogenesis without the issue of the maternal interference on the embryo’s response. Therefore, for this study we have chosen the chicken embryo, an animal model with a well-characterized developmental pattern of heat control, and for which the hypometabolic response to acute hypoxia is also well documented (1, 5, 24, 28, 37, 39, 40).

MATERIALS AND METHODS

Experiments were performed on fertilized White Leghorn chicken eggs, obtained from a local supplier. Eggs were stored at 15°C until the start of incubation, and for no longer than 7 days. The eggs were weighed and placed in incubators (Hova-Bator, Savannah, GA) around midday (day 0). The incubators maintained a steady temperature (T) of about 38°C and 60% relative humidity and provided a 45° egg rotation four times a day. At first, all eggs were incubated in normoxic conditions. Then, at embryonic day E5, they were separated into two groups. Some continued in normoxia (21% O2, normoxia, Nx), others were transferred into a hypoxic incubator kept at 15–16% O2 (on average, including the periods of incubator opening for egg transfer and cleaning, 15.48 ± 0.01) (hypoxia, Hx). The desired level of hypoxia was obtained by leaking a small stream of warmed and humidified N2 into the incubator from a pressurized tank, under the control of a flowmeter. The O2 concentration within the incubator was continuously sampled by a calibrated fuel cell gas analyzer (Foxbox, Sable Systems, Las Vegas, NV) and displayed on a computer monitor. A T-data logger and a hygrometer were inside each incubator; the former collected the T value every 10 min, while humidity was read daily.

Body growth was measured from embryonic day E8 to hatching, at 3-day intervals. The metabolic responses to changes in T were measured in normoxia, in the Nx and Hx embryos at the same chronological day (E11, E20), and in the hatchlings on the day of hatching (H1), either within the first 8 h (<8 h), on average at 4.2 ± 0.4 and 3.9 ± 0.4 h in Nx and Hx, respectively) or later during the day (8–24 h, on average 14.7 ± 0.8 and 14.3 ± 0.8 h in Nx and Hx, respectively).

Egg weight at the start of incubation, embryo weight, and daily water loss (equal to the egg weight loss) were measured for each egg. At the end of the experiment, the embryo was killed by exposure to CO2 and cold, the egg was opened, the embryo was examined to exclude gross anatomical abnormalities, and its body weight (W) was measured on a digital scale.

Metabolic rate was measured in normoxic conditions by indirect calorimetry (oxygen consumption, V˙O2, and carbon dioxide production, V˙CO2), with an open-flow methodology (10) adapted to the chicken embryo (16, 17, 28). Measurements were performed either in sets of two (E11, E20) or individually (H1) in a respirometer, which consisted of a 300-ml plastic container maintained at the desired T by a water bath. A steady gas flow of either 100 (E11) or 200 ml/min (E20 and H1) was continuously delivered through the respirometer, under the control of a precision flowmeter. The inflow and outflow O2 and CO2 concentrations were monitored by gas analyzers (OM-11, Beckman and CD-3A, Applied Electrochemistry) arranged in a series, after the gas had passed through a drying column. The outputs of the analyzers were displayed on a computer monitor during on-line acquisition. V˙O2 and V˙CO2 were computed from the flow rate and the inflow-outflow concentration difference. The values, calculated at standard temperature, pressure, and dry conditions, are presented in milliliters per minute.

Protocol 1: V˙O2 at steady ambient temperatures. These measurements were performed on day 11 (E11) and day 20 (E20) embryos, and in H1 during the first (H1 < 8 h) or late hours (H1 > 8 h). Each age group consisted of different animals. Embryos were studied in sets of two, and the hatchlings were studied individually. Measurements of gaseous metabolism were collected at T = 39-36-33-30°C. Each T was maintained for 1 h, and data refer to the last 5 min of each exposure. T was changed either in ascending or descending sequence, alternating the order among animals. Because of the thermal inertia of the water bath, any change in T required about 15 min to reach complete equilibrium.

