Ventilation changes associated with hatching and maturation of an endothermic phenotype in the Pekin duck, Anas platyrhynchos domestica

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Sirsat TS, Dzialowski EM. Ventilation changes associated with hatching and maturation of an endothermic phenotype in the Pekin duck, Anas platyrhynchos domestica. Am J Physiol Regul Integr Comp Physiol 310: R766–R775, 2016. First published January 27, 2016; doi:10.1152/ajpregu.00274.2015.—Precocial birds begin embryonic life with an ectothermic metabolic phenotype and rapidly develop an endothermic phenotype after hatching. Switching to a high-energy, endothermic phenotype requires high-functioning respiratory and cardiovascular systems to deliver sufficient environmental oxygen to the tissues. We measured tidal volume (VT), breathing frequency (f), minute ventilation (VE), and whole-animal oxygen consumption (VO2) in response to gradual cooling from 37.5°C (externally pipped paranates, EP) or 35°C (hatchlings) to 20°C along with response to hypercapnia during developmental transition from an ectothermic, EP parame in entothermic hatching. To examine potential eggshell constraints on EP ventilation, we repeated these experiments in artificially hatched early and late EP paranates. Hatchlings and artificially hatched late EP paranates were able to increase VO2 significantly in response to cooling. EP parame had high f that decreased with cooling, coupled with an unchanging low VT and did not respond to hypercapnia. Hatchlings had significantly lower f and higher VT and VE that increased with cooling and hypercapnia. In response to artificial hatching, all ventilation values quickly reached those of hatchlings and responded to hypercapnia. The timing of artificial hatching influenced the temperature response, with only artificially hatched late EP animals, exhibiting the hatching ventilation response to cooling. At the end of incubation, oxygen limitation occurs across the eggshell because of a limited number of eggshell pores and reliance on the vascular choioallantoic membrane (CAM) for oxygen uptake. During the hatching period, the transition exists from reliance on gas exchange at the CAM to exchanges at the lungs as the embryo first externally and then internally pips the eggshell with its beak. Whittow and Tazawa (30) proposed a model for endothermic development in precocial birds that describes phases experienced during the transformation from ectothermy to endothermy. Initially, developing embryos are in a limiting stage where embryo metabolism is limited by oxygen conductance across the eggshell (30). During the paranatal hatching period, the embryo begins to breathe with its lungs as it first internally pips through the inner membrane of the shell and then externally pips the eggshell with its beak. Whittow and Tazawa (30) proposed that during the external pipping stage, precocial species are no longer oxygen limited because they are beginning to breathe with their lungs, but instead are power-limited by immature metabolic capacity of the tissues, thus limiting endothermy. Endothermic capacity is reached once tissues and organ systems express a metabolic phenotype with the ability to increase aerobic respiration and generate enough heat to maintain homeothermy.

At the end of incubation, oxygen limitation occurs across the eggshell because of a limited number of eggshell pores and reliance on the vascular choioallantoic membrane (CAM) for oxygen uptake. During the hatching paranatal period, a transition exists from reliance on gas exchange at the CAM to exchanges at the lungs as the embryo first internally and then externally pips (13, 19). As the lungs increase their contribution to oxygen delivery, limits placed on oxygen uptake by eggshell conductance should decrease. One might expect that the ability to increase oxygen delivery by changing breathing patterns in externally pipped paranates might allow for hypercapnic and endothermic ventilatory responses when faced with high CO2 or cooling ambient temperatures.

The ventilator chemosensitivity to hypoxia and hypercapnia differs between externally pipped paranates and hatchlings (16, 27). In chicken hatchlings, a strong hypoxic and hypercapnic response of breathing frequency (f), tidal volume (VT), and pulmonary ventilation (VE) exists. Chemosensitivity in VE appears to be lacking in externally pipped chicken paranates: there is little change in response to either 10% hypoxia or 4% hypercapnia (27). Externally pipped paranates are apparently the cardiovascular system may place the greatest limits on maximal oxygen transport associated with aerobic metabolism of adult vertebrates (11). Whether this holds true for developing life stages is unknown. It may be more likely that during ontogeny, multiple levels of the oxygen cascade develop together, and no one level is limiting.

