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

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ABSTRACT

How the determinants of hematocrit (Hct) – alterations in mean corpuscular volume (MCV) and/or red blood cell concentration ([RBC]) – are influenced by acid–base balance 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%CO_2, 15%O_2) or hypoxia alone (0%CO_2, 15%O_2), but decrease in response to hyperoxia (0%CO_2, 40%O_2). Further, age-related differences in acid–base disturbances and Hct regulation may arise, because the O_2 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 O_2 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 $\dot{M}_{O_2}$ and immature physiological functions of younger embryos.

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 O_2 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 O_2 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 hyperoxia in situations where sufficient O_2 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 much 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 $[HCO_3^-]$ 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 O_2 levels (as in Tazawa et al., 1988a) may be required.

The present study investigates how Hct responds to moderately altered environmental O_2 and CO_2 concentration ($[O_2]$ and $[CO_2]$) 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 O_2 transport, which would otherwise be impeded by hypoxia or a decrease in O_2 affinity (resulting from the Bohr shift in hypercapnia – *e.g.*, Tazawa and Mochizuki, 1976). During hyperoxia, we hypothesize that Hct will decrease because O_2 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 $[O_2]$ and $[CO_2]$ 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 O_2 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 $T_a = 37.5 \pm 0.1\,^{\circ}C$ 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 $T_a = 37.5 \pm 0.1\,^{\circ}C$ (and relative humidity ~25%). The embryos were exposed to an experimental gas mixture within a gas-exposure bag (relative humidity ~15%, see below and Appendix A for justification of flow and humidification 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 ($ABL^{TH}5$, Radiometer Medical A/S, Copenhagen, Denmark) was measured for pH, P_{CO_2} and $[HCO_3^-]$ (calculated by the analyzer from pH and P_{CO_2}) at 37 °C. [RBC] and [Hb] were measured on 0.01 mL of blood with a Coulter analyzer ($A^c{\cdot}T$, Beckman Coulter Inc., CA, USA) and the mean corpuscular indices (mean corpuscular hemoglobin (MCH), MCV and mean corpuscular hemoglobin concentration ([MCHb])) were calculated (following Tazawa et al., 2011). Lactate concentration ($[La^-]$) 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, $[HCO_3^-]$, P_{CO_2}), hematological respiratory variables (Hct, [RBC], [Hb], MCV, MCH, [MCHb]), Osm and $[La^-]$ 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 chorioallantoic membrane (CAM). Piiper et al. (1980) referred to this as arterialized, and we adopt that nomenclature.)

2.3. In vitro effects of altered $[O_2]$ and $[CO_2]$ 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 Andrewartha 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, Andrewartha et al., 2011b) to avoid water evaporation from the blood. The blood was equilibrated *in vitro* with one of 12 different gas mixtures (6 $[O_2] \times 2$ $[CO_2]$: 0% or 5%CO_2 ($P_{CO_2} = 0$ or 36 mmHg, respectively) and one of 0, 10, 15, 20, 40 or 100%O_2 (P_{O_2} = 0–760 mmHg), or 95%O_2 in case of 5%CO_2 with N_2 balance. After 1.5 h, the tonometer was removed from the water-bath and the blood immediately assessed for pH, $[HCO_3^-]$ and P_{CO_2} at 37 °C and Osm and Hct (again measured in duplicate and the mean determined) at room temperature; referred to as pH_{eq}, $[HCO_3^-]_{eq}$, $P_{CO_2(eq)}$, Osm_{eq} and Hct_{eq}. Changes in Hct resulting from equilibration to altered $[O_2]$ and/or $[CO_2]$ were expressed as $\Delta Hct = 100 \times (Hct_{eq} - Hct_a)/Hct_a$ (in %) to emphasize changes from arterialized values and to provide a convenient measure for comparing percentages across groups.