Protocol 2: time-course of response to cold. These measurements were performed at H1. After at least a half-hour of acclimatization to the respirometer, gaseous metabolism was measured continuously for 30 min at 39°C and for 1 h after an abrupt drop in water bath temperature to 30°C.

Protocol 3: preferred temperature. These measurements were performed on Nx and Hx hatchlings at <8 h and >8 h, studied individually in a thermocline. This consisted of a plastic tube 70 cm long with an inner diameter of 10 cm, closed by lids at either end, with steady airflow of 400 ml/min. An internal platform about 7 cm wide ran through the whole length. Two roller pumps circulated the water from separate water baths through long polyethylene tubing coiled around the thermocline, each coil a few centimeters apart. By regulating the temperature of the baths, the water flows and the relative distance of the coils, it was possible to generate a temperature difference between the two ends of the thermocline, from about 43 to 31°C, with an internal linear gradient of 5 cm°C. Four tungsten constantan thermocouples were permanently placed at various locations, to make sure that the prefixed T gradient was stable throughout the recording; the correlation coefficient of the distance-temperature regression line was never below 0.96, and averaged 0.984 ± 0.001.

The hatching was placed in the middle of the thermocline, and was left undisturbed for at least 1 h. Then, the chick’s position was noted (± 1.5 cm) continuously every min for 30 min. The average minute-by-minute difference in position represented the average distance traveled over the 30 min of the measurements. Position data were transformed into thermocline T from the slope and intercept of the position-T linear regression.

Number of animals, data normalization, and statistics. For each experiment, numbers of animals are provided in the RESULTS, Table 1, and figure captions. All group data are presented as means ± SE. Linear regression through the data points was used to compute the Q10 values of E11 and E20, according to the van’t Hoff equation Q10 = (V˙O2'/V˙O2)10/(T’−T), where V˙O2’ and V˙O2 are the values of V˙O2 at ambient temperature 39°C (T’) and 30°C (T’). Statistically significant differences between the two groups of data (e.g., body weights at any given age, Q10 values, V˙O2 at a given T) were evaluated by two-tailed t-test. Comparisons of the responses to the thermocline were done by two-way ANOVA (one grouping factor being age, the second being the normoxic or hypoxic incubation), with post hoc Bonferroni’s limitations for the four comparisons. In all cases, a difference was considered statistically significant at P < 0.05.

RESULTS

Number of animals, egg weights, age, postnatal hours of the experiments, body weights of embryos, and hatchlings are presented in Table 1.

Embryo’s growth and hatching. The body weights (W) of the embryos growing in hypoxia, collected every 3 days from day 5 to hatching, were lower than in Nx (Fig. 1). At E20, all of the hypoxic embryos had the yolk separated, while more than half of the Nx embryos studied had the yolk already incorporated in the abdomen, increasing their W by about 12 g. The hatching age of Hx occurred was 21.04 ± 0.07 days, an age slightly, yet significantly, longer than that of Nx (20.59 ± 0.07; P < 0.001). During the H1, the W of the Hx hatchlings (39.7 ± 0.5 g, n = 53) was close to that of Nx (40.5 ± 0.5 g,

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Table 1. Number of animals, egg weight, age, and body weight