Avian embryos begin life with an ectothermic metabolic phenotype and are incapable of regulating body temperature by internal heat production (30), but precocial species, like the Pekin duck (Anas platyrhynchos domestica), develop an endothermic metabolic phenotype rapidly upon hatching. Whittow and Tazawa (30) proposed a model for endothermic development in precocial birds that describes phases experienced during the transformation from ectothermy to endothermy. Initially, developing embryos are in a limiting stage where embryo metabolism is limited by oxygen conductance across the eggshell (30). During the paranatal hatching period, the embryo begins to breathe with its lungs as it first internally pips through the inner membrane of the shell and then externally pips the eggshell with its beak. Whittow and Tazawa (30) proposed that during the external pipping stage, precocial species are no longer oxygen limited because they are beginning to breathe with their lungs, but instead are power-limited by immature metabolic capacity of the tissues, thus limiting endothermy. Endothermic capacity is reached once tissues and organ systems express a metabolic phenotype with the ability to increase aerobic respiration and generate enough heat to maintain homeothermy.

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unable to alter ventilation to a great extent in response to CO₂ and O₂.

During hatching and development of endothermy, the response of the maturing respiratory system to cold thermal stress in the paranatal, externally pipped stage and hatching stages is unknown. Could one constraint on attaining an endothermic metabolic phenotype in externally pipped paranatal embryos be due to a limit placed by the eggshell on their ability to increase V˙E and oxygen delivery to the lungs? In this study, we examined the response of the developing respiratory system of externally pipped paranates and day 0 hatchlings to cold thermal stress and hypercapnia. We then looked at ventilation patterns in animals as externally pipped paranates in the egg and then after artificial hatching to address the possible constraints placed on ventilation by the eggshell during the externally pipped stage.

**MATERIALS AND METHODS**

**Animals**

Pekin duck (*Anas platyrhynchos*) eggs were obtained from Blanco Industries (McKinney, TX) and incubated at 37.5°C and 60% relative humidity (RH). Sampling of multiple respiratory chambers, allowing inflow gas to be sampled at least every 7.5 min. To hatching, internally pipped eggs were moved into a clear Lyon drier. Data were recorded with LabChart 7 and a PowerLab 16SP (ADInstruments). Oxygen and nitrogen were mixed with a microprocessor control unit (controller model 0154, Brooks Instruments, Hatfield, PA) to achieve an inflow gas mixture between 20.9 and 21.3% oxygen with a balance of nitrogen. Oxygen and nitrogen were mixed with a microprocessor control unit (controller model 0154, Brooks Instruments, Hatfield, PA) to achieve an inflow gas mixture between 20.9 and 21.3% oxygen with a balance of nitrogen. The gas flow rate to the chambers was measured with a calibrated FlowBar1 mass flow meter (Sable Systems International, Las Vegas, NV). A sample stream of the outflow gas was pulled by an R1 flow controller (AEI Technologies, Pittsburgh, PA) through a nafion tube (ADInstruments, Colorado Springs, CO) surrounded by Drierite, a column of Sodasorb, and another nafion tube drier (for water and CO₂ removal) before it was pulled through a FC-1B O₂ analyzer (Sable Systems International) to measure oxygen levels. In some experiments, CO₂ in the inflow and outflow gas was also measured using a CA-10 CO₂ analyzer (Sable Systems International). In this case, the gas stream was dried by a single nafion tube drier. Data were recorded with LabChart 7 and a PowerLab 16SP (ADInstruments). Sampling of multiple respirometer chambers outflow gas was automatically controlled by a custom-built four-channel solenoid multiplexer controlled by LabChart 7. At most, three animals were measured at any given time, and each chamber was sequentially sampled for 120 to 150 s during the course of an experimental run. An example trace shows that 120 s is sufficient for the oxygen signal to stabilize in our system (Fig. 1). An additional solenoid allowed for sampling of inflow O₂ and CO₂ levels between sampling of the animal chambers, allowing inflow gas to be sampled at least every 7.5 min.

Rates of oxygen consumption (V˙O₂, ml O₂/min) were calculated using the following equations derived from Withers (31):

\[
V\dot{O}_2 = V_1 \times \frac{(F_{O_2} - F_{EO_2}) - (F_{CO_2} - F_{ECO_2})}{1 - F_{EO_2}}
\]

(1)

\[
V\dot{O}_2 = V_1 \times \frac{(F_{O_2} - F_{EO_2})}{1 - F_{EO_2}}
\]

(2)

where V₁ is incurrent flow rate (ml/min), F₁O₂ and F₁CO₂ are incurrent O₂ and CO₂ fractions of dry gas, and FEO₂ and FECO₂ are excurrent O₂ and CO₂ fraction in dry gas. When CO₂ was not recorded, but scrubbed with Sodasorb, Eq. 2 was used.

