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RESEARCH ARTICLE

Thermal tachypnea in avian embryos

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ABSTRACT

Many adult mammals and birds respond to high surrounding temperatures with thermal tachypnea – an increase in breathing frequency accompanied by shallow tidal volume, with minimal increase in oxygen consumption ($\dot{V}_{O_2}$). This pattern favors heat dissipation by evaporative water loss (EWL) through the respiratory tract. We asked to what extent this response was apparent at the earliest stages of development, when pulmonary ventilation initiates. Measurements of pulmonary ventilation ($\dot{V}_E$; barometric technique), $\dot{V}_{O_2}$ (open-flow methodology) and EWL (water scrubbers) were performed on chicken embryos at the earliest appearance of pulmonary ventilation, during the internal pipping stage. Data were collected, first, at the normal incubation temperature (37.5°C); then, ambient and egg temperatures were increased to approximately 44°C over a 2 h period. Other embryos of the same developmental stage (controls) were maintained in normothermia for the whole duration of the experiment. During heat exposure, the embryo's $\dot{V}_{O_2}$ and carbon dioxide production increased little. In contrast, $\dot{V}_E$ more than doubled (~128% increase), entirely because of the large rise in breathing frequency (~132% increase), with no change in tidal volume. EWL did not change significantly, probably because, within the egg, the thermal and water vapor gradients are almost nonexistent. We conclude that chicken embryos respond to a major heat load with tachypnea, like many adult mammals and birds do. Its appearance so early in development, although ineffective for heat loss, signifies that thermal tachypnea represents an important breathing response necessary to be functional from hatching.

KEY WORDS: Heat stress, Breathing pattern, O_2 consumption, CO_2 production, Evaporative water loss, Breathing frequency

INTRODUCTION

In adult birds and mammals, the high basal metabolic rate supports high values of core body temperatures (T_b), maintained at an almost constant level over a wide range of ambient temperatures (T_a) through an active balance between heat gain and heat loss (see Bícego et al., 2007). By contrast, in embryos and fetuses, T_b is entirely dependent on the surrounding T_a. Hence, in birds and mammals (Mortola, 2009), including the human infant (Hey, 1969), there is an ontogenetic transition from an initial ectothermic embryonic state to the full endothermic postnatal pattern, with implications for the response to cold or heat exposures. With respect to cold, brown adipose tissue thermogenesis (Cannon and Nedergaard, 2004) is one important mechanism of eutherian neonates for the protection of T_b when T_a drops. In a precocious mammal like the sheep, brown adipose tissue is functional in the fetus toward end-gestation (Symonds et al., 2015). Depending on the species, in precocious birds, which do not possess brown adipose tissue (Bícego et al., 2007), endothermy and cold-induced thermogenesis are apparent at end-incubation and become established after hatching (Kuroda et al., 1990; Khandoker et al., 2004; Dzialowski et al., 2007; Szdzuy et al., 2008). This occurs in parallel with the increased activity of regulatory enzymes involved in the oxidative metabolism pathways (Seebacher et al., 2006), and in the oxidative phosphorylation capacity of skeletal and cardiac muscles (Sirsat et al., 2016).

In contrast to the response to cold, much less information is available on the ontogenesis of the responses to heat exposure. The fact that, at birth, neonates are subjected to a fall in T_a is probably the reason why, traditionally, the developmental responses to cold (and the prevention of cold exposure in the neonatal clinical setting) have attracted far more attention than the response to heat (Power and Blood, 2011; Sahni, 2016). Yet, the safety margin beyond which protein denaturation occurs is much narrower for high than it is for low T_b (Tansey and Johnson, 2015) and the vulnerability of newborns in a hot environment probably exceeds that in the cold because of their limited mechanisms for thermolysis (Mortola, 2001). Similarly to animal studies (Edwards, 1967), epidemiological studies in humans have indicated that short-lasting elevation of T_b of pregnant women can be responsible for a number of fetal developmental defects (Smith et al., 1978; Edwards et al., 2003; Power and Blood, 2011); in infants, a high T_a is considered a cofactor of sudden infant death syndrome (Leiter and Böhm, 2007). In a warm environment, increasing skin perfusion favors heat dissipation by radiation; however, when T_a equals or exceeds T_b, heat loss can only occur through evaporation, which, in neonates, may not be as efficient as in adults because of the lower epidermal water control (Harpin and Rutter, 1982; Rutter, 2000).

The present study takes advantage of the chicken embryo as a model to investigate the mechanisms of defense against a heat load in the perinatal phase. Avian embryos are suitable models because their T_a can be manipulated experimentally much more easily than in mammalian fetuses. Furthermore, the interpretation of the avian embryo's response to heat is free of the confounding factors typical of mammalian preparations, which include the protective responses offered by the mother, uterus and placental circulation.

In chicken embryos close to hatching, a rise in incubation temperature increases the blood flow of the chorioallantoic membrane (Holland et al., 1998), a highly vascular structure that envelops the inside of the eggshell for the main purpose of gas exchange. This extraembryonic vasodilatation may be considered a prenatal response to promote heat loss. Postnatally in birds, especially in Galliformes and Passeriformes, rapid and shallow breathing, i.e. thermal tachypnea, is the predominant form of evaporative heat loss against a rise in T_a (Richards, 1970a; Bouverot et al., 1974; Bícego et al., 2007; Mortola and Maskrey, 2011;

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List of symbols and abbreviations

EWL	evaporative water loss
f	breathing frequency
f_{inst}	instantaneous breathing frequency
IP	internal pipping
T_a	ambient temperature
T_b	body temperature
T_{egg}	egg temperature
$\dot{V}_{CO_2}$	carbon dioxide production
$\dot{V}_E$	pulmonary ventilation
$\dot{V}_{O_2}$	oxygen consumption
V_T	tidal volume

McKechnie et al., 2016). Like in mammals (Adolph, 1947), also in birds the breathing pattern during thermal tachypnea (and its association with gular flutter; Bartholomew et al., 1968) enhances the ventilation of the dead space over that of the respiratory zone; hence, it permits a rise in evaporative heat loss of the upper airways without major disturbances to gas exchange. Eventually, as the heat load progresses, thermal tachypnea wanes into thermal hyperventilation, a deep and slow breathing pattern oriented to boost heat dissipation at the expense of blood gas homeostasis (Mortola and Maskrey, 2011). Interestingly, several lizard species also use thermal tachypnea or gaping (increased heat loss with opened mouth) to protect the brain from a rise in temperature (Tattersall et al., 2006; Scarpellini et al., 2015).