Blood buffer value was determined using blood collected from d13, d15 and d17 control embryos. pH, $[HCO_3^-]$ and P_{CO_2} were immediately determined, then the remaining blood was equilibrated for 1.5 h in a tonometer with one of the following gas mixtures; air (0.3%CO_2, P_{CO_2} = 2 mmHg), 3%CO_2 with 21%O_2 or 6%CO_2 with 21%O_2 balanced with N_2 (supplied by pre-mixed gas cylinders; Air Liquide). After 1.5 h, the blood gas parameters were immediately determined. The pH_{eq} and $[HCO_3^-]_{eq}$ values of individual blood samples were plotted on a Davenport diagram which was constructed by plotting P_{CO_2} isopleths calculated from the Henderson–Hasselbalch equation using a CO_2 solubility factor of 0.0308 mmol L mmHg^{-1} and a serum carbonic acid pK′ which varied with pH (Severinghaus et al., 1956a,b). Least squares regression equations expressing a relation between $[HCO_3^-]$ and pH were determined for each incubation day and the mean gradient used as the buffer value.

2.4. *In vivo responses of Hct and blood-gases to changes in $[O_2]$ and $[CO_2]$*

Chicken embryos were exposed *in vivo* to one of four N_2-balanced gas mixtures; hypercapnia (5%CO_2, 20%O_2), hypercapnic hypoxia (5%CO_2, 15%O_2), hypoxia (0%CO_2, 15%O_2) or hyperoxia (0%CO_2, 40%O_2). One day prior to the target incubation days (d13, d15 or d17), eggs were placed on a cardboard holder inside a 3.78 L (26.8 cm × 29.7 cm) plastic Ziploc® bag with inlet and outlet conduits at diagonally opposite corners (gas-exposure bag) in the incubator (37.5 ± 0.1 °C) for ~24 h on d12, d14 or d16. Gas ventilating the bag was supplied via a Wösthoff (oHG, Bochum, Germany) gas-mixing pump at a rate ~600 mL min^{-1} and was not humidified in order to maintain driving pressure and ensure complete washout of respiratory gases from the embryos. Although the preliminary study demonstrated that the relatively high flow rate resulted in increased water loss from the egg in experimental embryos, there was no significant difference in wet or dry embryo mass or in blood gas or hematological respiratory variables between control and experimental animals (see Appendix A).

On d13, d15 or d17 of incubation, eggs were removed from the gas-exposure bag (experimental) or the incubator (control) for blood collection. Experimental eggs were immediately wrapped in aluminum foil to prevent eggs being exposed to normoxia during blood collection (see Appendix B). Arterialized blood was sampled (as above) and immediately assessed for blood gas (pH_a, $[HCO_3^-]_a$, Pa_{CO_2}), hematological respiratory variables (Hct_a, $[RBC]_a$, $[Hb]_a$, mean corpuscular indices), $[La^-]$ and Osm_a (see Section 2.2).

The change in Hct_a of experimental (*e.g.*, hypoxia) eggs from the control was expressed as ΔHct to allow comparisons of a % and with other variables ([RBC] and MCV); *e.g.*, $\Delta Hct\ (in\ \%) = 100 \times (Hct_{hypoxia} - Hct_c)/Hct_c$, where Hct_c is control value and $Hct_{hypoxia}$ is the value obtained by hypoxic exposure. Likewise, changes in [RBC] and MCV were expressed by $\Delta[RBC]\ (in\ \%) = 100 \times ([RBC]_{hypoxia} - [RBC]_c)/[RBC]_c)$ and $\Delta MCV\ (in\ \%) = 100 \times (MCV_{hypoxia} - MCV_c)/MCV_c$, 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 $[O_2]$ at 0%CO_2 or 5%CO_2 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 O_2 at either 0%CO_2 ($N = 54$) or 5%CO_2 ($N = 48$). 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 $[CO_2]$ groups. Although there was no group difference in initial blood pH prior to tonometry (pH_a 7.60 ± 0.01 for both groups), $[HCO_3^-]_a$ and Pa_{CO_2} were slightly higher in the 0%CO_2 group (29.0 ± 0.5 mmol L^{-1} and 29.3 ± 0.5 mmHg) prior to tonometry compared with the 5%CO_2 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 Osm_a (275 ± 1 *cf.* 276 ± 1 mmol kg^{-1}, $P = 0.133$) or Hct_a (28.5 ± 0.3 *cf.* 28.0 ± 0.3%, $P = 0.162$). Further, no control parameter varied significantly amongst the six $[O_2]$ groups. Because the experiment with 5%CO_2 was made later on eggs laid by a different flock of hens, different control acid–base status between 0% and 5%CO_2 groups might be attributed to the difference of flocks.