During measurements of V˙O₂, animal temperature was continuously recorded. Body surface temperature was measured in externally pipped paranates just under the shell. The eggshell was cleaned with 70% isopropyl alcohol, and a small hole was made with an 18-gauge needle or dental drill. The egg was candled prior to inserting the thermocouple to ensure that no major chorioallantoic blood vessels were damaged. A 36-gauge copper constantan thermocouple was placed just under the shell on the animal’s skin and secured in place with dental wax (Kerr, Czech Republic). Shell temperature during gradual cooling of three infertile eggs warmed to 40.4°C prior to cooling were also measured to show the cooling response of an egg without CAM blood flow or internal heat production. Body temperature of hatchlings was measured in the cloaca. Thermocouples were placed in the cloaca and held in place with a small plastic disk glued to the feathers of the animal, as in Ricklefs and Williams (20). Temperatures were measured using temperature pods and recorded with a PowerLab 16SP and LabChart 7 (ADInstruments). Wet thermal conductance (C; ml O₂·h⁻¹·g⁻¹·°C⁻¹) was calculated for some of the hatchlings in experiment 4 using the equation: V˙O₂/[Tb - T Estate mass] using whole hatchling mass, including the yolk sac.

**Pulmonary Ventilation**

Tidal volumes (V₁) were estimated during the cooling trials and CO₂ exposure using a modification of the barometric technique (6, 27). Changes in pressure associated with breathing were measured with a spirometer (ADInstruments) connected in-line with each metabolic chamber (14). Volume calibration was conducted on each metabolic chamber after each trial by injecting known volumes of air (Vcal) into the system using a Hamilton syringe (Hamilton, Reno, NV). The corresponding change in pressure (Pcal) was used to calibrate the system (K = Vcal/Pcal as in Ref. 27), where K is a calibration constant. Respirometer chamber relative humidity was measured on experiment air with a relative humidity sensor (HH 4021, Honeywell, Minneapolis, MN) and was used to estimate water vapor pressure in
the chamber. All data were recorded with a PowerLab 16SP and LabChart 7 (ADInstruments). Breathing frequency (f, breaths/min) was determined from the pressure waves using the cycle measurements function in LabChart 7. Tidal volume (μL/min BTPS) was estimated from K, chamber and body temperature, water vapor pressure, and the measured pressure changes as in Szdzuy and Mortola (27). Minute ventilation (VE, ml/min BTPS) was calculated as VT·f.

**Experimental Design**

**Experiment 1.** Ventilation and VO₂ patterns were measured in externally pipped paranates (n = 7), and day 0 hatchlings (n = 5; between 0 and 24 h old) during gradual cooling. After the resting period, temperature of the incubation chamber was gradually decreased at a rate of 9.2°C/h until an air temperature of 20°C was reached. During these runs, flow through the chambers was high enough to maintain chamber CO₂ below 1.5%. A second set of early externally pipped paranates was exposed to 40% O₂ during cooling (n = 8).

**Experiment 2.** To ensure the hatchling ventilation changes that we observed in experiment 1 were not in response to elevated CO₂, ventilation patterns were estimated in response to 0, 1.5, and 4% CO₂ in another set of day 0 hatchlings (n = 4). Animals were maintained at 35°C for the entire CO₂ exposure experiment. After the resting period, gas flowing into the chamber was changed to 21% O₂ and 1.5% CO₂ balanced with nitrogen for 15 to 20 min. Inflow CO₂ level was then increased to 4% CO₂ for an additional 15 to 20 min. Inflow gas was mixed with the Brooks flow controllers, as previously mentioned.

**Experiment 3.** To determine whether limits to ventilation chemosensitivity are influenced by the eggshell, we measured hypercapnic ventilation responses in externally pipped paranates in the eggshell and then after artificially removing them from the egg (artificially hatched). Animals were maintained at 37.5°C for this experiment. Ventilation patterns were measured in externally pipped paranates for 30 min. After recording the baseline ventilation response, the current CO₂ level was increased to 4%, and ventilation was recorded for an additional 20 min. The externally pipped paranates were then quickly removed from the metabolic chamber, helped out of the eggshell, and dried with a hair drier (Conair, Stamford, CT). In some cases, the CAM artery and vein were tied off at the belly of the animal. The artificially hatched animals were placed back into the metabolic chamber, and baseline ventilation was measured for 45–60 min. This was followed by an increase in the current CO₂ level to 4% for 20 min. Shell and cloacal temperatures were measured as noted above.

