Interactions of acid–base balance and hematocrit regulation during environmental respiratory gas challenges in developing chicken embryos (Gallus gallus)

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A B S T R A C T

How the determinants of hematocrit (Hct) – alterations in mean corpuscular volume (MCV) and/or red blood cell concentration ([RBC]) – are influenced by acid–base adjustments across development in the chicken embryo is poorly understood. We hypothesized, based on oxygen transport needs of the embryos, that Hct will increase during 1 day of hypercapnic hypoxia (5%CO2, 15%O2) or hypoxia alone (0%CO2, 15%O2), but decrease in response to hyperoxia (0%CO2, 40%O2). Further, age-related differences in acid–base disturbances and Hct regulation may arise, because the O2 transport and hematological regulatory systems are still developing in embryonic chickens. Our studies showed that during 1 day of hypoxia (with or without hypercapnia) Hct increased through both increased MCV and [RBC] in day 15 (d15) embryo, but only through increased MCV in d17 embryo and therefore enhancement of O2 transport was age-dependent. Hypercapnia alone caused a ∼14% decrease in Hct through decreased [RBC] and therefore did not compensate for decreased blood oxygen affinity resulting from the Bohr shift. The 11% (d15) and 14% (d17) decrease in Hct during hyperoxia in advanced embryos was because of an 8% and 9% decrease, respectively, in [RBC], coupled with an associated 3% and 5% decrease in MCV. Younger, d13 embryos were able to metabolically compensate for respiratory acidosis induced by hypercapnic hypoxia, and so were more tolerant of disturbances in acid–base status induced via alterations in environmental respiratory gas composition than their more advanced counterparts. This counter-intuitive increased tolerance likely results from the relatively low MDo2 and immature physiological functions of younger embryos.

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1. Introduction

The regulation of hematocrit (Hct) – a function of red blood cell concentration ([RBC]) and mean corpuscular volume (MCV) – is innately complex in avian embryos. Across the last half of embryonic chicken development (days 10–19), Hct increases due to an increase in [RBC] with relatively constant MCV (Tazawa et al., 2011). Mean corpuscular hemoglobin concentration ([MCHb]) remains constant and accordingly hemoglobin concentration ([Hb]) increases in proportion to the sigmoid increase in Hct (Tazawa, 1971; Tazawa et al., 1971a, 2011). Hemoglobin (Hb) is important not only as a facilitator of O2 transport (which during the course of embryonic development increases in parallel to increases in [RBC]), Hct, chorioallantoic membrane diffusing capacity and oxygen consumption (Tazawa, 1980; Tazawa and Whittow, 2000 for review), but also as a non-carbonate buffer for acid–base compensation. Concurrently, the cardiovascular, acid–base, metabolic, renal and thermoregulatory systems are also developing and each system must be regulated to provide sufficient conditions for embryonic development and to advance along the trajectory toward successful hatching/birth and adulthood (see e.g., Tazawa, 2005; Mortola, 2009; Andrewartha et al., 2011a for review; Burggren and Reyna, 2011). We know that the developing systems must be intrinsically interrelated, but the relationships and causal effects of perturbations of one system (e.g., blood acid–base balance) on the regulation of other systems (e.g., hematological respiratory variables) are still largely unknown. Further, changes in ambient gas composition have the potential to alter Hct either directly or through changes in blood acid–base balance. Thus, altering the environment is likely to provide insight into Hct regulation.

It has long been understood that Hct increases during environmental hypoxia in adult vertebrates (see Banchero, 1987; Kanagy, 2009 for review). However, the ontogeny of this response is poorly understood. Erythropoiesis is stimulated by chronic (e.g., days, weeks) hypoxic incubation in chicken embryos (e.g., Xu and Mortola, 1989; Dzialowski et al., 2002; Dragon and Baumann,

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2003). It is likely, however, that even acute (hours, day) hypoxic exposure will alter Hct, since elevating Hct through increased [RBC] (either release of potentially sequestered RBCs or actual erythropoiesis) during environmental hypoxia will improve O2 transport. RBCs are energetically expensive to produce and the additional RBCs produced (or released) may result in increased blood convection costs due to increased blood viscosity. Therefore, it may be advantageous to reduce [RBC] (sequester and/or down-regulate RBC production) in hypoxia in situations where sufficient O2 transport can occur with less RBCs.

The avian embryo provides a simplified (yet sufficiently complex) model for examining the interactions of acid–base and blood cell responses to environmental respiratory gas disturbances. Developing within an eggshell, the avian embryo lacks mechanical ventilation, instead exchanging gases with the environment by diffusion through the eggshell. The renal system is also relatively underdeveloped for most of avian development, with development of the metanephros continuing after hatching (Romanoff, 1960; Carretero et al., 1995). Consequently, avian embryos may not have available renal compensation to combat perturbations in acid–base balance. Embryonic blood acid–base balance has been previously manipulated via alterations in either environmental respiratory gas composition (e.g., Dawes and Simkiss, 1969; Tazawa, 1982, 1986; Bruggeman et al., 2007; Everaert et al., 2008; Andrewartha et al., 2011b) or eggshell conductance (e.g., Tazawa, 1981; Tazawa et al., 1981, 1988a). One day of decreased eggshell conductance (analogous to hypercapnic hypoxia) increased Hct in day 17 (d17) embryos, through an increase in MCV associated with decrease in pH and increase in [HCO3\(^{-}\)] without erythropoiesis. More advanced embryos (d19) increased both MCV and [RBC] when exposed to the same protocol (Tazawa et al., 1988a), highlighting differences in Hct regulation at different stages of development. Further, Hct was maintained constant during acute (~4–6 h) hypercapnic exposure in d13–17 embryos (Andrewartha et al., 2011b). Potentially, a longer hypercapnic exposure period might alter Hct, but concurrent alteration of O2 levels (as in Tazawa et al., 1988a) may be required.

The present study investigates how Hct responds to moderately altered environmental O2 and CO2 concentration ([O2] and [CO2]) and the resulting changes in acid–base balance in d13, d15 and d17 chicken embryos. We hypothesize that Hct will increase through increased [RBC] during 1 day exposure to hypercapnic hypoxia, hypercapnia or normocapnic hypoxia to enhance O2 transport, which would otherwise be impeded by hypoxia or a decrease in O2 affinity (resulting from the Bohr shift in hypercapnia – e.g., Tazawa and Mochizuki, 1976). During hypoxia, we hypothesize that Hct will decrease because O2 transport needs can be sufficiently met by transporting less RBCs in the circulation and thereby incurring less cost of transport. Additionally, blood will be equilibrated in vitro to a range of [O2] and [CO2] to help evaluate the in vivo findings. Hct changes in vitro can only occur through changes in MCV and may be influenced by changes in ambient respiratory gas composition more than in vivo where homeostatic mechanisms are integral to regulating Hct and other blood parameters. Further, age-related differences in acid–base disturbances and Hct regulation may be evident because the O2 transport system and hematological respiratory variables are still developing in embryonic chickens (see e.g. Tazawa et al., 2011).

2. Materials and methods

2.1. Incubation of eggs and blood collection

Fertile eggs of the domestic fowl (Gallus gallus) were obtained once a week from Texas A&M University (College Station, TX, USA). Eggs were weighed (±0.01 g) and then incubated at T\(_\text{r}\) = 37.5 ± 0.1 °C and relative humidity ~55% in a forced draught incubator (Model 1502, G.Q.F. Manuf. Co., GA, USA). The eggs were placed vertically on an automatic turning tray which rotated the eggs every 3 h. Two days prior to blood collection and analysis, the eggs were candled to locate the allantoic vein. On the following day (d12, d14 or d16), eggs were transferred to a desk-top incubator (Hova-Bator incubator, G.Q.F. Manuf. Co.) at a T\(_\text{r}\) = 37.5 ± 0.1 °C (and relative humidity ~25%). The embryos were exposed to an experiment gas mixture within a gas-exposure bag (relative humidity ~15%, see below and Appendix A for justification of flow and experimental regime) for ~24 h (experimental) or allowed to develop in air in the desk-top incubator until the target day (control).