<table>
<thead>
<tr>
<th>N</th>
<th>Egg Weight at E0, g</th>
<th>Day of Hatching</th>
<th>Posthatch, h</th>
<th>Body Weight, g</th>
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<tr>
<td>Protocol 1: Steady ambient temperatures (30–33–36–39°C)</td>
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<tr>
<td>E11</td>
<td>Nx 10 sets*</td>
<td>57.0±0.6</td>
<td>4.2±0.1</td>
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<tr>
<td></td>
<td>Hx 10 sets*</td>
<td>60.3±1.0 (&lt;0.001)</td>
<td>3.6±0.1 (&lt;0.001)</td>
<td></td>
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<tr>
<td>E20</td>
<td>Nx 10 sets*</td>
<td>56.2±0.8</td>
<td>40.5±1.6</td>
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<tr>
<td></td>
<td>Hx 10 sets*</td>
<td>60.9±0.7 (&lt;0.001)</td>
<td>27.2±1.0 (&lt;0.0001)</td>
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</tr>
<tr>
<td>H1 &lt; 8 h</td>
<td>Nx 7</td>
<td>54.1±1.7</td>
<td>20.7±0.3</td>
<td>3±1</td>
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<tr>
<td></td>
<td>Hx 7</td>
<td>58.8±0.7 (&lt;0.05)</td>
<td>21.0±0.0</td>
<td>3±1</td>
</tr>
<tr>
<td>H1 &gt; 8 h</td>
<td>Nx 7</td>
<td>59.8±1.8</td>
<td>20.9±0.2</td>
<td>13±3</td>
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<td></td>
<td>Hx 11</td>
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<td>13±1</td>
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<td>Protocol 2: Time course of response to 30°C</td>
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<td>H1 &lt; 8 h</td>
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<td>20.8±0.2</td>
<td>4±1</td>
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<td></td>
<td>Hx 8</td>
<td>59.9±1.6</td>
<td>20.9±0.1</td>
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<td>H1 &gt; 8 h</td>
<td>Nx 10</td>
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<td>Hx 7</td>
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<td>Protocol 3: Preferred temperature in thermocline</td>
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<td>H1 &lt; 8 h</td>
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<td>20.4±0.1</td>
<td>5±1</td>
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<td>Hx 10</td>
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<td>21.3±0.1 (&lt;0.001)</td>
<td>4±0.4</td>
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<td>H1 &gt; 8 h</td>
<td>Nx 10</td>
<td>55.5±1.2</td>
<td>20.4±0.1</td>
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<tr>
<td></td>
<td>Hx 10</td>
<td>54.2±1.5</td>
<td>21.2±0.2 (&lt;0.001)</td>
<td>16±1</td>
</tr>
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</table>

Values are means ± SE. Nx, normoxia-incubated; Hx, hypoxia-incubated; E11, E20, embryos at age 11 and 20; H1, first day posthatching. In parentheses, P value of the statistical comparison between Hx and Nx. *Each set comprises two embryos. Posthatch hours indicate the hours following hatching at the time of the measurements.

n = 52; P > 0.05). Specifics of body weight, days of hatching and posthatch hours for the H1 studied in the various protocols are given in Table 1.

Sustained changes in ambient temperature. The W of the E11 embryos used for these measurements averaged 4.2 ± 0.1 g, and 3.6 ± 0.1, in Nx and Hx, respectively (P < 0.001). At any given T, the V̇O₂ values of the Hx were lower than in Nx (Fig. 2, top). This was due to the differences in embryo’s W; in fact, after W normalization, the curves overlapped. The shape of the individual curves could be compared readily after expressing the T-V̇O₂ relationships in percent of the values at 39°C (bottom); they did not differ significantly from each other. Also, the Q₁₀ values of the two groups did not differ significantly (Nx, 1.92 ± 0.075; Hx, 1.94 ± 0.10).

The E20 of the Hx group used for these measurements weighed 27.2 ± 1.0 g, significantly less than the Nx embryos (40.5 ± 1.6 g). In either group of embryos, the decrease in T lowered V̇O₂. At any given T, the V̇O₂ values of Hx were lower than in Nx (Fig. 3, top left). After expressing the T-V̇O₂ relationships in percent of the values at 39°C (Fig. 3, bottom left), the Hx values were significantly lower than in Nx at T = 33°C (P < 0.05) and 30°C (P < 0.01). The steeper T-V̇O₂ curve in Hx than in Nx was reflected by the differences in Q₁₀ values, which averaged 1.41 ± 0.06 in Nx and 1.79 (± 0.08) in Hx (P < 0.005).