Pulmonary ventilation initiates at the internal pipping (IP) phase of the hatching process; that is, after the embryo has pierced through the chorioallantoic membrane and gained access to the air cell. Then, until hatching is completed, the embryo uses both pulmonary ventilation and the chorioallantoic membrane as paths for gas exchange. This bimodal format, homologous to the brief overlapping of placenta and lung gas exchange during the birth of a mammal, in the hatching bird lasts several hours (Mortola, 2009). Whether thermal tachypnea as a mode to dissipate heat appears at this early developmental stage remains unknown; if it did, it would mean that this important mechanism against heat stress initiates before hatching is completed. One may argue that thermal tachypnea in the embryo close to hatching, at the IP stage, would serve no purpose because the high water content of the egg precludes airway evaporation. Hence, an additional question arises regarding the extent of evaporative heat loss through pulmonary ventilation. Therefore, this study on chicken embryos at the IP phase addressed two questions relevant to the development of the heat dissipation capacity in the perinatal phase: (1) does thermal tachypnea occur at this stage; and (2) how efficient is respiratory evaporation in these embryos?

MATERIALS AND METHODS

Animals

Experiments were conducted on chicken embryos [*Gallus gallus* (Linnaeus 1758)] of the White Leghorn variety. Freshly laid fertilized eggs were obtained from a local supplier. After noting the mass, the eggs were placed in incubators set at 37.5°C and 60% relative humidity, monitored every 10 min by a data logger, with a 90 deg egg rotation four times a day. Embryonic day 0 (E0) was the day of the onset of incubation. Measurements were collected toward the end of incubation, at the pre-IP and IP phases of the hatching process, which corresponded, respectively, to E19 and E20. The stage of the embryo was determined by transillumination and confirmed by visual inspection after opening the egg upon completion of the measurements.

Egg preparation

Fig. 1 is a schema of the experimental setup. On the day of the experiment, a small hole was drilled through the eggshell in the hemisphere opposite to the air cell. The size of the hole was just sufficient to thread a fine tungsten-constantan thermocouple (<0.2 mm o.d.) through, secured a few mm under the eggshell, for monitoring egg temperature (T_{egg}). A second larger hole (~2 mm^2) was made at the blunted end of the egg over the air cell. A small cap (~1 ml volume) was glued to the shell with dental cement to cover the hole and portion of the air cell region (Impregum™ F Polyether Impression Material, 3 mol l^{-1}, Neuss, Germany). The cap had four leads, two of which permitted the delivery of a steady airflow, the third one for the recording of the breathing-related pressure oscillations by a sensitive pressure transducer (Data Instruments Honeywell #DUXL3OD), and the last lead was for volume calibration by injecting a known volume with a micro syringe. The egg was positioned in a ~110 ml plastic container ('respirometer') with leads for the passage of a steady flow of air. A transmitter powered by an external energizer-receiver unit (4000E, Minimitter, Sunriver, OR, USA) was inside the respirometer to monitor its temperature (T_a). The respirometer was placed inside a tight-fitting container, the temperature of which was controlled by a water bath (Julabo F25ME, Allentown, PA, USA) (Fig. 1). At the start, all eggs were maintained at 37.5–38°C, which corresponded to the normal incubation temperature for chicken eggs.

Measurements of $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and evaporative water loss

Gaseous metabolism [oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$)] was measured by an open-flow

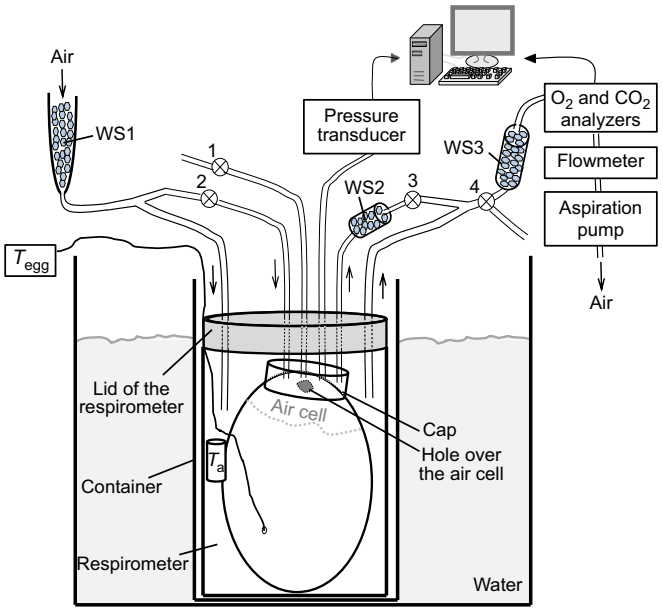

Fig. 1. Schematic representation of the setup. The egg was positioned in a respirometer completely submerged into water, for the control of ambient temperature (T_a). T_{egg}, egg temperature. By turning the four stopcocks (numbers 1, 2, 3 and 4) appropriately, the airflow was directed either through the cap of the air cell or through the respirometer. Water scrubbers were positioned at the entrance of the circuitry (WS1) and at the outflow (WS2 and WS3). A catheter connected to a pressure transducer permitted us to monitor the pressure oscillations due to breathing, from which tidal volume (V_T) and breathing rate were computed according to the barometric technique. All signals were displayed on a computer monitor and saved for later analysis. For further explanations, see Materials and methods.

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methodology (Frappell et al., 1992) adapted to the chicken embryo (Menna and Mortola, 2003). Three water scrubbers (WS; anhydrous $CaSO_4$, 8 mesh; Indicating Drierite®, Xenia, OH, USA) were placed at the inlet and outlet of the circuitry (respectively, WS1 and WS3 in Fig. 1) and in series with the outlet of the cap circuitry (WS2). A steady air flow (104±0.4 ml min^{-1}) was maintained through the respirometer and the outflow O_2 and CO_2 concentrations were recorded continuously by calibrated gas analyzers (FoxBox, Sable Systems, NV, USA). The inflowing gas concentrations were checked periodically by switching stopcock 4 (Fig. 1) to the inflow path. The concentrations of O_2 and CO_2, flow rate and T_a were continuously collected and displayed on a computer monitor. After mathematical correction of the gas concentrations for a respiratory quotient different from unity (Depocas and Hart, 1957; Mortola and Besterman, 2007), $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were computed [at standard temperature pressure and dry conditions (STPD)] from the flow rate and the difference between the respective O_2 and CO_2 inflow and outflow concentrations.