On the target developmental day (one of d13, d15, d17), blood was collected from the allantoic vein. A 6–8 mm diameter region of the eggshell was removed and the underlying allantoic vein gently lifted by forceps through the hole in the eggshell. Approximately 0.4 mL of blood was sampled using a 25-gauge needle mounted on a 1 mL plastic syringe, flushed in advance with heparinized saline (100 mg in 100 mL saline).

After blood collection, embryos were euthanized by cold exposure, the yolk and extra-embryonic membranes were removed, and the embryo’s wet body mass determined (±0.01 g) with an electronic balance. The embryos were then placed in a desiccating chamber at 65 °C and body mass was determined every 24 h for 5 days. Dry mass was recorded at 48 h in d13 and d15 embryos and 72 h in d17 embryos, when in both instances body mass subsequently varied less than 0.01 g over 24 h.

2.2. Blood analysis

Arterialized blood drawn from the allantoic vein was inverted several times in hermetically sealed syringes to ensure sample mixing. The blood was gently transferred through the syringe needle into a 2-mL conic-ended plastic vial. Blood (0.12 mL) then drawn from this vial to a blood gas analyzer (ABL 725, Radiometer Medical A/S, Copenhagen, Denmark) was measured for pH, P\(_\text{O2}\), and HCO3\(^{-}\) (calculated by the analyzer from pH and P\(_\text{O2}\)) at 37 °C. [RBC] and [Hb] were measured on 0.01 mL of blood with a Coulter analyzer (A\(_\text{E}\)-T, Beckman Coulter Inc., CA, USA) and the mean corpuscular indices (mean corpuscular hemoglobin (MCH), MCV and mean corpuscular hemoglobin concentration (MCHC)) were calculated (following Tazawa et al., 2011). Lactate concentration ([L\(^{-}\)]\(_\text{a}\)) was determined on one drop of blood using a Lactate Plus Meter (Nova Biomed. Co., MA, USA). Osmolality (Osm) was determined on 0.01 mL of blood using a vapor pressure osmometer (Vapro 5520, Wescor, Inc., USA). Duplicate 0.06 mL samples of blood were transferred into hematocrit tubes, sealed and centrifuged for 4 min at 10,000 rpm and the mean Hct determined (±0.1%, Readacrit centrifuge, Becton Dickinson, MO, USA). Blood gas and acid–base variables (pH, [HCO3\(^{-}\]), P\(_\text{CO2}\)), hematomlar respiratory variables (Hct, [RBC], [Hb], MCV, MCH, [MCHC]), Osm and [L\(^{-}\)]\(_\text{a}\) were determined immediately after blood collection, and were referred to as “arterialized values”. (Note that blood collected from the allantoic vein is equivalent to adult arterial blood or pulmonary venous blood, because it returns oxygenated from the choroidal allantoic membrane (CAM). Piiper et al. (1980) referred to this as arterialized, and we adopt that nomenclature.)

2.3. In vitro effects of altered [O2] and [CO2] on Hct and osmolality

Approximately ~0.7 mL of blood was sampled from d15 embryos. After initially determining blood gas variables, Hct and Osm, the remainder of each well-mixed sample was then
transferred into a tonometer consisting of a glass, concave-ended vial (5.5 cm height, 2.8 cm diameter) with inlet and outlet conduits to allow gas mixtures to pass through the sample (see Andrewsartha et al., 2011b). The tonometer was sealed with a rubber cap and ventilated for 2 min with an experimental gas mixture (see below). The hermetically sealed tonometer was then placed in a 37 °C water-bath for 1.5 h (exposure period determined from a previous study, Andrewsartha et al., 2011b) to avoid water evaporation from the blood. The blood was equilibrated in vitro with one of 12 different gas mixtures (6 [O2] × 2 [CO2]: 0% or 5%CO2 (P O2 = 0 or 36 mmHg, respectively) and one of 0, 10, 15, 20, 40 or 100%O2 (P O2 = 0–760 mmHg), or 95%O2 in case of 5%CO2 with N2 balance. After 1.5 h, the tonometer was removed from the water-bath and the blood immediately assessed for pH, [HCO3−], PaCO2, PaO2, 37 °C and Osm and Hct (again measured in duplicate and the mean determined) at room temperature; referred to as pHeq, [HCO3−]eq, PaCO2(eq), Osmeq and Hcteq. Changes in Hct resulting from equilibration to altered [O2] and/or [CO2] were expressed as ΔHct = 100 × (Hct hypoxia − Hct)/Hct, where Hct is control value and Hct hypoxia is the value obtained by hypoxic exposure. Likewise, changes in [RBC] and MCV were expressed by Δ[RBC] (in %) = 100 × ([RBC] hypoxia − [RBC])/[RBC] and ΔMCV (in %) = 100 × (MCV hypoxia − MCV)/MCV, respectively.

2.5. Statistical analysis

All data were tested for normality and equal variance. An un-paired t-test or a Mann–Whitney rank sum test was used to determine differences between two group means. Differences between multiple-group means were examined by one-way ANOVA. The effect of varied [O2] at 0%CO2 or 5%CO2 on in vitro blood gas variables, Osm and Hct were determined using two-way ANOVA with post hoc multiple comparison (Holm–Sidak method) analysis to determine differences between individual treatment groups. Significance was assumed at P < 0.05. All data are presented as mean ± 1 S.E.M.

3. Results

3.1. In vitro Hct and osmolality responses

The blood of 102 embryos in total was equilibrated to six different concentrations of O2 at either 0%CO2 (N = 54) or 5%CO2 (N = 48). Neither fresh egg mass (55.01 ± 0.37 and 54.45 ± 0.39 g, respectively) nor embryo body mass (13.95 ± 0.17 and 13.49 ± 0.19 g, respectively) differed between the two [CO2] groups. Although there was no group difference in initial blood pH prior to tonometry (pH eq 7.60 ± 0.01 for both groups), [HCO3−] eq and PaCO2 were slightly higher in the 0%CO2 group (29.0 ± 0.5 mmol L−1 and 29.3 ± 0.5 mmHg) prior to tonometry compared with the 5%CO2 group (25.4 ± 0.6 mmol L−1 and 26.1 ± 0.6 mmHg) (P < 0.001 for both). Despite these small differences, there was no difference prior to tonometry in either Osmeq (275 ± 1 cf. 276 ± 1 mmol kg−1, P = 0.133) or Hcteq (28.5 ± 0.3 cf. 28.0 ± 0.3; P = 0.162). Further, no control parameter varied significantly amongst the six [O2] groups. Because the experiment with 5%CO2 was made later on eggs laid by a different flock of hens, different control acid–base status between 0% and 5%CO2 groups might be attributed to the difference of flocks.

After tonometry, significant differences in Hcteq existed between the two [CO2] groups and amongst the six [O2] groups (Fig. 1). The mean ΔHct for 0%CO2 group (across all O2 levels) was −2.6 ± 0.4% (N = 54) compared with a mean of 2.4 ± 0.5% (N = 48) for the 5%CO2 group (P < 0.001). The ΔHct at 0%O2 and both [CO2] groups (8.7 ± 0.8%) was significantly larger than the ΔHct at 10–100%O2 (e.g., −0.9 ± 0.8% at 10%O2) (Fig. 1).