The W of H1 used for these measurements did not differ significantly between Nx and Hx, neither for the groups studied in the first hours (<8 h) nor for the groups studied in the later hours (8–24 h) (Table 1). In Nx, V̇O₂ at 33 and 36°C was higher than at 39°C. With further cooling to 30°C, this thermogenic response was not maintained in the younger hatchlings, whereas it was well maintained in the older group (Fig. 3, middle and right). This general pattern was also apparent in the Hx hatchlings, with two main differences. At the warmer temperatures, the absolute values (ml/min) of V̇O₂ of the Hx hatchlings exceeded the Nx values (Fig. 3, top, middle and right), the difference reaching statistical significance in the younger group. After expressing each curve in percent of the corresponding 39°C value (Fig. 3, bottom, middle and right), the T-V̇O₂ functions of the Hx were below Nx. At each T, this difference was statistically significant at 33 and 36°C (younger group) and at 30°C (older group). A statistical analysis of all the data points of the thermogenic response (i.e., the 30–36°C range) indicated a significantly lower response in Hx than in Nx, both for the <8 h group (Hx: 107% ± 5; Nx: 140% ± 8, P < 0.001) and the >8 h group (Hx: 131% ± 7; Nx: 158% ± 10, P < 0.05).
Response to thermocline. During the 30-min recording in the thermocline, on average, hatchlings moved at 4.0 ± 0.5 cm/min, with no significant differences between groups or age periods. In Nx, the most frequently chosen T was 37.6°C ± 0.4 and 37.4°C ± 0.4 in the younger and older groups, respectively. The preferred T of Hx did not differ significantly from Nx at either age (Fig. 5, left; two-way ANOVA, *P > 0.05*). When the same statistical analysis was applied to the 30-min data points of all animals combined (Fig. 5, right), a small significant difference emerged between the young Hx and Nx groups and between the younger and the older Hx groups (two-way ANOVA, post hoc Bonferroni’s limitations, *

**DISCUSSION**

The main information obtained from this study is that sustained embryonic hypoxia blunts the development of the thermogenic mechanisms. Previously, it was noticed that high-altitude infants had a lower thermogenic capacity than sea-level gestated infants (11). However, because the measurements were performed at high altitude, it was unclear to what extent the infant’s blunted thermogenesis was a consequence of the hypoxic gestation or was part of the acute response to hypoxia, which, in neonates, is characterized by hypometabolism (27). Measurements of the metabolic response to cooling temperatures in neonatal rats studied in normoxia after hypoxic gestation circumvented this problem (30). However, they could not eliminate the confounding issue of the maternal hypoxia, a stress-evoking condition that has implications on fetal and newborn development, including temperature control (2, 20, 34, 36). The current results in an avian model, therefore, give substantial weight to the possibility that prenatal hypoxia by itself, and independently of maternal responses, disturbs the normal development of thermogenesis.

Prenatal hypoxia may interfere with the normal development of thermogenesis through a variety of mechanisms. Chronic prenatal hypoxia blunts embryonic growth (Fig. 1) (5, 8), and during cold exposure, a small-sized animal decreases metabolic rate (through the Q10 effect) more readily than a large-size animal because of its smaller heat capacitance and larger surface-to-mass ratio. However, the possibility that the smaller body size may be the main reason for the differences in thermogenesis between Hx and Nx cannot apply to the current results, either at E20 or at H1. At E20, egg mass, not embryo mass, is the pertinent factor determining heat capacitance, and the Hx eggs were not smaller than the Nx eggs. The two groups of hatchlings did not differ in body size; in fact, the slightly longer incubation time allowed the Hx embryos to incorporate their yolk, so that, by hatching time, they had reached a body size comparable to Nx, meaning that they also had a comparable surface-to-mass ratio. A similar phenomenon was seen in embryos incubated in the cold, in which the lower embryo’s weight was compensated by a longer incubation time (23).

Another possibility is the existence of cardiopulmonary malformations in the hatchlings of the Hx group causing hypoxemia, a situation that would decrease their metabolic rate and impede a calorigenic response (28). However,
although embryonic hypoxia can modify the growth of various organs, neither the lungs nor the heart are affected by the level of hypoxia (15% O2) adopted in this study (see Ref. 5, also for review). In addition, the resting V\O2 (at 39°C) of the Hx hatchlings was not lower than in Nx. On the contrary, in the younger group, it was slightly higher than in Nx, presumably to pay back some lactic acid accumulation and O2 debt contracted during the hatching efforts in hypoxia or for the compensatory (catch-up) growth upon return to normoxia. Hence, it does not seem probable that the Hx chicks had hypoxemia and that this was the cause of their poor thermogenesis.