**Statistics**

Ventilation data, VO₂, and animal temperature were analyzed within and between each age group using two-way repeated-measures ANOVA with air temperature or CO₂ level as the repeated variable, followed by a Sidak post hoc test. Values from experiment 3 were log transformed prior to analysis due to unequal variability between treatments. To examine differences between externally pipped and day 0 hatchlings, we used a t-test comparing resting thermoneutral values at 37.5°C for externally pipped paranates with those at 35°C for day 0 hatchlings. Mean minimum wet thermal conductance was determined at air temperatures below 30°C and examined with a t-test. Statistical analysis was carried out with Prism 6 (GraphPad Software, La Jolla, CA) and SigmaPlot 12.0 (Systat Software, San Jose, CA). For all statistical tests, level of significance was set at P < 0.05. All data are presented as means ± SD.

**RESULTS**

Body mass of developing animals did not differ between external pipping and after hatching (Table 1). There was no difference between externally pipped paranatal and hatchling whole body mass (P = 0.064), residual yolk sac mass (P = 0.21), or yolk-free body mass (P = 0.05).

**Externally Pipped Paranate and Hatchling Responses to Cooling**

An endothermic phenotype developed rapidly upon hatching (Fig. 2). Hatchlings had a significantly higher VO₂ in their thermoneutral zone than did externally pipped paranates at the same air temperatures (Table 1). During external pipping, VO₂ remained constant during cooling from 37° to 20°C (Fig. 1A; P = 0.97). Upon hatching, the hatchlings were able to increase VO₂ in response to gradual cooling. Oxygen consumption

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<th>Table 1. Resting values in externally pipped paranates and day 0 posthatching Pekin duck from experiment 1</th>
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<tr>
<td><strong>Age</strong></td>
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<td><strong>Tₑ</strong>, °C</td>
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<tr>
<td>EP (n = 7)</td>
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<td>40.2 ± 1.3</td>
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<td>65.2 ± 5.8</td>
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<td>Residual yolk mass, g</td>
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<td>f, breaths/min</td>
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<td>VO₂, ml O₂/min</td>
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Values are presented as means ± SD. EP, externally pipped; Tₑ, body temperature. *Data for Muscovy duck and chicken hatchlings from Mortola and Toro-Velasquez (16). †EP chickens from Szdzuy and Mortola (27). ‡Wedge-tailed Shearwater from Petit and Whittow (18) and Zhang and Whittow (33). For correct comparisons with published values, mass specific values are based on whole body mass including yolk. *Estimated using externally pipped and hatchling body masses from Zhang and Whittow (33).
began to increase significantly at 27°C and continued to increase in a linear fashion during the rest of the cooling exposure ($P < 0.001$).

Surface body temperature of externally pipped paranates was not significantly different from hatching cloacal temperature at the highest air temperatures (Table 1). Upon cooling, both externally pipped paranates and hatchlings showed significant decreases in surface and cloacal temperatures, respectively ($P < 0.001$). Surface body temperature of externally pipped paranates began to decrease significantly around an air temperature of 27°C (Fig. 2D). Hatchling cloacal temperature did not drop significantly until 27°C. At the lowest air temperature, hatchlings had a significantly higher body temperature (∼36.7°C) than externally pipped paranates (∼32.1°C).

Externally pipped paranates and hatchlings exhibited significantly different ventilation patterns (Table 1; Fig. 1, C–E). Ventilation rate was significantly higher in externally pipped paranates compared with hatchlings (Table 1; $P < 0.001$). Upon cooling, externally pipped paranates’ $f$ decreased significantly (Fig. 2C; $P < 0.001$). In contrast, hatchling $f$ was initially constant during the first portion of cooling in the thermal neutral zone and then increased significantly at temperatures below 27°C ($P < 0.001$).

The estimated $V_T$ was significantly higher in hatchlings than externally pipped paranates, while both were within the air temperature of the hatchlings thermoneutral zone (Table 1; $P < 0.001$). There was a significant effect of temperature on estimated $V_T$ in the externally pipped paranate with $V_T$ being higher at air temperatures below 24°C compared with $V_T$ at 37.5°C (Fig. 2D; $P = 0.007$). Hatchlings exhibited a significant increase in $V_T$ only at the lowest air temperature (20°C; $P = 0.015$).

The $V_E$ differed significantly between the two ages (Table 1; Fig. 2E): $V_E$ was significantly higher in hatchlings compared with externally pipped embryos. During cooling, $V_E$ did not change in externally pipped paranates (Fig. 2E; $P = 0.56$). Hatchling $V_E$ remained constant during the first portion of cooling and then increased significantly in a linear fashion (Fig. 2E; $P < 0.001$). Below an air temperature of 28°C, hatchling $V_E$ was significantly higher than that of externally pipped paranates.