After tonometry, significant differences in Hct_{eq} existed between the two $[CO_2]$ groups and amongst the six $[O_2]$ groups (Fig. 1). The mean ΔHct for 0%CO_2 group (across all O_2 levels) was −2.6 ± 0.4% ($N = 54$) compared with a mean of 2.4 ± 0.5% ($N = 48$) for the 5%CO_2 group ($P < 0.001$). The ΔHct at 0%O_2 at both $[CO_2]$ groups (8.7 ± 0.8%) was significantly larger than the ΔHct at 10–100%O_2 (*e.g.*, −0.9 ± 0.8% at 10%O_2) (Fig. 1).

Equilibration of the blood to 0%CO_2 resulted, as expected from the previous study (Andrewarsa et al., 2011b), in a large increase in pH from pH_a 7.60 ± 0.01 to pH_{eq} 8.31 ± 0.02 ($P < 0.001$) with no difference amongst the six O_2 groups ($P = 0.804$). Similarly, there were no differences in $[HCO_3^-]$ between O_2 groups ($P = 0.353$) during equilibration to 0%CO_2. $[HCO_3^-]$ decreased sharply from $[HCO_3^-]_a = 29.0 \pm 0.5$ mmol L^{-1} to $[HCO_3^-]_{eq} = 20.0 \pm 0.8$ mmol L^{-1} ($P < 0.001$). Accordingly, a comparable degree of respiratory alkalosis occurred in all O_2 groups irrespective of concentrations. However, the ΔHct of blood equilibrated to 0%O_2 (7.6 ± 0.8%) was significantly different (larger) from ΔHct at $[O_2] > 10\%$ (*e.g.*, −4.3 ± 0.8% at 10%O_2) (Fig. 1).

Equilibration of blood with 5%CO_2 resulted in significant decrease from pH_a 7.60 ± 0.01 to pH_{eq} 7.53 ± 0.01 ($P < 0.001$) with

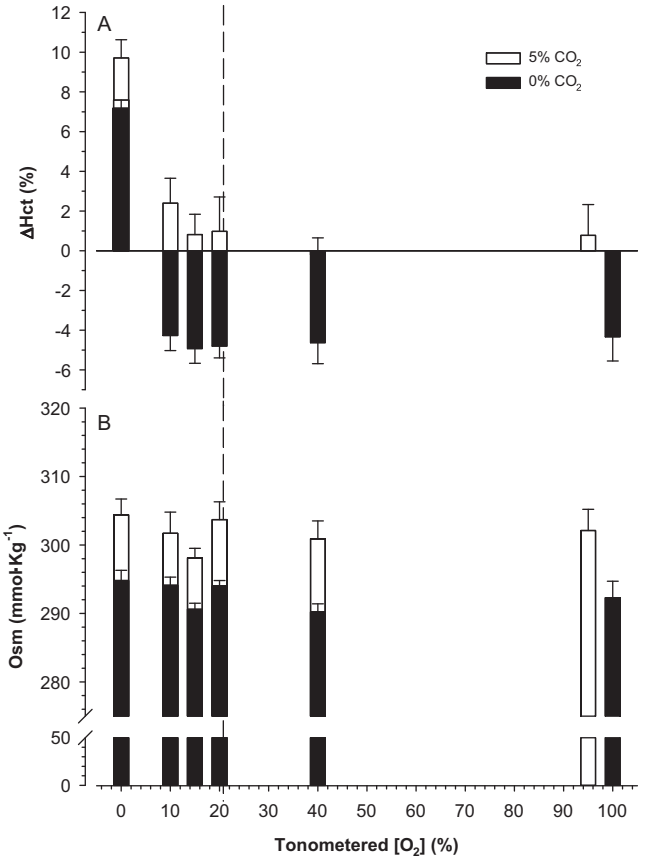

Fig. 1. (A) *In vitro* change in hematocrit (Hct) from *in vivo* arterialized values ($\Delta Hct = 100 \times (Hct_{eq} - Hct_a)/Hct_a$) and (B) osmolality (Osm) across a range of $[O_2]$ (%) and $[CO_2]$ (%) in blood of d15 chicken embryos. Positive and negative ΔHct values indicate an increase and decrease, respectively, in Hct values from *in vivo* values. The horizontal solid line indicates ΔHct = 0 and the vertical dashed line indicates normoxia (20.9%O_2). Mean values ± 1 S.E.M. are presented. $N = 9$ embryos for each $[O_2]$ except $N = 7$ for 10, 15 and 40%O_2 at 5%CO_2.