Equilibration of the blood to 0%CO2 resulted, as expected from the previous study (Andrewsartha et al., 2011b), in a large increase in pH from pH eq 7.60 ± 0.01 to pHeq 8.31 ± 0.02 with no difference amongst the six O2 groups (P = 0.804). Similarly, there were no differences in [HCO3−] between O2 groups (P = 0.353) during equilibration to 0%CO2. [HCO3−] decreased sharply from [HCO3−] eq = 29.0 ± 0.5 mmol L−1 to [HCO3−] eq = 20.0 ± 0.8 mmol L−1 (P < 0.001). Accordingly, a comparable degree of respiratory alkalosis occurred in all O2 groups irrespective of concentrations. However, the ΔHct of blood equilibrated to 0%O2 (7.6 ± 0.8%) was significantly different (larger) from ΔHct at [O2] > 10% (e.g., −4.3 ± 0.8% at 10%O2) (Fig. 1).

Equilibration of blood with 5%CO2 resulted in significant decrease from pH eq 7.60 ± 0.01 to pHeq 7.53 ± 0.01 (P < 0.001) with
no difference amongst the six [O₂] groups (P=0.674). There were no differences in [HCO₃⁻] between O₂ groups (P=0.367) during equilibration to 5%CO₂. Further, there was no difference between initial arterial [HCO₃⁻]a (25.5±0.4 mmol L⁻¹) and [HCO₃⁻]eq after equilibration to 5%CO₂ (26.3±0.6 mmol L⁻¹) (P=0.256). Equilibration to 5%CO₂ resulted in a similar state of respiratory acidosis in all O₂ groups irrespective of concentrations. Again, ΔHct at 0%O₂ (9.7±0.9%) was significantly different (larger) from ΔHct at [O₂]>10% (e.g., 2.4±1.3% at 10%O₂) (Fig. 1).

Equilibration of the blood to a range of O₂ at 0%CO₂ resulted in a difference in Osm which changed from Osmₐ = 275±1 mmol kg⁻¹ to Osmeq = 293±1 mmol kg⁻¹ (P=0.001) with no Osmeq differences between the six O₂ groups (P=0.080) (Fig. 1). Similarly, equilibration at 5%CO₂ increased Osm from Osmₐ = 276±1 mmol kg⁻¹ to Osmeq = 302±1 mmol kg⁻¹ (P<0.001) with no difference in Osmeq between the six O₂ groups (P=0.488) (Fig. 1).

3.2. Blood buffer value

The blood buffer value of d13 embryos was described by the equation [HCO₃⁻]a = 156.3–17.9 × pHₐ (*r=0.997, N=48). For d15 embryos, [HCO₃⁻]a = 144.4–15.8 × pHₐ (*r=0.998, N=48) and for d17, [HCO₃⁻]a = 140.5–14.4 × pHₐ (*r=0.994, N=54). The mean buffer value across these 3 days was −16 mmol L⁻¹ pH unit⁻¹ and subsequently this value is used on all Davenport diagrams and for all further discussion.

Table 1

<table>
<thead>
<tr>
<th>Incubation day</th>
<th>13</th>
<th>15</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg mass (g)</td>
<td>57.46±0.36</td>
<td>57.91±0.42</td>
<td>58.45±0.40</td>
</tr>
<tr>
<td>Body mass* (g)</td>
<td>6.52±0.07</td>
<td>12.18±0.12</td>
<td>19.12±0.18</td>
</tr>
<tr>
<td>pHₐ*</td>
<td>7.671±0.003</td>
<td>7.588±0.004</td>
<td>7.559±0.004</td>
</tr>
<tr>
<td>PaCO₂* (mmHg)</td>
<td>17.5±0.2</td>
<td>28.4±0.4</td>
<td>34.9±0.5</td>
</tr>
<tr>
<td>[HCO₃⁻]ₐ*</td>
<td>20.3±0.2</td>
<td>27.2±0.3</td>
<td>31.0±0.3</td>
</tr>
<tr>
<td>Hctₐ (%)</td>
<td>27.4±0.2</td>
<td>27.4±0.2</td>
<td>30.9±0.3</td>
</tr>
<tr>
<td>Osmₐ (mmol kg⁻¹)</td>
<td>265±1</td>
<td>268±1</td>
<td>266±1</td>
</tr>
</tbody>
</table>

| N              | 138    | 135    | 140    |

Values are mean±1 S.E.M. Asterisk (*) indicates a significant effect of incubation day on the variable.

3.3. In vivo blood gas and Hct responses

3.3.1. Continuous normoxic incubation (control embryos)

No significant difference in control egg fresh mass (57.94±0.23 g, N=413) compared with experimental fresh mass (58.35±0.22 g, N=423) was observed at the start of the experiments (t=−1.304, P=0.192). There was also no significant difference in fresh egg mass designated to the 3 age-groups (P=0.202, Table 1). Similarly, Osmₐ was statistically different between the 3 age-groups of control embryos, averaging 266±0.4 mmol kg⁻¹ (P=0.055). However, pHₐ decreased by 0.11 unit, while PaCO₂ approximately doubled from d13 (~18 mmHg) to d17 (~35 mmHg) (P<0.001 for both variables). Consequently, [HCO₃⁻]ₐ increased by ~11 mmol L⁻¹ across the same developmental period. Additionally, Hctₐ significantly increased from 23.4±0.2% to 30.9±0.3% (P<0.001, Table 1).

3.3.2. Hypercapnia

Exposure to hypercapnia (5%CO₂, 20%O₂) for the last day of 13, 15 or 17 days of incubation resulted in a significant decrease in Hctₐ in embryos of all three ages compared with controls (Fig. 2). No concurrent changes occurred in Osmₐ, which averaged 264±1, 266±1 and 266±1 mmol kg⁻¹ in d13, d15 and d17 embryos, respectively. Hctₐ decreased with ΔHct values of 1%, 13% and 15% in d13, d15 and d17 embryos. [RBC] decreased by ~11% in embryos of all ages during hypercapnic exposure, whereas MCV decreased by ~4% in d17 embryos only (Fig. 3). [Hb] decreased by ~12%, 10% and 9% in d13, d15 and d17 embryos respectively, with MCH remaining constant and [MCHb] increasing by ~3%, 4% and 7% (Table 2). Blood [La⁻] decreased by ~25–35% in 3-age groups as a result of hypercapnic exposure (Table 2).

During hypercapnic exposure, PaCO₂ increased by 23.0 mmHg, 27.4 mmHg and 30.4 mmHg, while pHₐ decreased by 0.14, 0.11 and 0.11 unit in d13, d15 and d17 embryos, respectively (Fig. 4). [HCO₃⁻]ₐ increased in all embryos by 14 mmol L⁻¹, 14 mmol L⁻¹ and 13 mmol L⁻¹ in d13, d15 and d17 embryos, respectively. The increase in [HCO₃⁻]ₐ markedly exceeded the value predicted by the buffer value in all three-age groups, indicating that embryos encountered a respiratory acidosis that was partially compensated by metabolic alkalosis (Fig. 4). A metabolic compensation of ~50% occurred in all embryos.

No change in wet or dry body mass occurred as a result of 1 day of hypercapnic exposure (Table 2, Fig. 2).

3.3.3. Hypercapnic hypoxia

Exposure to hypercapnic hypoxia (5%CO₂, 15%O₂) for the last day of incubation produced embryos with a lower wet body on d15 and d17 and lower dry body mass on d17 only compared with control embryos incubated continuously in air (Table 2, Fig. 2). Hct remained unchanged in d13 embryos, but increased with ΔHct.
Table 2
Masses and hematological respiratory variables of chicken embryos exposed to environmental gas mixtures for 1 day.