**Hatching Response to Hypercapnia**

Hatchlings significantly increased $f$ ($P = 0.0007$), $V_T$ ($P = 0.0038$), and $V_E$ ($P = 0.0002$) in response to hypercapnic
conditions (4% CO₂; Fig. 3). In response to increasing CO₂ from 0% to 1.5%, f, VT, and V˙E did not change. Increasing CO₂ from 1.5% to 4% produced a significant increase in f, VT, and V˙E.

**Ventilatory Response to Hypercapnia after Artificial Hatching**

Artificial hatching during the externally pipped stage resulted in significant changes in ventilation without changes in VT. The V˙O₂ did not differ between the externally pipped paranate in the eggshell, and once it was artificially removed from the shell (Fig. 4A; P = 0.53). During external pipping in the eggshell, there were no changes in f, VT, and V˙E in response to hypercapnia (Fig. 4, B–D). Upon artificial hatching, there was a significant increase in normocapnic VT and V˙E and significant decrease in f compared with the same animal as an externally pipped paranate in the eggshell. In response to 4% CO₂, the artificially hatched animals significantly increased V˙E. This was accomplished mainly by significantly increasing f (Fig. 4B). The response in VT was variable, increasing in some and remaining constant or slightly decreasing in others (Fig. 4C).

**Ventilatory and Metabolic Response to Cooling after Artificial Hatching**

The endothermic V˙O₂ response to cooling depended upon the length of time an animal had been in the externally pipped stage (Fig. 5). Externally pipped paranates with initial eggshell star fractures exhibited an ectothermic V˙O₂ response to cooling, while those that had been in the externally pipped stage for longer had an intermediate V˙O₂ response to cooling. With increased length of time in the externally pipped stage, there was an increase in V˙O₂ with cooling that was not maintained at the coolest air temperatures. Early externally pipped paranates were unable to increase V˙O₂ during cooling when exposed to 40% O₂ (Fig. 6).

An endothermic V˙O₂ response in artificially hatched animals depended upon the length of time the animal had been in the externally pipped stage (P < 0.001 for V˙O₂, f, and V˙E; P = 0.01 for VT, Fig. 7). Animals that were removed from the shell with star fractures maintained V˙O₂, f, or V˙E and significantly increased VT, in response to gradual cooling (Fig. 7). If allowed to stay in the eggshell for longer before artificial hatching, V˙O₂, f, VT, and V˙E all increased significantly with cooling after the artificial hatching (Fig. 7). At air temperatures below 30°C, cloacal temperatures were significantly higher in the late externally pipped hatchlings compared with the early externally pipped hatchlings (P < 0.001; Fig. 7B). Mean minimum wet thermal conductance was lower in the early externally pipped hatchlings (0.10 SD 0.02 ml O₂·h⁻¹·g⁻¹·°C⁻¹) compared with the late externally pipped hatchlings (0.15 SD 0.02 ml O₂·h⁻¹·g⁻¹·°C⁻¹) across the lowest air temperatures (P < 0.001).

**DISCUSSION**

**Development of an Endothermic Metabolic Response**

Upon hatching, Pekin duck’s metabolic response to cooling transitioned rapidly from an ectothermic phenotype to an endothermic phenotype (Fig. 2A). The mean response of externally pipped paranates in the eggshell was to not change V˙O₂ and heat production during to gradual cooling. This is a typical response for a precocial embryo at this stage. Whittow and Tazawa (30) proposed that at the end of incubation, the embryo’s ability to increase V˙O₂ becomes limited by the oxygen conductance of the eggshell. During the paranatal hatching period, the embryo begins to ventilate the lungs first during internal pipping and then after the eggshell is broken at external pipping. As the lungs become a more prominent site of gas exchange (13, 19), Whittow and Tazawa (30) predicted that embryos become power-limited as the limits of eggshell conductance are countered by increasing lung ventilation. Pekin...
duck embryos exhibited a varying ability to increase $\dot{V}_O_2$ during external pipping depending on the extent of pipping (Fig. 5). Newly externally pipped paranates were unable to increase $\dot{V}_O_2$, while older externally pipped paranates were able to increase $\dot{V}_O_2$ to a limited extent during gradual cooling. Internally and externally pipped chicken embryos were unable to increase $\dot{V}_O_2$ in response to cooling at 30°C for 1 h (26). Even when the blunt end of the eggshell covering the air cell was removed, chicken embryos were unable to increase $\dot{V}_O_2$ to a great extent (26).

Once the paranate has hatched, resting $\dot{V}_O_2$ increased, and the ability to increase $\dot{V}_O_2$ upon cooling appeared rapidly. A strong endothermic response upon hatching, as in the duck, has been observed in other water birds (9, 24), while other species, such as the chicken, may not show as strong an endothermic response until a day or two after hatching (1, 26, 32). The rapid appearance of an endothermic $\dot{V}_O_2$ response to cooling upon hatching led us to examine changes in ventilation patterns associated with obtaining an endothermic phenotype.