no difference amongst the six $[O_2]$ groups ($P = 0.674$). There were no differences in $[HCO_3^-]$ between O_2 groups ($P = 0.367$) during equilibration to 5%CO_2. Further, there was no difference between initial arterial $[HCO_3^-]_a$ (25.5 ± 0.4 mmol L^{-1}) and $[HCO_3^-]_{eq}$ after equilibration to 5%CO_2 (26.3 ± 0.6 mmol L^{-1}) ($P = 0.256$). Equilibration to 5%CO_2 resulted in a similar state of respiratory acidosis in all O_2 groups irrespective of concentrations. Again, ΔHct at 0%O_2 (9.7 ± 0.9%) was significantly different (larger) from ΔHct at $[O_2] > 10$% (*e.g.*, 2.4 ± 1.3% at 10%O_2) (Fig. 1).

Equilibration of the blood to a range of O_2 at 0%CO_2 resulted in a difference in Osm which changed from $Osm_a = 275 \pm 1$ mmol kg^{-1} to $Osm_{eq} = 293 \pm 1$ mmol kg^{-1} ($P < 0.001$) with no Osm_{eq} differences between the six O_2 groups ($P = 0.080$) (Fig. 1). Similarly, equilibration at 5%CO_2 increased Osm from $Osm_a = 276 \pm 1$ mmol kg^{-1} to $Osm_{eq} = 302 \pm 1$ mmol kg^{-1} ($P < 0.001$) with no difference in Osm_{eq} between the six O_2 groups ($P = 0.488$) (Fig. 1).

3.2. Blood buffer value

The blood buffer value of d13 embryos was described by the equation $[HCO_3^-]_a = 156.3–17.9 \times pH_a$ ($r = 0.997$, $N = 48$). For d15 embryos, $[HCO_3^-]_a = 144.4–15.8 \times pH_a$ ($r = 0.998$, $N = 48$) and for d17, $[HCO_3^-]_a = 140.5–14.4 \times pH_a$ ($r = 0.994$, $N = 54$). 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
Fresh egg and embryo body mass, and *in vivo* blood gas variables (pH_a, Pa_{CO_2}, $[HCO_3^-]_a$), hematocrit (Hct_a) and osmolality (Osm_a) of control chicken embryos on days 13, 15 and 17 of incubation.

Incubation day	13	15	17
Egg mass (g)	57.46 ± 0.36	57.91 ± 0.42	58.45 ± 0.40
Body mass* (g)	6.52 ± 0.07	12.18 ± 0.12	19.12 ± 0.18
pH_a*	7.671 ± 0.003	7.588 ± 0.004	7.559 ± 0.004
Pa_{CO_2}* (mmHg)	17.5 ± 0.2	28.4 ± 0.4	34.9 ± 0.5
$[HCO_3^-]_a$* (mEq L^{-1})	20.5 ± 0.2	27.2 ± 0.3	31.0 ± 0.3
Hct_a* (%)	23.4 ± 0.2	27.4 ± 0.2	30.9 ± 0.3
Osm_a (mmol kg^{-1})	265 ± 1	268 ± 1	266 ± 1
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 egg 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, Osm_a was statistically similar between the 3 age-groups of control embryos, averaging 266 ± 0.4 mmol kg^{-1} ($P = 0.055$). However, pH_a decreased by 0.11 unit, while Pa_{CO_2} approximately doubled from d13 (~18 mmHg) to d17 (~35 mmHg) ($P < 0.001$ for both variables). Consequently, $[HCO_3^-]_a$ increased by ~11 mmol L^{-1} across the same developmental period. Additionally, Hct_a 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%CO_2, 20%O_2) for the last day of 13, 15 or 17 days of incubation resulted in a significant decrease in Hct_a in embryos of all three ages compared with controls (Fig. 2). No concurrent changes occurred in Osm_a, which averaged 264 ± 1, 266 ± 1 and 266 ± 1 mmol kg^{-1} in d13, d15 and d17 embryos, respectively. Hct_a decreased with ΔHct values of 14%, 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, Pa_{CO_2} increased by 23.0 mmHg, 27.4 mmHg and 30.4 mmHg, while pH_a decreased by 0.14, 0.11 and 0.11 unit in d13, d15 and d17 embryos, respectively (Fig. 4). $[HCO_3^-]_a$ 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 $[HCO_3^-]_a$ 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%CO_2, 15%O_2) 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.