<table>
<thead>
<tr>
<th>Age (d)</th>
<th>Exp</th>
<th>Egg mass g</th>
<th>Wet body mass g</th>
<th>$[\text{La}]$ mmol L$^{-1}$</th>
<th>Osm mmol kg$^{-1}$</th>
<th>$\text{Hb}$ g%</th>
<th>MCH pg</th>
<th>$\text{MCHb}$ g%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypercapnia (5%CO₂ 20%O₂)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>C</td>
<td>57.97 ± 0.68 (36)</td>
<td>6.58 ± 0.17 (22)</td>
<td>1.4 ± 0.14 (10)</td>
<td>264 ± 2 (36)</td>
<td>7.4 ± 0.1 (28)</td>
<td>46.2 ± 0.4 (28)</td>
<td>31.5 ± 0.2 (28)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>58.44 ± 0.64 (36)</td>
<td>6.24 ± 0.21 (22)</td>
<td>1.0 ± 0.05 (10)</td>
<td>263 ± 1 (36)</td>
<td>6.5 ± 0.1 (29)</td>
<td>46.6 ± 0.4 (29)</td>
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<tr>
<td>15</td>
<td>C</td>
<td>59.14 ± 0.74 (40)</td>
<td>11.64 ± 0.30 (22)</td>
<td>0.9 ± 0.08 (10)</td>
<td>266 ± 1 (40)</td>
<td>9.9 ± 0.2 (29)</td>
<td>45.3 ± 0.5 (29)</td>
<td>33.9 ± 0.4 (29)</td>
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<td>E</td>
<td>58.89 ± 0.72 (38)</td>
<td>12.20 ± 0.25 (22)</td>
<td>0.7 ± 0.03 (10)</td>
<td>266 ± 1 (38)</td>
<td>8.4 ± 0.1 (29)</td>
<td>46.1 ± 0.5 (29)</td>
<td>35.3 ± 0.4 (29)</td>
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<td>17</td>
<td>C</td>
<td>59.18 ± 0.78 (38)</td>
<td>18.64 ± 0.34 (20)</td>
<td>1.3 ± 0.11 (10)</td>
<td>267 ± 2 (28)</td>
<td>10.4 ± 0.2 (32)</td>
<td>43.6 ± 0.4 (32)</td>
<td>33.0 ± 0.4 (32)</td>
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<tr>
<td></td>
<td>E</td>
<td>58.17 ± 0.84 (37)</td>
<td>19.25 ± 0.55 (20)</td>
<td>0.8 ± 0.02 (10)</td>
<td>266 ± 2 (37)</td>
<td>9.5 ± 0.2 (31)</td>
<td>44.6 ± 0.3 (31)</td>
<td>35.2 ± 0.4 (31)</td>
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<td>Hypercapnic hypoxia (5%CO₂ 15%O₂)</td>
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<tr>
<td>13</td>
<td>C</td>
<td>56.38 ± 0.71 (41)</td>
<td>5.83 ± 0.14 (21)</td>
<td>1.9 ± 0.28 (21)</td>
<td>263 ± 1 (41)</td>
<td>7.0 ± 0.1 (21)</td>
<td>45.9 ± 0.6 (21)</td>
<td>31.3 ± 0.2 (11)</td>
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<td></td>
<td>E</td>
<td>58.72 ± 0.72 (41)</td>
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<td>263 ± 1 (41)</td>
<td>6.9 ± 0.2 (20)</td>
<td>44.8 ± 1.0 (20)</td>
<td>29.7 ± 0.7 (20)</td>
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<td>C</td>
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<td>9.7 ± 0.2 (21)</td>
<td>43.5 ± 0.5 (21)</td>
<td>30.7 ± 0.3 (21)</td>
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<td>264 ± 1 (35)</td>
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<td>44.0 ± 0.5 (20)</td>
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<td>C</td>
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<td>264 ± 1 (37)</td>
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<td>Hyperoxia (0%CO₂ 40%O₂)</td>
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Values are mean ± 1 S.E.M. (N). Bold values indicate a significant experimental (E) difference from control (C).
values of ∼17% and 14% in d15 and d17 embryos, respectively (Fig. 2). No concurrent changes in Osmo occurred in any age group (Table 2). A significant increase in [RBC] by ∼11% occurred only in d15 embryos. MCV increased by 6% and 14% in d15 and d17 embryos, respectively (Fig. 3). [Hb] remained unchanged. MCH, however, decreased in d13 and d15 embryos and consequently [MCHb] decreased by ∼5%, 10% and 14% in d13, d15 and d17 embryos, respectively (Table 2). [La\(^-\)] decreased by ∼10–30% in all embryos (Table 2).

Hypercapnic hypoxia exposure increased \(\text{PaCO}_2\) and decreased \(\text{pH}_a\) by 0.13 unit in all embryos (Fig. 4). The subsequent increase in \([\text{HCO}_3^-]_a\) was similar to values predicted from the buffer value in d15 and d17 embryos, indicating that the embryos encountered respiratory acidosis without compensatory metabolic alkalosis (Fig. 4). Day 13 embryos, however, increased \([\text{HCO}_3^-]_a\) in excess of buffer value predictions, indicating that the respiratory acidosis was partially compensated by metabolic alkalosis (Fig. 4). The degree of metabolic compensation for pH was ∼38%.

**Fig. 2.** Dry body mass and arterIALIZED hematocrit (Hcta) of embryos exposed to air (control) or altered [CO₂] and/or [O₂] for the last day of 13, 15 or 17 days of incubation. Mean values ± 1 S.E.M. are presented and N values are reported at the bottom of the bars.
3.3.4. Hypoxia

A reduction in both wet and dry body mass was only observed in d15 embryos exposed to hypoxia (0%O₂, 15%O₂) for 1 day (Fig. 2, Table 2). Hct significantly increased in all embryos exposed to hypoxia with no concurrent alterations in Osm, which averaged 264 ± 1 mmol kg⁻¹ in d13 embryos, 267 ± 2 mmol kg⁻¹ and 269 ± 1 mmol kg⁻¹ in d15 and d17 embryos, respectively. The ΔHct was ~8%, 13% and 11% compared with control in d13, d15 and d17 embryos, respectively. [RBC] increased by ~8% in d15 embryos only, whereas MCV significantly increased by ~4%, 5% and 6% in d13, d15 and d17 embryos (Fig. 3). [Hb] remained unchanged, but both MCH and [MCHb] decreased in all embryos by ~5% and 10%, respectively (Table 2). [La⁻] increased by ~120%, 80% and 110% in d13, d15 and d17 embryos, respectively, while Osm remained constant (Table 2).

pH decreased with ΔpH 0.04, 0.08 and 0.07 units in d13, d15 and d17 embryos, respectively. There was no significant change in PaCO₂. Consequently, [HCO₃⁻]ₐ decreased significantly in d15 and d17 embryos, indicating metabolic acidosis (Fig. 4).

3.3.5. Hyperoxia

Hyperoxia (0%O₂, 40%O₂) increased wet body mass of d17 embryos only (Table 2), but did not alter dry body mass at any...
4. Discussion

The avian embryo model allows exploration of acid–base responses without ventilatory and with limited renal contributions. One day exposure to environmental gases during incubation significantly affected blood acid–base balance, Hct and in some instances body mass of embryos, as will now be discussed.