The total air volume of the air-cell cap and of its four leads, including WS2 (measured by filling with distilled water), was 10.6 ml, whereas the air volume of the respirometer (with the egg inside) and connecting tubes was 70 ml. The wash-out time of the circuitry (respirometer, WS and connecting tubes) was measured as the time required for a complete wash out (~four time constants) of a bolus injection of CO_2; it averaged 6 min. Of the total gas flow, about 7% passed through the air-cell-cap circuitry. The masses of WS2 and WS3 were measured on a scale accurate to 10^{-4} g (AB104; Mettler Toledo, Switzerland). Because the inflowing gas was dry, the mass gain of WS2 over a fixed time was the evaporative water loss (EWL) of the air cell, and that of WS2+WS3 was the total water loss of the egg, both expressed as mass/time (mg min^{-1}). Cooling efficiency (%) was calculated as evaporative heat loss (J min^{-1})·100/heat production (J min^{-1}), taking the caloric equivalents of 2.426 J of heat transfer per mg of water evaporated and 20.083 J of heat produced per ml O_2 consumed (Arad and Marder, 1982).

Pulmonary ventilation and breathing pattern

Measurements of pulmonary ventilation ($\dot{V}_E$) were obtained by the barometric methodology applied to the chicken embryo (Szdzuy and Mortola, 2007), conveniently coupled to the measurement of gaseous metabolism (Fig. 1). With closure of all the stopcocks, the air-cell cap circuitry was momentarily sealed for 1–2 min; in such condition, changes in temperature and humidity due to breathing were responsible for the pressure oscillations. The conversion of the breathing-related pressure oscillation into tidal volume (V_T), in addition to the volume calibration, required the values of temperature and water vapor pressure of the egg and the corresponding effective values of the chamber, as described in detail elsewhere (Szdzuy and Mortola, 2007). Breathing frequency [f (breaths min^{-1})] was calculated from the number of breaths over the recording time (which lasted 100 breaths or 2 min, whichever came first), from which was calculated: $\dot{V}_E = V_T \cdot f$. In addition to f, the instantaneous breathing frequency (f_{inst}) was measured from the breath-by-breath computation of the breathing cycle duration; this is the sum of the inspiratory and expiratory times, defined as the times with, respectively, inspiratory and expiratory flows. Therefore, f_{inst} invariably exceeded f because f_{inst} does not include the inter-breath pauses, which, in the perinatal phase, can be frequent events.

Protocol

The main experiment was conducted on two groups of embryos at the IP stage of the hatching process, in which pulmonary ventilation had started: the controls (embryos maintained in normothermia throughout the experiment; N=10) and the experimental group (embryos subjected to heat stress; N=15). Five experimental IP embryos had reached the star fracture of the eggshell and, therefore, were almost entering the external pipping phase. A third group consisted of embryos just before the IP phase (pre-IP embryos; N=10) subjected to heat exposure as were the experimental group. The pre-IP embryos, which had not pierced the inner membrane of the air cell and therefore had not begun pulmonary ventilation (Mortola, 2009), were included in the study to examine whether the metabolic response to heat changed during the latest phases of embryonic development.

After egg preparation and once T_a had stabilized at the normothermic value (~37.5°C), the experiment started (time 0′). Twenty minutes later, the first measurements of gaseous metabolism and $\dot{V}_E$ were obtained. Then, in one set of embryos (experimental group), the bath temperature was set to 45°C; recordings were obtained every 20 min up to a total experimental time of 2 h (Fig. 2, red symbols). For the first 20 min, neither T_a nor T_{egg} changed significantly because of the thermal inertia of the water bath; thereafter, temperature rose from 37.5 to 44°C. In the other set of embryos (control group; Fig. 2, blue symbols), measurements were obtained every 20 min and bath temperature was constant throughout the whole duration of the experiment. In the experimental group, the rise in T_{egg} during heat exposure was the consequence of the increase in T_a; therefore, the difference (T_{egg}–T_a) was nearly 0. The similarity between T_{egg} and T_a indicated that the time allowed for heating (100 min) was sufficient to overcome the thermal resistance of the eggshell – the time constant of which averages 16 min – or about one third of the whole egg (Mortola and Gaonac'h-Lovejoy, 2016). In controls, T_{egg} remained stable between 38 and 38.5°C, which was ~1°C higher than T_a because of the heat of metabolic origin (Mortola et al., 2015; Fig. 2).

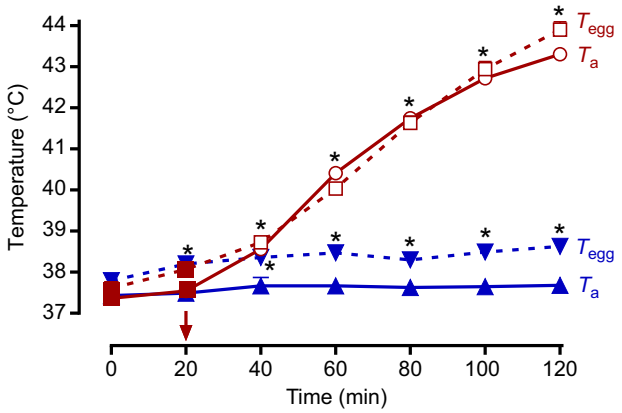

Fig. 2. Temperature profiles during the 120-min duration of the measurements. All embryos were at the internal pipping (IP) stage. Some embryos (control, N=10) were maintained at constant temperature throughout (blue upward- and downward-pointing triangles); others (experimental, N=15) were subjected to progressive heat exposure (red squares and circles). Values are group means±1 s.e.m.; where not indicated, s.e.m. was within the symbol's size. T_a, ambient temperature inside the respirometer; T_{egg}, egg temperature. Open symbols indicate statistical difference from the onset (0 min) within the same group. *Statistical difference from the constant T_a group (blue triangles) at any given time. The red arrow indicates the time when bath temperature was set to 45°C in the experimental groups.