Body temperature of all ages (externally pipped and hatchlings) decreased with the cooling exposure. As expected, surface eggshell temperature of externally pipped paranates experienced a greater drop than cloacal temperature of the hatchling (Fig. 2B). The externally pipped eggshell cooled in a similar fashion to the eggshell of infertile eggs. Tazawa et al. (29) found that chicken eggs tended to cool at similar rates with and without a developing embryo present. In hatchlings, while $\dot{V}_O_2$ and thus, heat production, increased, cloacal temperature dropped during the second half of the cooling bout. The capacity to maintain body temperature increased as the externally pipped paranatal stage progressed. Hatchlings that were helped out of the eggshell early in the externally pipped stage had a greater drop in body temperature than those artificially

Fig. 5. Examples of individual oxygen consumption (ml/min) from externally pipped paranates at various stages of hatching in response to gradual cooling.

Fig. 4. Breathing pattern response to exposure to 0% and 4% CO2 at 37.5°C in animals as externally pipped paranates and then after artificial hatching by removing the animal from the eggshell. Whole animal oxygen consumption (ml/min) (A), ventilation rate (breaths/min) (B), tidal volume (µl/breath) (C), and minute ventilation (ml/min) (D). Values with different letters are significantly different from each other at $P < 0.05$. Data points express means ± SD; $n = 7$. Gray lines indicate individual responses.
hatched later in external pipping (Fig. 7B) but had a lower wet thermal conductance (Fig. 7C). Lower wet thermal conductance during the first hours after hatching compared with older hatchlings have been observed in both Eider duck (25) and chicken (15) hatchlings. The difference observed in Eider duck minimum wet thermal conductance was suggested to be due to changes in temperature regulation of the periphery (25). From external pipping through hatching, the capacity to maintain body temperature may be limited by maturation of the insulation and the extent of aerobic and thermogenic capacity of the tissues (i.e., power-limited).

In externally pipped paranates, we measured body surface temperature just under the surface of the eggshell. Because body surface temperature is closer to the surface of the egg, a more rapid drop in externally pipped egg surface temperature is expected than that of the hatchling core temperature. Heat loss across the eggshell might be greater because of the lack of insulation. Core body temperature of externally pipped paranates most likely did not drop as much as the measured subsurface egg temperature and is probably closer to that of hatchlings. We did not measure core temperature of the externally pipped embryo in the eggshell and were not able to estimate minimum wet thermal conductance at this stage.

**Breathing Frequency, Tidal Volume, and Minute Ventilation**

Resting ventilation patterns undergo significant changes during hatching. Externally pipped paranates had a higher resting
f when compared with hatchlings (Fig. 2C), roughly three times higher than hatchlings. Similar high f of internally pipped Pekin duck embryos, ranging from 86 to 239, have been previously observed (23). The difference in f between Pekin duck externally pipped paranates and hatchlings was greater than in other species. The difference in resting f between externally pipped paranates and hatchlings was only a 1.2-fold (Table 1; Refs. 16 and 27). In wedge-tailed shearwater, f of externally pipped embryos and hatchlings was not different (Table 1; Ref. 33). However, the Pekin duck differs from other species in that externally pipped f is high, and upon hatching, f decreases significantly.

Pekin duck hatchlings have a lower f when compared with other precocial hatchlings (Table 1). Pekin duck hatchling f is 31, 27, and 47% lower than wedge-tailed shearwater, Muscovy duck, and chicken hatchlings (16, 33). At rest, hatchlings breathe at a rate of 33 breaths/min, which is roughly three times the breathing frequency of adult Pekin ducks (2, 5, 12). A similar difference between hatching and adult f is observed in chicken and Muscovy ducks (10, 12, 16).

Resting estimated VT differed significantly between externally pipped paranates and hatchlings. Externally pipped paranatal VT was less than 20% that of day-old hatchlings. Similar differences in VT have been observed in externally pipped paranates and hatchling chickens. Comparisons of similarly sized externally pipped paranates and hatchlings reveal a large difference in VT. As with the duck, VT in externally pipped chicken paranates is about 20% that of similar sized hatchlings (16, 27). Similar VT differences between externally pipped paranates and hatchlings are seen in developing chicken and wedge-tailed shearwater (Table 1). The Pekin duck hatchling has a larger absolute VT compared with Muscovy ducks and larger VT normalized to body mass than the Muscovy duck, chicken, and wedge-tailed shearwater (Table 1).