4.1. Embryo buffer line and Pco2 isopleths in the Davenport diagram

To evaluate and compare acid–base status in embryos exposed to different environmental gases, blood gas variables were depicted using a pH–[HCO3⁻] diagram (“Davenport diagram”), which requires quantification of both an embryo buffer line and Pco2 isopleths. Reported buffer values for chicken embryos have varied from −12.7 to −15 mmol L⁻¹ pH unit⁻¹ for embryos at d16 (Tazawa et al., 1981; Tazawa, 1980, 1981, 1982, 1986) and −18 mmol L⁻¹ pH unit⁻¹ for embryos from d14 to d18. (Tazawa et al., 1983). Hemoglobin, which serves as the important non-carbonate buffer in blood, increases during the last half of incubation. Therefore, the buffer value is also expected to increase as development progresses. Indeed some studies have demonstrated buffer values increasing from −8 to 10 mmol L⁻¹ pH unit⁻¹ on d9–10 to −17 mmol L⁻¹ pH unit⁻¹ on d15–18, reflecting the time-course of Hct and [Hb] increase (Erasmus et al., 1970–1971; Tazawa and Piiper, 1984). However, other studies (Tazawa et al., 1983; Andrewartha et al., 2011b) and our current study did not find increasing buffer values across development. Accordingly, the mean buffer value of −16.0 mmol L⁻¹ pH unit⁻¹ across the three age groups was used in all Davenport diagrams and the buffer line was positioned at an arbitrary elevation indicating buffering capacity.

4.2. Acid–base balance during environmental respiratory gas challenges

As expected, 1 day of hypercapnic exposure (5%CO2, 20%O2) increased Paco2 and decreased pH in all embryos (Fig. 4). This respiratory acidosis was partially compensated by a metabolic alkalosis of ~50% at all embryonic stages examined (Fig. 4). Lactate concentration decreased in all embryos during hypercapnic exposure and even during hypoxic exposure in the presence of hypercapnia. These data suggest a possible inhibitory role of CO2 on anaerobic pathways, although this speculation requires further testing.
Similar patterns of respiratory acidosis with partial compensatory metabolic alkalosis have been demonstrated in embryos with reduced eggshell gas conductance (Tazawa et al., 1971b; Tazawa, 1981), in embryos in a 20%O2/80%SF6 atmosphere (Tazawa et al., 1981) and in embryos exposed to a range of [CO2] (4.8 or 9%) in air for 3 h to 3 days (Dawes and Simkiss, 1969; Tazawa, 1982, 1986). However, chronic hypercapnic (12 days at ~4%CO2) exposure produced a different acid–base status, with metabolic alkalosis occurring in d12 and d16 embryos without respiratory acidosis (Everaert et al., 2008). Additionally, challenging embryos by hypercapnic hypoxia (5%CO2, 15%O2) for 1 day abolished the compensatory metabolic alkalosis in d15 and d17 embryos. However, a metabolic compensation of ~37% was still observed in d13 embryos (Fig. 4), although it was not sufficient to mitigate changes in pH. Thus, it appears that a high O2 level is required for metabolic compensation to occur. Potentially, lower M02 (i.e., less O2 leaving the circulation) and an overall higher allantoic PaO2 (in air; Tazawa, 1971, 1980; Tazawa et al., 1971a,b) in d13 embryos compared with more advanced (d15, d17) embryos may preserve the metabolic compensation during hypoxic hypercapnia.

Movement of a relatively large number of HCO3– ions will be required for the observed metabolic compensations to occur during hypercapnia and hypoxic hypercapnia. The developing kidney presumably does not play an important role in excreting proteins and creating the extra [HCO3–] in embryos, because titratable acids and ammonium in the allantoic fluid do not increase in response to hypercapnia (Dawes and Simkiss, 1971). Instead, re-absorption of eggshell minerals has been proposed as the HCO3– source (e.g. Dawes and Simkiss, 1971; see Gabrielli and Accili, 2010 for review). However, the relatively rapid time-course of the increase in non-respiratory HCO3– (within 4–6 h, H. Tazawa, unpublished data) makes eggshell re-absorption an unlikely candidate, in our view. More likely, the elevated PaCO2 results in increased hydration of CO2 in the blood. This will increase the [H+] (which is excreted from the blood) and [HCO3–] which accumulates in the blood. The choioallantoic membrane (CAM) actively transports Na+ from the allantoic fluid to the blood (Stewart and Terepka, 1969; Boutillier et al., 1977; Hoyt, 1979; Graves et al., 1986; Davis et al., 1988) and consequently H+ is excreted from the blood to bind the anion in the allantoic fluid and/or remain in it. Potentially, metabolic compensation does not occur in advanced embryos in a hypoxic environment (e.g., d15, d17), because the active transport of Na+ may be limited in such environment (i.e., a high enough M02 cannot be maintained for active transport).

Potentially, down-regulated M02 (see Mortola, 2009 for review), paired with constant gas conductance during hypoxia, could have created a respiratory alkalosis. However, hypoxic exposure for 1 day resulted in a metabolic acidosis in d15 and d17 embryos (Fig. 4). Whether the immature kidney can function sufficiently to excrete enough HCO3– from the blood into the allantoic fluid is as yet unknown. Potentially, the extensive CAM may play a role in ion regulation and the metabolic acidotic response. In hypoxia, M02 is reduced without anaerobic energy compensation (Bjénnès et al., 1987; Mortola and Besterman, 2007). However, once a lower threshold M02 is reached, embryos turn to anaerobic glycolysis and the blood lactate concentration increases (Grabowski, 1961, 1966; Bjénnès et al., 1987). Although d11 and d16 embryos do not incur an O2 debt during 40 min hypoxic exposure (Mortola and Besterman, 2007), longer exposure (such as the 1 day in this study) resulted in M02 reaching the lower threshold and the induction of anaerobic respiration causing an accumulation of lactate and ensuing decrease in pH. However, the increase in [La–] of ~1 mmol L~1 only accounts for ~1/6th of the decrease in [HCO3–] (~6 mmol L~1) in the present study (Table 2). Hypoxia exposure (1 day) in d13 embryos did not result in metabolic acidosis (Fig. 4; Tazawa, 1986) likely due to the lower M02 requirements of younger embryos resulting in a lower (unachieved in this study) threshold M02 where anaerobic metabolism is implemented.

Respiratory acidosis was also a consequence of 3 h or 1 day of hyperoxic exposure (Tazawa, 1986 and Fig. 4, respectively). M02 (and consequently M02) is increased when embryos are exposed to hyperoxia (Piiper et al., 1980; Visschedijk et al., 1980; Hailby et al., 1983; Stock et al., 1985; Tazawa et al., 1992). Increased CO2 is accumulated in the blood, due to a fixed gas conductance of the eggshell, and hydrated to create H+ and HCO3– according to buffer capacity. The age-specific difference in magnitude of the acidosis is likely due to hyperoxia resulting in a large up-regulation of M02 in older embryos (e.g. Stock et al., 1985; Tazawa et al., 1992).

Overall, the resulting acid–base status of d13 embryos exposed to altered environmental gas mixtures tended to differ from the more advanced d15 and d17 embryos (Fig. 4). D13 embryos demonstrated an ability to metabolically compensate for the respiratory acidosis induced by hypercapnic hypoxia, a compensation that was lacking in the advanced embryos. Further, metabolic acidosis did not result from hypoxic exposure in d13 embryos. Thus, it appears that d13 embryos are more resilient to alterations in acid–base status induced via alterations in environmental gas composition than their more advanced counterparts. The reasons for this increased tolerance are largely unknown, but are likely a result of the relatively low M02 and immature physiological functions of younger embryos.