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Table 1. Normothermic values of the chicken embryos

	Pre-IP phase	IP phase	
Protocol	Heat exposure	Heat exposure	Constant T_a
No. embryos	10	15	10
Fresh egg mass (g)	59.3±0.6	58.8±0.5	59.8±0.8
Egg mass at experiment (g)	53.3±0.5	53.4±0.7	53.4±0.8
Embryo age (days)	18.6±0.3[b]	20.1±0.1a	20.0±0.0[a]
$\dot{V}_{O_2}$ (µl O_2 STPD)	402.4±22.5[b]	464.0±22.9[a,b]	514.2±23.4[a]
$\dot{V}_{CO_2}$ (µl O_2 STPD)	253.6±11.1[b]	301.0±14.2[a,b]	331.3±15.0[a]
$\dot{V}_E$ (µl min^{-1})	No breathing	2058.8±452.6	2482.3±483.9
V_T (µl)	No breathing	29.7±5.3	34.4±5.6
f (breaths min^{-1})	No breathing	69±5	70±7
f_{inst} (breaths min^{-1})	No breathing	104±7	102±7

Values are means±1 s.e.m. IP, internal pipping; T_a, temperature inside the respirometer (ambient temperature); $\dot{V}_{O_2}$, oxygen consumption; $\dot{V}_{CO_2}$, carbon dioxide production; $\dot{V}_E$, pulmonary ventilation; V_T, tidal volume; f, breathing frequency; f_{inst}, instantaneous breathing frequency; STPD, standard temperature pressure and dry conditions. Different superscript letters indicate statistical difference among groups.

Analysis

All data are presented as means±s.e.m. The comparisons of normothermic resting values among three experimental groups (IP constant T_a, IP heat exposure, pre-IP heat exposure) were done using one-way ANOVA (Table 1). The comparisons of the ventilatory response to heat between two experimental groups (IP constant T_a, IP heat exposure) were done using *t*-tests (Table 1). Two-way repeated measures (RM) ANOVA was used to evaluate the differences in breathing, metabolic rate and EWL between the 'IP constant T_a' and 'IP heat exposure' groups. The same analysis was done to evaluate the differences in gaseous metabolism between pre-IP and IP embryos. In both cases, the two factors were the groups (conditions; see Table 2) and the experimental time. If differences were detected, the following step of the analysis involved the *post hoc* multiple comparisons test according to the Holm–Šidák method, which is considered more powerful for multiple comparisons than the Bonferroni or Tukey methods (Seaman et al., 1991). The tests were performed with SigmaPlot-Stats® v. 13 (Systat Software Inc., San Jose, CA, USA). In all cases, differences were considered statistically significant at $P<0.05$.

Table 2. Results of the two-way RM-ANOVA testing for the effects of time and experimental condition (heat exposure and constant T_a)

Variable	Factors	d.f.	*F*	*P*
$\dot{V}_{O_2}$	IP heat exposure versus IP constant T_a			
	Time	5	4.07	0.0019*
	Condition	1	0.14	0.7137
	Condition×time	5	2.60	0.0286*
	IP heat exposure versus pre-IP heat exposure			
	Time	5	5.13	0.0003*
	Condition	1	5.99	0.0224*
	Condition×time	5	1.96	0.0905
$\dot{V}_{CO_2}$	IP heat exposure versus IP constant T_a			
	Time	5	23.67	<0.0001*
	Condition	1	0.05	0.8249
	Condition×time	5	15.01	<0.0001*
	IP heat exposure versus pre-IP heat exposure			
	Time	5	43.45	<0.0001*
	Condition	1	8.76	0.0070*
	Condition×time	5	4.29	0.0013*
f	IP heat exposure versus IP constant T_a			
	Time	5	9.88	<0.0001*
	Condition	1	10.58	0.0035*
	Condition×time	5	9.57	<0.0001*
f_{inst}	IP heat exposure versus IP constant T_a			
	Time	5	4.58	0.0008*
	Condition	1	15.05	0.0008*
	Condition×time	5	6.14	<0.0001*
V_T	IP heat exposure versus IP constant T_a			
	Time	5	1.16	0.3318
	Condition	1	2.58	0.1220
	Condition×time	5	1.04	0.3979
$\dot{V}_E$	IP heat exposure versus IP constant T_a			
	Time	5	8.28	<0.0001*
	Condition	1	0.08	0.7770
	Condition×time	5	3.28	0.0083*

T_a, ambient temperature; IP, internal pipping; $\dot{V}_{O_2}$, oxygen consumption; $\dot{V}_{CO_2}$, carbon dioxide production; f, breathing frequency; f_{inst}, instantaneous breathing frequency; V_T, tidal volume; $\dot{V}_E$, pulmonary ventilation. For $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, the comparisons were also performed between pre-IP and IP embryos, both exposed to heat. *Statistically significant difference.

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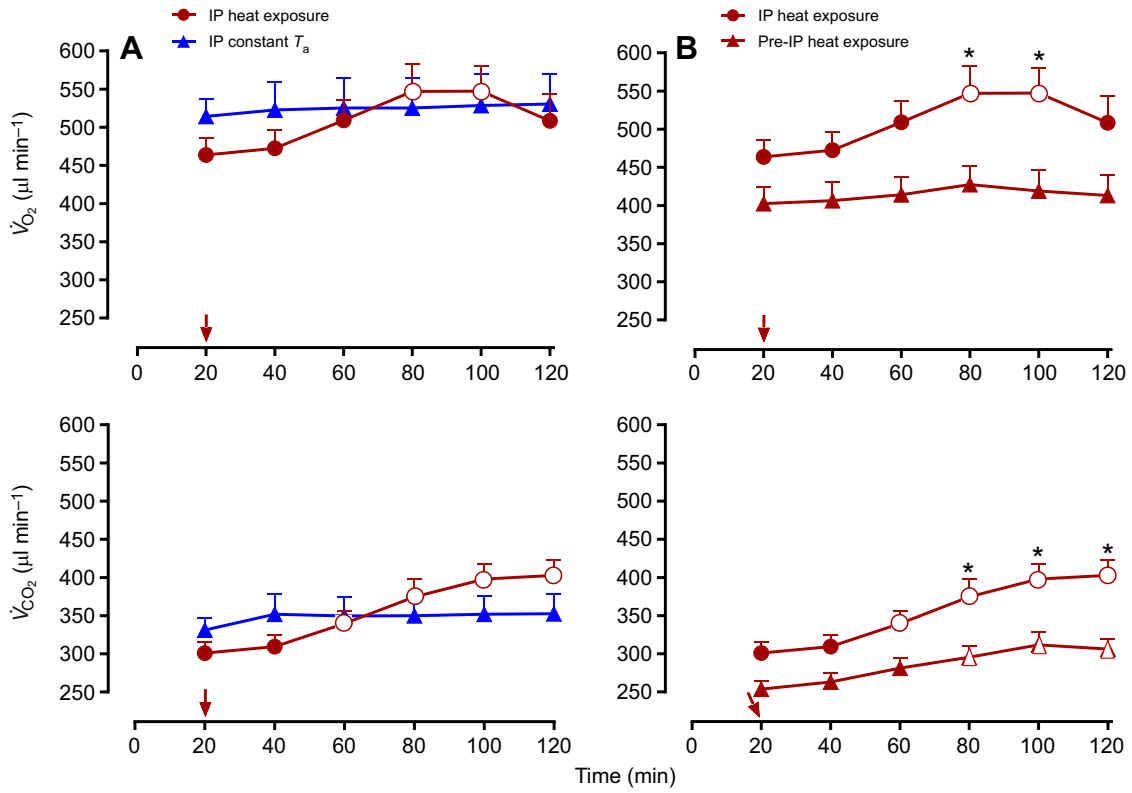