At each stage of development and environmental condition, an animal needs to ensure that minute ventilation provides adequate oxygen supply at the lungs to support resting or active VO2. Externally pipped paranate resting VE was only 57% that of a hatching, while VO2 was 73%. At this stage, the externally pipped paranate is also relying on the CAM for a portion of oxygen exchange. When compared with hatchlings, VE was lower in externally pipped paranates due to the significantly lower VT, even though f was three times greater in the externally pipped paranate. Similar differences in VE have been observed between externally pipped and hatchling chickens (Table 1; Refs. 16 and 27) and wedge-tailed shearwater (18). As with Pekin duck, differences in VT between the two stages were the main factor driving the different VE in these other species.

Differences in resting f, VT, and VE between externally pipped paranates and hatchlings appear to be due to limits placed on VT by the eggshell. This is supported by the observation that artificially hatching externally pipped paranates resulted in a rapid change in f, VT, and VE to hatching levels, regardless of the age of the externally pipped paranate. Ventilation in birds involves filling of multiple air sacs within the animal that require an expansion and increase in the volume of the animal (21). The eggshell may hinder full air sac filling, resulting in low VT that was compensated in externally pipped paranates by an elevated f. Tidal volume increased six-fold upon artificial hatching compared with their externally pipped levels in the eggshell (Fig. 4C). While we did not measure lung volume, chicken lung volumes were not significantly different between externally pipped paranates and day-old hatchlings (22).

Pulmonary Ventilation and Endothermy

In response to cooling, externally pipped paranates were on average unable to increase VO2 and ventilation. There was a significant decrease in f during cooling that was compensated by a small, but significant, increase in VT at the lowest temperature. This allowed the animal to maintain VE at resting levels, but not increase it. A similar change was observed in externally pipped Japanese quail f that decreased from 82 to 41 breaths/min when cooled from 38°C to 28°C for 40 min (17). During external pipping in the egg, f may be near the maximal frequency and unable to increase further.

Thermoregulatory heat production in externally pipped paranates may be limited at this stage by their inability to increase VE when exposed to cold. As noted above, externally pipped embryo resting VT is only 20% of the hatching value at rest. If VT of externally pipped embryos is limited by the animal’s ability to expand the air sacs, then they should not be able to exhibit an endothermic metabolic response to cooling. Upon hatching, the constraint of the eggshell on VT is removed and the hatchling responds to cooling by increasing VE. During initial cooling in the thermal neutral zone, there is no increase in ventilation in the duck. At around 28°C, the hatching increases VO2 and VE in a typical endothermic response. During gradual cooling to 20°C, f, VT, and VE continue to increase, providing oxygen to the lungs to meet the increasing thermoregulatory VO2 demand. This increase in VE occurs by increasing both f and VT during cooling. Hatchling f at the coldest temperature measured (20°C) had increased to that of the cold externally pipped embryo. This was lower than the maximal measured in the resting externally pipped embryo.

The low VT in externally pipped paranates may explain the limited capacity of thermogenesis during this stage, but there may also be limits on the metabolic machinery to generate heat during the externally pipped stage. To test this, we artificially hatched externally pipped paranates, either early or late in the external pipping stage. During external pipping, the length of time in this stage influenced an animal’s ability to increase VE in response to cooling. Animals artificially hatched early in the externally pipped stage were unable to increase f, VT, VE, and VO2 in response to cooling; instead, they only maintained resting levels. When artificial hatching occurred later in the externally pipped stage, the hatchlings were able to respond to cooling with increases in f, VT, VE, and VO2. Therefore, during the externally pipped stage, there is maturation of the ventilatory response possibly due to maturation of the thermosensors in the animal. Additionally, early externally pipped embryos with only small star fractures exposed to 40% O2 were unable to increase VO2 during cooling (Fig. 6). This is unlike the emu, in which VO2 increased during cooling in a hyperoxic environment as early as the internally pipped stage (7). Our data suggest that along with maturation of the ventilatory response, there is a maturation of the cardiovascular system and metabolic capacity of the shivering muscles required before the animal exhibits an endothermic phenotype.
The ventilatory response to cooling in the hatchling Pekin ducks was different from that found in adult Pekin ducks (2). Bech et al. (2) found that when measured at 20°C, 0°C, and -20°C, the adult Pekin ducks increase VO₂ by increasing VE, mainly through increasing VT. In contrast, we found that during cooling, the hatchlings increased VT and, to a greater extent, f.