4.3. Hct responses to altered environmental respiratory gas composition

4.3.1. In vitro Hct changes to altered O2 and CO2

Changes in Hct are inherently less complex in vitro than in vivo, because [RBC] is fixed and alterations in Hct can only be achieved through changes in MCV. Although equilibration of the blood to 0%CO2 produced the same acid–base status (i.e., comparable degree of respiratory alkalosis) between all O2 groups irrespective of [O2], the ΔHct of blood equilibrated to 0%O2 differed significantly from ΔHct equilibrated to >10%O2. Similarly, equilibration to 5%CO2 resulted in the same acid–base status between all O2 groups irrespective of [O2], but the ΔHct of blood equilibrated to 0%O2 was larger than the ΔHct of blood equilibrated to >10%O2. Consequently, Hcteq was not influenced by pH and [HCO3–] in vitro. Nonetheless, [O2] did play an important role. Hcteq was greater after anoxic equilibration (0%O2) irrespective of the presence or absence of CO2 (Fig. 1). There was no change in Hct when blood was equilibrated to any gas mixture containing O2 (10–100%O2) in the absence of CO2 (0%CO2). Equilibration with 5%CO2 mitigated the decreases in MCV, resulting in a smaller ΔHct which was similarly unchanged by increases in >10%O2 (Fig. 1). Thus, the RBCs likely hydrate in anoxia and dehydrate in the presence of O2. These alterations occurred without changes in blood osmolality. In anoxia, RBC hydration was augmented by the addition of CO2 and, conversely, the dehydration of the RBCs was mitigated by the addition of CO2. These in vitro results suggest that it may be possible for Hct to increase in vivo through increased MCV in embryos with low PaO2. Additionally, this could account for a constant low Hct (due to increased MCV) across a wide range of high PaO2. Indeed, Hct increased in vivo through increased MCV in hypoxic environments irrespective of [CO2]. MCV decreased in d15 and d17 embryos in hyperoxia, however, likely due to dehydration in the presence of O2.

When interpreting these results, it is important to bear in mind that an arterialized PaCO2 = 28 mmHg (~4%) is normocapnic for the blood of d15 embryos (Table 1). Thus, equilibration with 5%CO2 is not expected to alter MCV, and hence Hct, significantly from in vivo arterIALIZED values, as observed. Altering PaCO2, in this case below
normocapnia in vivo values (i.e. 0%CO₂) resulted in changes in Hct through MCV. Thus, the Hct changes during altered P Cô (i.e. hypercapnia) in vivo are potentially the result of MCV. Further, alterations in P Cô, above normoxia did not elicit changes in MCV (and Hct) at 5%CO₂ (similarly to in vivo normocapnia). In vitro, it is likely, then, that the decrease in Hct during hyperoxia exposure in vivo (Fig. 2) was the result of changes in [RBC] as well as alterations in MCV in vivo (see more below).

4.3.2. Increased Hct in hypoxia through increased MCV in vivo

Hypoxia, regardless of the presence or absence of CO₂, increased Hct in all treatments, with the sole exception of d13 embryos exposed to hypercapnic hypoxia [Fig. 2]. The increased Hct was due to an increase in MCV in all circumstances (supported by the in vitro experiments), with d15 embryos additionally increasing [RBC] in both hypoxia and hypercapnic hypoxia. Similarly, previous studies have reported an increase in [RBC] in response to 1 day of hypoxic exposure on d15 and d17, but not embryos younger than d13 (Ackerman, 1970; Nakazawa and Tazawa, 1988). In d16 embryos with eggshells covered with gas-impermeable material, increased Hct was attributed to an increase in both MCV and [RBC]. Contrary to expectation, O₂ delivery may not be significantly improved during 1 day of hypoxia because the increase in Hct is, in general, attributable to increased MCV (as opposed to [RBC]). In fact, total [Hb] remained unchanged and MCH decreased (contrary to expectation) and, due to the increase in MCV, [MCHb] also decreased (Table 2). Further, it is interesting to note that although increased MCV resulted in increased Hct during both hypoxia and hypercapnic hypoxia, no corresponding alterations in Osm occurred. Indeed none of the gas compositions changed blood osmolality.

Hypoxia-induced changes in Hct manifest differently during chronic and acute exposure and also are dependent upon embryonic age. Chronic exposure to 15%O₂ from d5–20 and d12–18 of incubation failed to increase Hct compared with control embryos incubated in air (Dzialowski et al., 2002; Azzam and Mortola, 2007). However, hypoxic exposure (15%O₂) during d6–12 increased Hct at d12, although Hct returned to control levels by d18 in normoxia (Dzialowski et al., 2002). An acclimation response may occur during chronic exposure, particularly in advanced embryos, as it may not be advantageous for embryos to sustain elevated Hct due to increased blood viscosity and the reduction in cardiac output this might produce. Moreover, an increase in Hct during hypoxic exposure of early embryos may not be necessary, because diffusion through the body wall rather than convective blood transport is sufficient for meeting O₂ requirements (Burggren et al., 2000, 2004).

Respiratory acidosis occurred in all embryos exposed to all experimental gas mixtures except hypoxia [Fig. 4]. A reduction in pH has potential implications for Hct because changes in MCV are influenced by ion transport through ion channels, which are generally pH sensitive (see Cossins and Gibson, 1997; Nakimma, 1992; Hoffmann et al., 2009 for review). The general trend of decreased pH did not uniformly affect Hct, though, producing a variety of MCV responses.

4.3.3. Hyperoxia and hypercapnia alter Hct by decreasing [RBC] in vivo

RBCs were sequestered (i.e., decrease in [RBC]) during 1 day of hyperoxia, reducing Hct. For example, Hct decreased from 27.6% to 24.6% during hyperoxia in d15 embryos due to a decrease in [RBC] from 2.04 million cells/µL to 1.88 × 10⁶ µL⁻¹ (Fig. 3). In addition, the MCV of advanced embryos (d15 and 17) decreased. In d15 embryos, for example, MCV decreased from 135µL to 131µL during hyperoxia, further contributing to the decrease in Hct. The in vivo findings are in contrast to the day 15 in vitro findings, where alterations in O₂ levels did not elicit changes in MCV (and Hct) at 5%CO₂ (similar to in vivo normocapnia). These data suggest that there are more factors involved in altering Hct and MCV than just oxygen partial pressure (Fig. 1). Cell volume is regulated by a multitude of interacting mechanisms including potassium-chloride co-transport, taurine transport and sodium-dependent beta-amino acid transport systems. These systems are modulated by a variety of mechanisms including neuronal, hormonal and autocrine stimulation and via changes in osmolality (Osm), P Cô, P CO₂ and pH (see Nikimma, 1992; Cossins and Gibson, 1997; Lambert et al., 2008 for review, Haase, 2010). The determination of the relative contribution of these many mediators to MCV regulation during environmental perturbations is beyond the scope of the present study. Yet, it is obvious from the discrepancies between in vitro and in vivo findings in this study that changes in MCV during hyperoxia are due to far more than simple passive ion fluxes.

In hyperoxia, fewer RBCs are likely to be required to achieve effective O₂ transport. Thus, it may be energetically advantageous to reduce blood viscosity and cardiac output by removing RBCs from the circulation. MCH did not change during hyperoxia and accordingly blood [Hb] decreased in parallel with [RBC] (Table 2). Although [MCHb] increases with decreasing MCV, this overall pattern simply demonstrates the reduction in potential O₂ carrying capacity when RBCs are sequestered, i.e., a match of potential to supply with demand. Using the mean MCV of 133µL (MCV decreased from 135µL in control to 131µL in hyperoxia; Fig. 3), the volume of RBCs sequestered during hyperoxia in d15 embryos was 30 × 10⁻³ µL/µL blood. Sequestration of ~400 million RBCs (with a total volume of 75 µL) from a blood volume of 2.5 mL in d15 embryos would be required to produce this decrease in [RBC] (i.e., from 2.04 million cells/µL in control embryos to 1.88 million cells/µL in hyperoxia). The location of the non-circulating vascular compartment where this large number of RBCs is sequestered is as yet unknown. RBCs are sequestered and released by the spleen in adult vertebrates (see e.g. Brendolan et al., 2007 for review). The spleen may also play a similar role in embryos. However, the estimated number and volume of RBCs sequestered and released during hypoxia and hyperoxia may exceed the capacity of the still-developing spleen, so other potential sequestration sites, such as the CAM, may be important.