Fig. 3. Effects of heat exposure on oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) of chicken embryos. The IP embryos exposed to heat (experimental, red circles) are compared with those maintained at constant ambient temperature (T_a) throughout (controls, blue triangles) in A, and with the pre-IP embryos exposed to heat (red triangles) in B. Values are group means ±1 s.e.m. The red arrows indicate the time when bath temperature was set at 45°C to initiate the heat exposure. Within any given group, open symbols indicate a statistically significant difference from the values measured at 20 min. (A) At any given time, the values of the experimental and control IP embryos did not differ significantly. (B) *Statistical difference between 'IP heat exposure' and 'pre-IP heat exposure' at the same time point.

RESULTS

All the eggs used in the present study had similar fresh mass (Table 1). Because they lost a similar amount of mass between E0 and the time of the measurements, their eggshell must have had similar gas conductance. In normothermia, $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, $\dot{V}_E$ and breathing pattern did not differ significantly between 'constant T_a' and 'heat exposure' IP embryos. In both groups, f_{inst} exceeded f by about 32%, owing to the frequent occurrence of inter-breath pauses. As expected, pre-IP embryos, being 1.5 days younger (F=25.44; P<0.0001), had the lowest $\dot{V}_{O_2}$ (F=4.892; P=0.014) and $\dot{V}_{CO_2}$ (F=6.682; P=0.0038) (Table 1).

Metabolic and breathing responses to heat exposure

The results of the two-way RM-ANOVA testing for the data presented in Figs 3 and 4 are shown in Table 2. In IP embryos at constant T_a during the 2 h in which measurements were obtained, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ (Fig. 3A) and the breathing pattern (Fig. 4) showed no statistically significant changes. In the IP embryos exposed to heat, $\dot{V}_{O_2}$ increased at 80 and 100 min (Holm–Šidák test: P<0.0001; Fig. 3A) and $\dot{V}_{CO_2}$ increased over the 60- to 120-min phase (Holm-Šidák test: P<0.0001; Fig. 3A). These changes were not sufficient to reach a statistically significant difference between groups at any time during the protocol. Pre-IP embryos showed no change in $\dot{V}_{O_2}$ during heat exposure (Table 2) but an increase in $\dot{V}_{CO_2}$ over the 80- to 120-min phase (Holm–Šidák test: P<0.0001; Fig. 3B); their average values were constantly lower than in the IP group, and significantly so at 80–100 min (Holm–Šidák test: P<0.05) and at 80–120 min (Holm–Šidák test: P<0.01) for $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, respectively.

Heat exposure caused an obvious, and sometimes very striking, increase in the rate of breathing (Fig. 5). On average, this was significant at 100–120 min (Fig. 4; Holm–Šidák test: P<0.0001), when f and f_{inst} had increased by ∼132 and ∼68%, respectively. The larger increase in f signifies that a substantial component of its rise was the decrease in inter-breath interval. Because V_T had not changed, the significant rise in $\dot{V}_E$ (by ∼128% during the last 20 min of exposure) was entirely due to the tachypnea (Fig. 6, right panel). As a result of these changes, the ventilatory equivalent ($\dot{V}_E$ / $\dot{V}_{O_2}$) in severe hyperthermia was about twice the normothermic value (Fig. 6, left panel). When plotted as a function of T_{egg}, it appeared that the threshold for the tachypnea was at ∼41°C, or approximately 3°C above normothermia (Fig. 7).

EWL and cooling efficiency

The increased temperature did not modify significantly the EWL of the whole egg (effect of time: F=0.2835, P=0.755; effect of experimental condition: F=0.5357, P=0.4748; time×experimental condition: F=0.9983, P=0.3797) or of the air-cell compartment (effect of time: F=0.9245, P=0.4071; effect of experimental condition: F=1.287, P=0.2734; time×experimental condition: F=1.89, P=0.1675) (Table 3). Because of the small increase in $\dot{V}_{O_2}$ (Fig. 3), during maximal hyperthermia the cooling efficiency of the whole egg and of the air cell averaged, respectively, 17.3 and 2.2%, in comparison to 27.7 and 4.5% of the normothermic eggs at the same experimental times. The individual values were highly variable, and a statistically significant difference between the two groups occurred in the air-cell cooling efficiency (effect of time:

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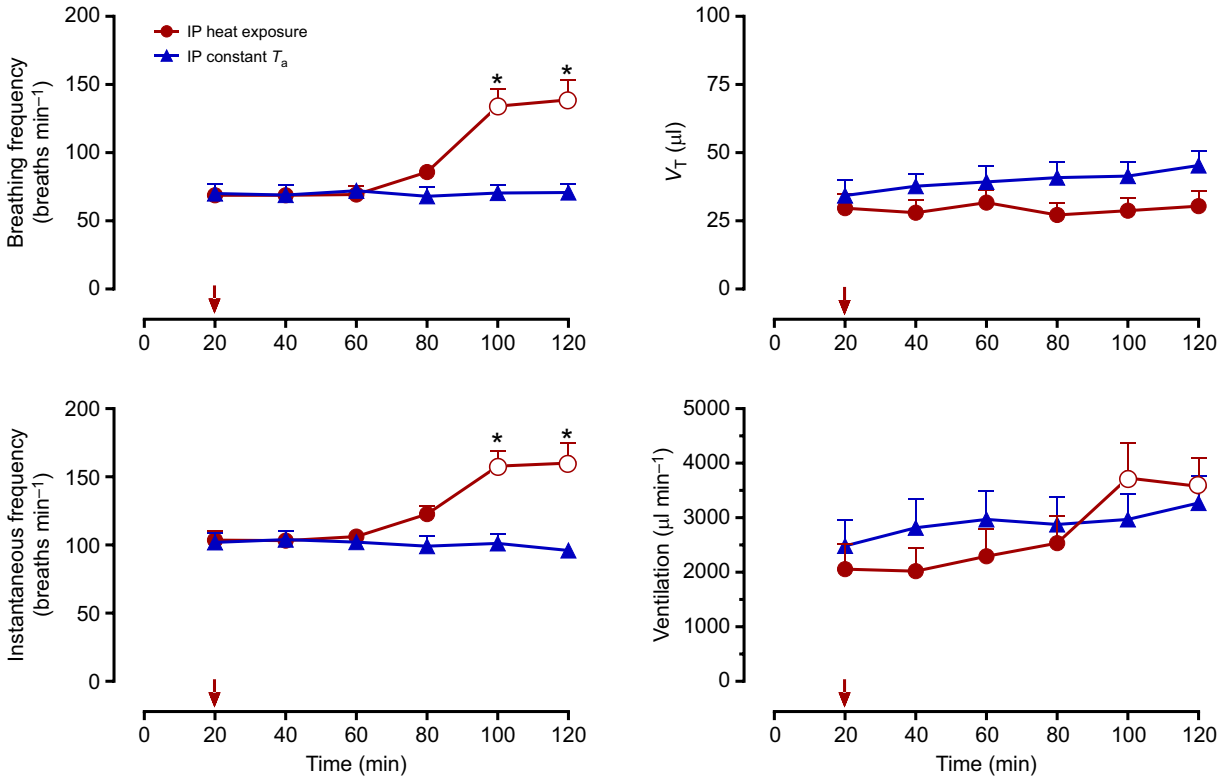