At E11, that is, at an age when the embryo is purely ectothermic, the lower metabolic rates of Hx were undoubtedly a consequence of their blunted body growth. In fact, the differences in V\O2 from Nx could be entirely corrected after normalization by weight or after expressing the curves in percent of the values attained at 39°C (Fig. 2). Also the Q10 values were similar between the two groups, and close to 2, which is the normal value for ectothermic embryos (23, 28, 39, 43). Because thermogenic mechanisms begin to function toward the last days of incubation, as apparent by a decrease in Q10, a developmentally retarded embryo retains the ectothermic pattern for a longer time during incubation, with higher Q10 values than normal. This is what was observed previously in normoxic embryos growth-retarded by a low incubation temperature (23) and could explain the differences between Hx and Nx at E20 and H1. Hatching is a biological event, not linked to a specific chronological age, and hypoxia delayed its occurrence by about 11 h. Therefore, the lower thermogenesis in Hx than Nx may reflect a generalized blunting effect of embryonic hypoxia on growth and a delay on the timing of hatching. Alternatively, the similar body weight at a time when thermogenesis was decreased may be interpreted as indicating that embryonic hypoxia did not affect body growth and the processes surrounding hatching as much as it affected thermogenesis. A disturbance in the relative timing of the onset of different physiological processes has been termed “heterokairy” (38), which is a special case of “epigenetic adaptation”. This latter expression refers to the long-term effects of early environmental perturbations on gene expression and the development of physiological mechanisms. Cold exposure during prenatal development has been quoted in the past as an example of epigenetic adaptation for the postnatal manifestation of thermogenesis (23, 32, 33, 42). The current results may suggest that embryonic hypoxia is another cause of epigenetic adaptation of thermogenesis. Because both hypoxia and cold during most of embryonic development cause hypometabolism, it would be of interest to study other

Fig. 3. Ambient temperature-oxygen consumption (V\O2) relationships in 20-day-old embryos (left) and in hatchlings, during the first 8 h from hatching (middle), or during the remaining hours of the day (right). V\O2 is expressed in absolute values (top) and in percent of the values attained at 39°C (bottom). ○, normoxia-incubated animals. ▲, hypoxia-incubated animals. Symbols are the averages of 10 sets of two embryos each, or of 10 hatchlings. Values are means ± SE. *Statistically significant differences between the two groups (P < 0.05).
interventions resulting in sustained metabolic changes, such as caloric restriction or pharmacological interventions, to test the possibility that embryonic hypometabolism represents the common event for the blunting in the development of thermoregulation. Equally, it would be informative to know whether the effects of hypoxia on the development of thermogenesis have specific time windows during embryonic growth, as it is the case for body and organ development (5, 8).

The preferred ambient T of the Hx hatchlings essentially coincided with that of Nx. Only the younger Hx group showed a small tendency for lower temperatures, but the difference from Nx was minute and, presumably, was dictated by their higher resting VO$_2$. Our interpretation of these results, therefore, is that prenatal Hx did not cause a long-term modification of the normoxic setpoint of thermoregulation. This is different from the drop in setpoint known to occur in the presence of hypoxia, and demonstrated in many classes of animals (6, 7, 15, 25, 44).

In conclusion, embryonic hypoxia delayed the establishment of thermogenic mechanisms. The blunted thermogenesis was manifest also after hatching, at a time when the hypoxia-incubated animals had reached normal body weight. It is possible that the blunted thermogenesis is one aspect of a generalized hypoxia-mediated slowing of all embryonic developmental trajectories. Alternatively, it may indicate that prenatal hypoxia has differential impact on growth, the timing of hatching, and thermoregulation. If these data were extrapolated to humans, they would suggest that fetal hypoxia, as with maternal smoking or high-altitude pregnancy, could endanger the heat control mechanisms of the neonate, not only because of the infant’s small size (26, 35), but also because of the epigenetic effects on neonatal thermogenesis.
REFERENCES


GRANTS

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