Hct decreased (∆Hct = ~13–15%) when d12–16 embryos were exposed to hypercapnia (5%CO₂, 20%O₂) for 1 day (Fig. 2). Similar to hyperoxia, a decrease in [RBC] (∆[RBC] = ~11%) was predominantly responsible with a small contribution of MCV only in d17 embryos (∆MCV = 4%) (as reported by Nakazawa and Tazawa, 1988). It is possible that the changes in Hct were due to acute, rapid fluid fluxes between the circulating blood volume and non-vascular compartments rather than due to RBC sequestration (Khorrami et al., 2008). Whether changes in environmental gas composition can elicit changes in fluid fluxes with implications for Hct regulation is yet to be determined.

4.4. Alterations in embryo body mass

Interestingly, a reduction in wet body mass was apparent after only 1 day of exposure to hypoxia (d15) or hypercapnic hypoxia (d15 and d17). On the other hand, hypercapnia alone did not elicit changes in body mass (Table 2). A similar reduction in body mass during 1 day of hypoxia occurs in d11 embryos (Ackerman, 1970). However, no difference in body mass occurs during d7–9 or d13–19. In the present study, rigorous testing demonstrated that the methodology did not result in substantial water loss in the experimental embryos even though the relative humidity was lower (Appendix A). Further, dry body mass only decreased
significantly in d15 hypoxic and d17 hypercapnic hypoxic embryos by \(-13\) and \(18\%\), respectively (Fig. 2).

Exposure to 10\% \(O_2\) for 1–2 h reduces the \(M_O\) of prenatal (d12, d16 and d18) and perinatal (d20, EP) embryos to more than half of control (normoxic) values (Tazawa et al., 1992). This response is conserved across many other embryonic, neonatal and even adult animals from a broad range of taxa (see Mortola, 2008 for review). In neonatal mammals, this reduction in \(M_O\) is due to a reduced thermoregulatory set-point resulting in less energy expenditure for body temperature (Tb) maintenance (see Mortola et al., 1989 for review). Avian embryos have very limited thermogenic ability and it is thus more likely that hypoxia blunts tissue growth and development in all embryos and further compounds the \(O_2\) conductance limitations of advanced embryos (see Mortola, 2009 and Andrewartha et al., 2011a for review). Regardless of mechanism, an overall reduction in \(M_O\) may be related to the decrease in body mass during hypoxia and hypercapnic hypoxia exposure in d15 and d17 embryos, respectively, in chicken embryos or other animal species (see Mortola, 2004 for review). Hypoxic incubation potentially delays development during the prenatal period. Smaller hypoxic-incubated d19 embryos reach a similar Hamburger–Hamilton stage when compared to the larger control embryos during chronic exposure (Villamor et al., 2004). Thus, it is unlikely that hypoxia affected development as distinct from growth.

Chronic hypoxic incubation does not always produce consistent changes in embryo body mass. Significant decreases (Stock and Metcalfe, 1987; Burton and Palmer, 1992; Rouwet et al., 2002; Dzialowski et al., 2002; Miller et al., 2002; Villamor et al., 2004; Azzam and Mortola, 2007) or no effect on body mass (Chan and Burggren, 2005) have been reported during hypoxic exposure \((P_{O_2} = 97–104\text{mmHg}; \sim 12–13\%O_2\) for periods ranging between 3 days to all of incubation. Whether the decreased body mass observed after 1 day of hypoxic incubation would still be apparent at hatching and beyond is unknown. Prolonged \(\text{in ovo}\) hypoxia exposure produced no difference in d21 (externally piping) embryo and hatching body mass compared with controls (Miller et al., 2002; Villamor et al., 2004; Ruijtenbeek et al., 2003; Ferner and Mortola, 2009), indicating that compensation for reduced body mass may occur by hatching or simply due to abdominal incorporation of the remaining yolk, which warrants a future study.

While embryo body mass decreased during 1 day of hypoxic exposure (with or without hypercapnia), hyperoxic exposure increased the wet body mass of d17 embryos only (Table 2). Dry body mass was unchanged, so it is likely that an increase in \(P_{O_2}\) above normoxic levels did not increase \(M_O\) and that the supply of \(O_2\) was sufficient to meet demand (.\(M_O\)). Thus, the embryos were not conductance-limited, a situation that occurs in advanced embryos close to hatching (e.g., Høiby et al., 1983; Tazawa et al., 1988b; Dzialowski et al., 2007).

4.5. Summary

Detailed characterization of the ontogeny of \(Hct\) regulation and acid–base perturbations during mild environmental respiratory gas challenges provides an important framework likely to be reflective of all endotherms including developing humans. Respiratory acidosis resulted from exposure to hypercapnia, hypercapnic hypoxia or hyperoxia (with some differences between environmental gases and embryonic ages). Metabolic acidosis occurred in more advanced embryos (d15–17) exposed to hypoxia. Respiratory acidosis during hyperoxic exposure was likely a result of increased metabolism (except in young d13 embryos with low metabolism) coupled with constant eggshell gas conductance. In hypercapnia, the respiratory acidosis was partially compensated by an increase in non-respiratory \([HCO_3^-]\), while in hypercapnic hypoxia the acidosis was uncompensated. Thus, it is likely that \(O_2\) plays a role in metabolic compensation. Future studies will be directed toward the origin of the non-respiratory \([HCO_3^-]\): i.e., the potential organs responsible for the metabolic compensation such as the CAM. The timing and progressions of acid–base disturbances will be examined and the concentration ranges of the environmental exposure gases, increased and modified during exposure to vary the magnitude of the acid–base disturbance (i.e., respiratory acidosis and metabolic compensation). For example, low \(O_2\) (e.g., \(10\%\)) may also produce metabolic acidosis in association with possible progress of anaerobic glycolysis.

In general, \(Hct\) increased in response to hypoxia through an increase in MCV irrespective of the presence of \(CO_2\). Although the increase in \([RBC]\) was only significant in d15 embryos, \([RBC]\) tended to increase in d13 and d17 embryos and further study is required to fully understand the contribution of \([RBC]\) in \(Hct\) regulation during hypoxic challenges. \(Hct\) decreased during hyperoxic and hypercapnic exposure through a decrease in \([RBC]\) in all embryos with the trend of decreasing MCV, particularly during hyperoxic challenges. It appears MCV regulation is related to \([O_2]\) and \([RBC]\) regulation to sequestration/release rather than erythropoiesis. However, this phenomenon, and the actual site responsible for storage and release of a relative large number of \(RBCs\), is yet to be elucidated. In addition, a study concurrently measuring plasma volume with \(Hct\) is highly warranted.