Fig. 4. Effects of heat exposure on the breathing pattern of chicken embryos. All embryos were at the IP stage. The IP embryos exposed to heat (experimental, red circles) are compared to controls maintained at constant temperature (T_a) throughout (blue triangles). The red arrows indicate the time when bath temperature was set to 45°C in the experimental group. Values are group means±1 s.e.m. Within each group, the open symbols indicate statistical difference from the 20-min mark. *Statistical difference between experimental and control embryos at the same time point. The breathing pattern of the two groups did not differ significantly except for breathing frequency, which increased in the experimental embryos.

F=0.0175, P=0.9827; effect of experimental condition: F=4.607, P=0.0475; time×experimental condition: F=3.691, P=0.0361), but not in the total cooling efficiency (effect of time: F=0.4945, P=0.6144; effect of experimental condition: F=4.364, P=0.053; time×experimental condition: F=3.473, P=0.0431; Holm–Šidák test: P>0.05) (Table 3).

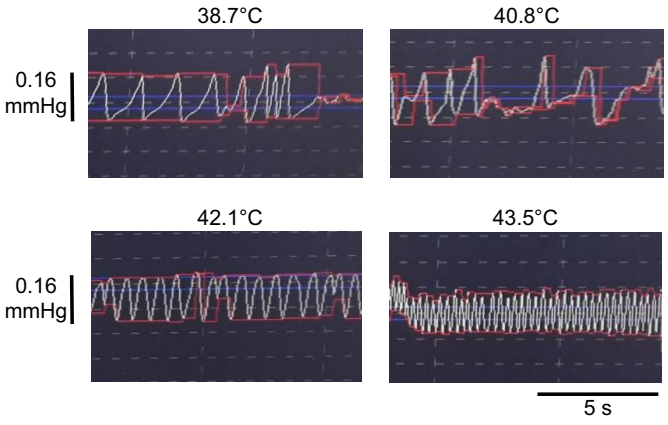

Fig. 5. Recordings of the breathing pattern during the IP phase at different T_a. The T_a is indicated above each panel. In this embryo, at ~41°C breathing rate was approximately as in normothermia (38.7°C); at ~42°C the tachypnea was becoming evident and at 43.5°C breathing rate was more than four times the normothermic value. Signal is pressure (mmHg) before the conversion to volume (see Materials and methods).

DISCUSSION

A sharp acceleration of breathing in response to a heat load is common in birds and mammals as a mechanism to lose heat via evaporation of the conductive airways. In fact, it was speculated to represent the most primitive form of thermolysis by evaporation, before sweating evolved as a supplementary mechanism in mammals (Hammel, 1968; Robertshaw, 2006). Most of the avian species studied (Galliformes and Passeriformes, but not Columbiformes) use respiratory EWL as a predominant heat dissipation mechanism (see McKechnie et al., 2016). The current results show the tachypneic response to heat to be present in the chicken embryo during the IP phase; that is, before hatching. To the best of our knowledge, this is the first indication that this typical thermal response is present very early during ontogenesis.

Thermal tachypnea

The two potential problems associated with the increase in $\dot{V}_E$ during thermal tachypnea are the hypocapnia and the increased respiratory work. In adult birds and mammals, tachypnea is often associated with a shallow breathing pattern, which permits a large increase in the ventilation of dead space with minimal changes in respiratory surface ventilation and blood gases. Because the frequency of breathing during thermal panting or gular flutter approaches the resonant frequency of the respiratory system, the work of breathing depends solely on airflow resistance (Crawford, 1962; Hales and Findlay, 1968). Hence, in adult mammals during panting, typically, the drops in the alveolar and arterial pressures of CO_2 and the rise in $\dot{V}_{O_2}$ are small (Mortola and Maskrey, 2011).

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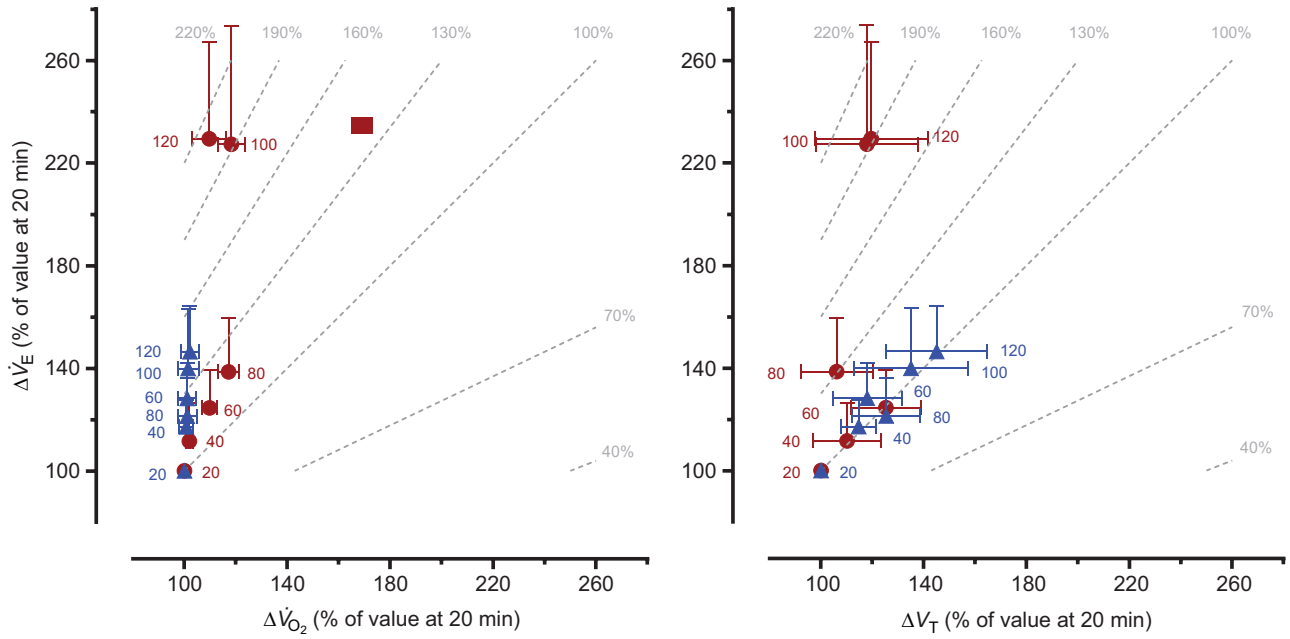