Hatchling ventilation patterns in response to cooling in altricial species differ from the precocial Pekin duck. The altricial rosly-faced lovebird does not show an endothermic ventilation response until 7 days posthatch, which increases through day 11 (4). On day 11, the nestling is able to increase VO₂ during 5°C cooling bouts. Increase in VO₂ is associated with an increase in VE due solely to an increase in f. This example contrasts with the duck hatchling that increased both f and VT.

**Development of Ventilatory Chemosensitivity**

To determine whether lack of a ventilatory response during external pipping is due to an insensitivity of ventilation, we examined hatchling ventilatory chemosensitivity in response to hypercapnia. In response to 1.5% CO₂, there was a small, insignificant increase in f, VT, and VE. This change in ventilation at 1.5% CO₂ was less than the changes in hatchling Muscovy ducks or chickens in response to 2% CO₂ (16). A level of 1.5% CO₂ was chosen here because that was the maximum CO₂ level measured in the outflow from our respirometry chambers at the lowest temperatures. The changes in f, VT, and VE at 1.5% CO₂ were all well below changes observed in the thermoregulating hatchling experiencing the coldest test temperatures (Figs. 2 and 3). Thus, we can rule out ventilatory changes caused by increased metabolic chamber CO₂ levels and instead suggest any change is a thermoregulatory response to cooling.

Pekin duck hatchling ventilatory changes in response to 4% CO₂ were of similar magnitude to changes occurring during cooling. Under both conditions, the significant increase in VE was due to both increased f and VT. The increase in VE from 0 to 4% CO₂ was larger than those observed in Muscovy ducks, but similar in magnitude to changes in the chicken hatchling (16). Pekin duck hatchlings had a similar ventilation rate at 4% CO₂ as the Muscovy duck, but a larger change in VT, thus producing a greater change in VE. The VE hypercapnic response of the chicken hatchling had a larger contribution of VT than in the Pekin duck (16, 28). This large contribution of VT in the chicken compared with the Pekin duck may be due to the higher resting f in the chicken when compared with the duck. Hatchling ventilation responses to hypercapnia are similar to those observed in the adult Pekin duck (5).

In contrast to hatchlings, externally pipped Pekin duck paranates were unable to alter ventilation in response to hypercapnia of 4% CO₂, but after undergoing artificial hatching, they showed a functioning hypercapnic chemosensitivity (Fig. 4). The f and VT of externally pipped paranates did not change significantly when exposed to hypercapnia. However, they may possess a chemosensitivity that can be observed in response to 4% CO₂: externally pipped paranates with lower f at 0% CO₂ increased f when exposed to 4% CO₂, resulting in similar values of 4% CO₂ f for all animals, which served to decrease variability compared with values at 0% CO₂ (Fig. 4B). Externally pipped chicken paranates VT increased only modestly in response to hypercapnia (8% CO₂) and hypoxia (5% O₂), increasing from 82 μl under control conditions to 147 μl (14) without increasing f. After artificial hatching of externally pipped paranates, the animals exhibited a clear chemosensitivity to elevated CO₂. This suggests that the externally pipped paranatal hypercapnic VT response may also be limited by the high f and physical inability to expand the air sacs.

**Perspectives and Significance**

An endothermic phenotype develops rapidly in the hatchling Pekin duck. Upon hatching, animals respond to cooling with a significant increase in VO₂ and heat production. The Pekin duck hatchling has both a ventilatory chemosensitivity and a ventilatory thermal sensitivity. In contrast, externally pipped paranates have high f coupled with low VT. Resting ventilation rate may be at a maximum during the externally pipped stage to compensate for the low VT. This low VT and inability of externally pipped paranates to increase VT and VE in response to either temperature or hypercapnia appears to be a constraint of the eggshell, as they both increase if the animal is helped from the eggshell. During the externally pipped stage, paranates appear limited by O₂ uptake across the CAM and at the lung, but they may also be power-limited as suggested by Whittow and Tazawa (30). At the end of in ovo development, one might predict that the systems develop together so that externally pipped paranates are limited by the respiratory and cardiovascular systems and capacity for aerobic metabolism in the muscles. Natural ontogeny of thermogenesis during external pipping and hatching include significant improvements in ventilation immediately after removal of eggshell, enabling paranates to establish endothermy.

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**AUTHOR CONTRIBUTIONS**

Author contributions: T.S.S. and E.M.D. conception and design of research; T.S.S. and E.M.D. performed experiments; T.S.S. and E.M.D. analyzed data; T.S.S. and E.M.D. interpreted results of experiments; T.S.S. and E.M.D. edited and revised manuscript; T.S.S. and E.M.D. approved final version of manuscript; E.M.D. prepared figures; E.M.D. drafted manuscript.

**REFERENCES**