Appendix A. Comparison between control and gas-exposure protocols

Twenty eggs were incubated as described in the methods until d14 to evaluate whether the airflow rate within the gas-exposure bag significantly affected water loss with subsequent consequences for blood gas or hematological variables. The eggs were randomly assigned to a control or experimental group \((N = 10\text{ for each})\) and no difference between control and experimental egg mass existed at either d0 \((57.02 \pm 1.07\text{ g for control}\); cf. 56.85 \(\pm 1.39\text{ g for experimental,}\); \(t = 0.010, P = 0.922\)) or d14 \((55.53 \pm 1.04\text{ g for control}\); cf. 52.88 \(\pm 1.26\text{ g for experimental,}\); \(t = 0.395, P = 0.698\)). Following the protocol outlined in Section 2, the control eggs were moved to a desk-top incubator at 37.5 °C with a relative humidity \((RH)\) of \(>25\%\) on d14 and experimental eggs were placed into the plastic gas-exposure bags ventilated with air provided by a Wösthoff pump resulting in \(RH\) of \(<15\%\). On the following target day (d15), egg mass, embryo wet and dry masses, blood gas variables and hematological respiratory variables were determined as outlined in the Methods and experimental and control values compared using unpaired \(t\)-test. Although water loss from the egg was higher in experimental embryos \((0.59\text{ g cf. 0.46 g in control})\), there was no difference in wet or dry embryo mass or in blood gas or hematological respiratory variables between control and experimental animals (Table 3). RH in the desk-top incubator \((25\%\) and gas-exposure bag \((15\%\)) was substantially lower than the incubator where the embryos developed from d0 to d14 \((55\%\) due to unhumidified air being supplied directly from a gas cylinder. Nevertheless, the present experiment demonstrates that although an \(\sim 28\%\) increase in water loss can be produced by an \(<10\%\) decrease in RH for 24 h, neither wet nor dry mass of the embryos are affected (Table 3). Further, the lack of any differences in physiological blood variables due to a reduction in RH for 24 h (and consequently increased water loss) allows the conclusion that any changes in mass, blood gas or hematological respiratory variable observed during the gas exposures in this study are a consequence of the gas exposure rather than a by-product of methodology.
Table 3
Water loss, mass, lactate concentration ([La−]), osmolality (Osm), blood acid–base status and hematological respiratory variables of control embryos (N = 10) and embryos within a gas-exposure bag ventilated with air for 1 day (N = 10). Data are mean ± 1 S.E.M.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Ventilated at 600 mL min−1</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water loss from egg d14-15 (g)</td>
<td>0.46 ± 0.02</td>
<td>0.59 ± 0.04</td>
<td>0.005</td>
</tr>
<tr>
<td>Embryos wet mass (g)</td>
<td>13.43 ± 0.46</td>
<td>14.07 ± 0.35</td>
<td>0.278</td>
</tr>
<tr>
<td>Embryo dry mass (g)</td>
<td>1.99 ± 0.11</td>
<td>2.19 ± 0.10</td>
<td>0.215</td>
</tr>
<tr>
<td>[La−] (mmol L−1)</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.343</td>
</tr>
<tr>
<td>Osm (mmol kg−1)</td>
<td>259 ± 1</td>
<td>260 ± 1</td>
<td>0.838</td>
</tr>
<tr>
<td>pHa</td>
<td>7.541 ± 0.007</td>
<td>7.555 ± 0.011</td>
<td>0.185</td>
</tr>
<tr>
<td>[HCO3−] [Hb]</td>
<td>28.6 ± 1.1</td>
<td>28.9 ± 0.7</td>
<td>0.815</td>
</tr>
<tr>
<td>Pco2</td>
<td>33.6 ± 1.5</td>
<td>32.6 ± 1.6</td>
<td>0.652</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>29.5 ± 1.1</td>
<td>29.1 ± 0.6</td>
<td>0.744</td>
</tr>
<tr>
<td>[RBC] (x106 µL)</td>
<td>2.09 ± 0.06</td>
<td>2.13 ± 0.04</td>
<td>0.574</td>
</tr>
<tr>
<td>MCV (µL)</td>
<td>141 ± 3</td>
<td>137 ± 2</td>
<td>0.150</td>
</tr>
<tr>
<td>[Hb] (g%)</td>
<td>9.4 ± 0.2</td>
<td>9.5 ± 0.2</td>
<td>0.779</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>45.0 ± 0.5</td>
<td>44.5 ± 0.3</td>
<td>0.413</td>
</tr>
<tr>
<td>[MCHb] (g%)</td>
<td>32.0 ± 0.5</td>
<td>32.6 ± 0.3</td>
<td>0.305</td>
</tr>
</tbody>
</table>

Appendix B. Validation of egg wrapping to preserve blood-gases

Blood gases alter rapidly in response to changes in environmental gases (Tazawa et al., 1981). Therefore, blood should ideally be collected whilst embryos are still exposed to an altered gas mixture (not atmospheric air), although this is often technically difficult. In the present experiment, environmental gas was conveniently and consistently altered and supplied to embryos within a gas-exposure bag (see Methods). However, blood could not be sampled whilst the embryos were still inside the gas-exposure bag. Instead, the eggs were immediately wrapped loosely with a gas-impermeable film when they were removed from the bag. Preliminary data were collected from d15 embryos that were wrapped either loosely with aluminum foil, tightly with parafilm or unwrapped (control, in atmospheric air) to evaluate the effect of wrapping on blood gas variables (Fig. 5). Additional embryos were exposed to hypercapnia (5%CO2, 20%O2) for the last day of 15 days incubation and subjected to the 3 wrapping treatments to validate the effectiveness of the wrapping sampling technique for preserving blood-gases. Blood samples were drawn within 2 min of wrapping, analyzed for blood gas variables (Pco2, pH, [HCO3−] and P02), Osm and Hct (as detailed in Section 2). One-way ANOVA with Tukey’s post hoc test was then used to analyze the data.

Wrapping eggs in aluminum foil in air for <2 min produced no significant difference in mean blood Pco2 (31.6 ± 1.1 cf. 28.4 ± 1.6 mmHg in control embryos), pH (7.560 ± 0.014 cf. 7.599 ± 0.014 in control embryos) and [HCO3−] (27.8 ± 1.0 cf. 27.5 ± 1.0 mmol L−1 in control embryos) (Fig. 5). Further, the wrapping procedure produced no differences in either Osm (266 ± 1 mmol kg−1 in aluminum wrapped cf. 268 ± 1 mmol kg−1 in control) or Hct (27.2 ± 0.5 cf. 27.0 ± 0.8% in control). In contrast, eggs wrapped in parafilm decreased pH and P02, and increased Pco2, with unchanged [HCO3−], Hct and Osm compared with both control and aluminum wrapped embryos (Fig. 5). CO2 is highly soluble and relatively large amounts are stored within the gas spaces of eggs. Elimination of CO2 stored in the gas-filled spaces in dead d19 embryos exposed to pure N2 lasted for ~10 min before the elimination of CO2 dissolved in the blood and tissues became predominant (Mortola and Besterman, 2007). Wrapping the eggs tightly with parafilm resulted in a very small gas reservoir between the parafilm and the eggshell. During the 2 min of blood sampling, O2 was depleted from the blood and CO2 accumulated increasing Pco2 by ~11 mmHg (Fig. 5). This highlights the greater efficiency of gas exchange due to blood perfusion of the CAM in contrast to CO2 elimination from dead eggs. Wrapping eggs loosely with aluminum foil allowed for a greater gas reservoir between the foil and the eggshell. Blood flowing through the chorioallantoic capillaries could maintain gas exchange with the gas reservoir and less O2 was depleted and less CO2 accumulated in the blood. Consequently, blood pH was maintained at a level similar to control (Fig. 5).

Hypercapnic exposure (5%CO2, 20%O2) resulted in respiratory acidosis partially compensated by metabolic alkalosis in all embryos (Fig. 6). The degree of respiratory acidosis was slightly smaller in unwrapped embryos compared with hypercapnic, aluminum-wrapped embryos. However, the respiratory acidosis in the parafilm-wrapped embryos was more severe than either aluminum-wrapped or unwrapped embryos (Fig. 6). The respiratory acidosis of unwrapped embryos was smaller than aluminum-wrapped embryos, likely due to the embryos being exposed to environmental air during sampling allowing acid–base status to recover toward control values.

Fig. 5. Effect of eggshell asphyxiation with aluminum foil and parafilm on arterialized blood (A) pH and Pco2 and (B) [HCO3−], and Osm, in d15 chicken embryos. Mean values ± 1 S.E.M. are presented (N = 13).
In conclusion, wrapping eggs in aluminum foil did not affect blood gases, Hct or Osm in environmental air (normocapnic normoxia) and, due to the large gas spaces between the foil and eggshell, allowed blood sampling to occur with the embryos still exposed to hypercapnia. Thus, foil wrapping was adopted as an effective convenient technique for sampling blood from embryos exposed to altered [CO₂] and [O₂].

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