Fig. 6. Changes in pulmonary ventilation ($\dot{V}_E$) plotted against $\dot{V}_{O_2}$ or against V_T. Results are shown for IP embryos maintained at constant temperature for the duration of the experiment (blue triangles) or exposed to heat (red circles). All values are expressed in percent of the corresponding values at the time 20 min. Dashed lines indicate the percent changes in ventilatory equivalent (left panel) or in breathing frequency (right panel). Symbols are mean values; bars represent 1 s.e.m. Numbers in red and blue indicate the time (min) of the measurements (see Fig. 2).

Whether or not this applies to the tachypnea of the embryo cannot be said for several reasons, as we now discuss.

Blood sample collection for gas analysis in chicken embryos requires windowing the eggshell (Tazawa et al., 1980); hence, we considered it incompatible with the measurements required for this study. V_T quantified by the barometric technique is based on the conversion of the pressure oscillations according to variables (T_{egg}, T_a and relative humidity) that could suffer some errors (Szdzuy and Mortola, 2007), especially when the difference (T_{egg}–T_a) is small because of the hyperthermia (Mortola and Frappell, 1998). Therefore, we cannot be as confident about the absolute values of V_T as we are for those of f. Nevertheless, placing more emphasis on relative changes than on absolute values, in the embryos in severe hyperthermia (100–120 min) we recorded only minimal, and insignificant, changes in V_T in comparison to controls (Fig. 6, right panel), whereas $\dot{V}_E$ and $\dot{V}_{CO_2}$ rose, respectively, by ∼128 and ∼33%; these changes were responsible for some 70% increase in $\dot{V}_E/\dot{V}_{CO_2}$. Such an increase in the ventilatory equivalent for CO_2 does not necessarily mean that the embryos experienced a profound hypocapnia because, during the IP phase, the chorioallantoic membrane participates in gas exchange together with the lungs (Menna and Mortola, 2002a) and in so doing acts as a gas exchange buffer to the CO_2 changes of pulmonary origin (Menna and Mortola, 2003).

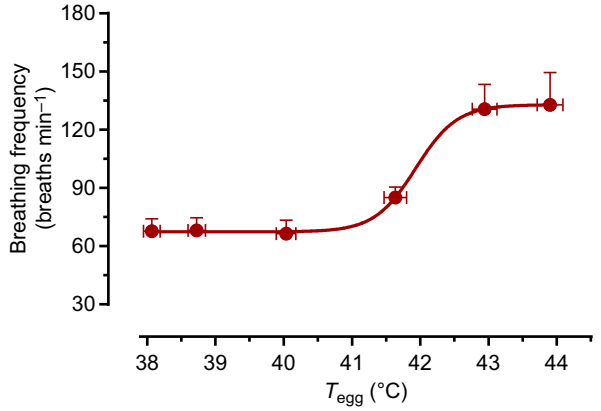

Fig. 7. Breathing rate as a function of egg temperature in IP embryos exposed to a progressively greater heat load. Symbols are mean values; bars represent 1 s.e.m.

Exposure to heat raised $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, albeit only slightly (respectively ∼13 and ∼33%) and less than the value (+42%) expected by a Q_{10} (Arrhenius) coefficient of 2 (Tazawa et al., 1989), which is the passive response to temperature in unregulated biological systems. The discrepancy between the Q_{10}-expected and the actual metabolic response was larger in pre-IP embryos, in which the increase in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ during severe hyperthermia was only 3 and 21%, respectively. The greater rise of $\dot{V}_{CO_2}$ than $\dot{V}_{O_2}$ (Fig. 3) most likely reflected the higher solubility and the much larger stores of CO_2 than O_2 (Mortola and Besterman, 2007). A rise in temperature decreases gas solubility in liquids; this implies that, for a given level of metabolism, the gases leaving the egg may have higher O_2 and CO_2 concentrations in hyperthermia than in normothermia, leading to spuriously low and high values of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, respectively. As mentioned above, this probably contributed to the mismatch in gas exchange during hyperthermia (Fig. 3) and could have led to values of Q_{10} lower than expected from passive reactions. The possibility of oxygen limitation toward end-incubation arose from the observation that a rise in O_2, either by exposure to hyperoxia or by opening the eggshell, produced some increase of the embryo's $\dot{V}_{O_2}$ (Tazawa et al., 1992; Szdzuy et al., 2008), suggesting that the O_2 supply poses a limit to $\dot{V}_{O_2}$. In this light, the higher $\dot{V}_{O_2}$ response of the IP embryos (in comparison to the pre-IP embryos) may reflect their better oxygenation after access to the air cell and the onset of pulmonary ventilation. To consider further the possibility of a limitation in O_2 availability, in three IP embryos we measured the $\dot{V}_{O_2}$ response to heat after fully opening

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Table 3. Total egg and air-cell evaporative water loss and cooling efficiency

	Total EWL (mg min^{-1})		Total cooling efficiency (%)		Air-cell EWL (mg min^{-1})		Air-cell cooling efficiency (%)	
Time (min)	Heat exposure	Constant T_a	Heat exposure	Constant T_a	Heat exposure	Constant T_a	Heat exposure	Constant T_a
40	1.05±0.15	1.07±0.13	21.1±3.1	25.5±2.8	0.13±0.03	0.17±0.02	2.7±0.6	4.0±0.5
80	0.96±0.13	1.14±0.11	16.7±3.5	27.3±3.1	0.15±0.03	0.18±0.03	2.6±0.6	4.1±0.5
120	1.01±0.11	1.17±0.10	17.3±3.0	27.7±2.7	0.13±0.03	0.20±0.03	2.2±0.6*	4.5±0.5

Values are means±1 s.e.m. EWL, evaporative water loss, from which evaporative heat loss was computed as 1 mg water=2.426 J. Heat production (J min^{-1}) was computed from oxygen consumption (1 ml O_2=20.083 J), from which cooling efficiency (%) was the percent ratio between evaporative heat loss and heat production. T_a, temperature inside the respirometer (ambient temperature). 'Heat exposure' refers to those embryos exposed to heat load (N=8); 'Constant T_a' refers to those embryos maintained at normothermic temperature for the whole protocol (N=10). *Statistical difference from the constant T_a group (Holm–Šidák *post hoc* test: P=0.0248).

the eggshell above the airspace (results not presented); the metabolic response of these embryos in severe heat averaged +27%, which was higher than the average increase of the experimental group (+13%) but still much less than expected (+42%). Another explanation for the low Q_{10} in hyperthermia is the embryo's $\dot{V}_{O_2}$ being close to its maximum already in normothermia and, therefore, with no possibility of further increase under heat exposure (Ide et al., 2017). In conclusion, the small metabolic response to heat (low Q_{10}) of the IP embryos could reflect the combination of numerous factors, of which the limitation in O_2 supply was only one, and perhaps not the most important one.

During thermal tachypnea, adult birds, including chickens, can breathe at rates 10–20 times the resting value (Bartholomew et al., 1968; Bouverot et al., 1974; Arad and Marder, 1982), probably because, at such high values, f approaches the resonant frequency of the respiratory apparatus. By comparison, the embryo's 138 breaths min^{-1} (~two times the resting f; Fig. 4) seems a relatively small tachypneic response. Because there is no information on the dynamic properties of the respiratory system in avian embryos under the mechanical constraints of the eggshell, yolk and membranes, it is impossible to estimate what the optimal f value would be from the viewpoint of the energetics of the respiratory apparatus. In addition, in mammals, tachypnea is triggered by thermal inputs from skin thermosensors, with no need for an increase in T_b (Richards, 1970a; White, 2006). Eventually, as T_b does increase, the thermal tachypnea gives way to thermal hyperventilation with a deep and slow breathing pattern (Richards, 1970a; White, 2006). In embryos, even when close to hatching like those of the present study, active thermolysis is almost absent, meaning that T_b unavoidably increases with T_a (Fig. 2). Therefore, in embryos, the breathing response to a thermal stimulus should result not only from the cutaneous thermal sensors at the body surface but also from inner areas, probably hypothalamic. Based on the data in mammals, such a scenario could explain the embryo's rather modest increase in f (in comparison to adults) and the maintenance of V_T, because this pattern would reflect the net result of the peripheral (cutaneous) stimulus to increase breathing frequency and the core stimulus to increase V_T of central origin. However, in contrast to this interpretation, there are data in adult chickens exposed to heat showing that a major (10 times) increase in f occurred only after colonic and hypothalamic temperatures have increased (Richards, 1970a,b). Finally, one should consider that, during heat, to obtain a relative increase in f like in adults (7–10 times resting values) the embryo should raise its f from the resting value of 69 breaths min^{-1} (Table 1) to 483–690 breaths min^{-1}, a rate which may be incompatible with the dynamic properties of their respiratory apparatus. Thus, a complex interplay of mechanisms participates to the ventilatory response to heat in birds, and many issues still need to be resolved in both adults and embryos.

It could be argued that the tachypneic response to heat was the only option available to the embryo if the increase in V_T was mechanically limited by the constraints imposed by its posture inside the eggshell. This may not be the case because it was noted previously that embryos during the external pipping phase increased V_T by at least 60–80% under hypoxia or hypercapnia (Menna and Mortola, 2003), and IP embryos were also able to increase V_T by about 60% under hypercapnia (data not published). The mechanical impedance of the embryo's respiratory system in quasi-static conditions (low inflation rates) was similar before and after exteriorization from the eggshell (Menna and Mortola, 2002b), but whether this remains true at the embryo's physiological f and in hyperthermia cannot be said.

Cooling efficiency

In adults, thermal tachypnea leads to significant increases in the EWL, which, in fowls, can be up to six times the normothermic value, with a substantial increase in cooling efficiency (Arad and Marder, 1982). In contrast, our embryos exposed to heat showed no change in EWL; because of the parallel increase in heat production, the tachypnea did not carry any improvement in cooling efficiency (Table 3). The water-saturated environment of the egg, with no thermal gradient (because of the similarity between T_{egg} and T_a; Fig. 2), explains why the tachypnea could not cause a substantial elevation of EWL.

Then, the question arises as to what the purpose of a tachypneic response may be when it carries no thermolytic advantage. In the egg it is unlikely that embryos face major hyperthermic challenges, because the heat capacitance of the water content buffers the oscillations in T_a (Mortola and Gaonac'h-Lovejoy, 2016). In addition, the embryo can use movements and vocalization as communication with the incubating parent for the purpose of thermoregulation (Gräns and Altimiras, 2007; Du et al., 2011). Hence, the hyperthermic tachypnea in the embryo close to end-incubation probably serves no purpose other than in preparation to the thermal challenges that may occur after hatching, when the protection of the egg and hen no longer exists. The prompt availability of this reflex since birth when needs arise attests to the value of pulmonary evaporation in response to a heat challenge (Richards, 1970a; McKechnie et al., 2016). Extremely small premature human infants, under assisted mechanical ventilation because they are unable to sustain their own, responded with rapid breathing to a 1.5°C increase in T_b (Rieger-Fackeldey et al., 2003); another hint is that thermal tachypnea is an entrenched breathing reflex established very early during development.

Conclusions

Chicken embryos during the last portion of incubation, at a time when pulmonary ventilation initiates, respond to a heat load with

minimal increases in $\dot{V}_{O_2}$ and with tachypnea, the response typical of adult mammals and birds. Although ineffective from the viewpoint of heat loss by evaporation while in the egg, the appearance of this breathing response so early in development probably signifies that tachypnea is an important mechanism for thermoregulation that hatchlings need to have fully functional at hatching.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.C.B., J.P.M.; Methodology: K.C.B., J.P.M.; Formal analysis: K.C.B., J.P.M.; Investigation: K.C.B., J.P.M.; Writing - original draft: K.C.B., J.P.M.; Writing - review & editing: K.C.B., J.P.M.; Funding acquisition: K.C.B., J.P.M.

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