	Age (d)	Exp	Egg mass g	Wet body mass g	$[La^-]$ mmol L^{-1}	Osm mmol kg^{-1}	[Hb] g%	MCH pg	[MCHb] g%
Hypercapnia (5%CO_2 20%O_2)	13	C	57.97 ± 0.68 (36)	6.58 ± 0.17 (22)	1.4 ± 0.14 (10)	264 ± 2 (36)	7.4 ± 0.1 (28)	46.2 ± 0.4 (28)	31.5 ± 0.2 (28)
		E	58.44 ± 0.64 (36)	6.24 ± 0.21 (22)	**1.0 ± 0.05 (10)**	263 ± 1 (36)	**6.5 ± 0.1 (29)**	46.6 ± 0.4 (29)	**32.4 ± 0.2 (29)**
	15	C	59.14 ± 0.74 (40)	11.64 ± 0.30 (22)	0.9 ± 0.08 (10)	266 ± 1 (40)	9.9 ± 0.2 (29)	45.3 ± 0.5 (29)	33.9 ± 0.4 (29)
		E	58.89 ± 0.72 (38)	12.20 ± 0.25 (22)	**0.7 ± 0.03 (10)**	266 ± 1 (38)	**8.4 ± 0.1 (29)**	46.1 ± 0.5 (29)	**35.3 ± 0.4 (29)**
	17	C	59.18 ± 0.78 (38)	18.64 ± 0.34 (20)	1.3 ± 0.11 (10)	267 ± 2 (28)	10.4 ± 0.2 (32)	43.6 ± 0.4 (32)	33.0 ± 0.4 (32)
		E	58.17 ± 0.84 (37)	19.25 ± 0.55 (20)	**0.8 ± 0.02 (10)**	266 ± 2 (37)	**9.5 ± 0.2 (31)**	44.6 ± 0.3 (31)	**35.2 ± 0.4 (31)**
Hypercapnic hypoxia (5%CO_2 15%O_2)	13	C	56.38 ± 0.71 (41)	5.83 ± 0.14 (21)	1.9 ± 0.28 (21)	263 ± 1 (41)	7.0 ± 0.1 (21)	45.9 ± 0.6 (21)	31.3 ± 0.2 (11)
		E	58.72 ± 0.72 (41)	5.61 ± 0.16 (20)	**1.3 ± 0.09 (20)**	263 ± 1(41)	6.9 ± 0.2 (20)	**44.8 ± 1.0 (20)**	**29.7 ± 0.7 (20)**
	15	C	56.80 ± 0.87 (29)	11.62 ± 0.32 (21)	0.9 ± 0.04 (21)	265 ± 1 (29)	9.2 ± 0.2 (21)	45.9 ± 0.7 (21)	34.3 ± 0.3 (21)
		E	57.87 ± 0.80 (31)	**10.82 ± 0.22 (21)**	**0.8 ± 0.03 (21)**	266 ± 1 (31)	9.7 ± 0.2 (21)	**43.5 ± 0.5 (21)**	**30.7 ± 0.3 (21)**
	17	C	57.72 ± 0.82 (35)	18.36 ± 0.49 (20)	1.1 ± 0.07 (20)	264 ± 1 (35)	10.4 ± 0.2 (20)	44.0 ± 0.5 (20)	34.6 ± 0.2(20)
		E	59.25 ± 0.81 (35)	**16.59 ± 0.40 (20)**	**0.9 ± 0.04 (20)**	264 ± 1 (35)	10.2 ± 0.2 (20)	43.7 ± 0.3 (20)	**29.7 ± 0.2(20)**
Hypoxia (0%CO_2 15%O_2)	13	C	58.18 ± 0.69 (37)	6.23 ± 0.17 (20)	1.3 ± 0.10 (20)	264 ± 1(37)	7.4 ± 0.2 (20)	45.6 ± 0.5 (20)	31.5 ± 0.3 (20)
		E	58.32 ± 0.58 (41)	6.21 ± 0.16 (20)	**2.9 ± 0.38 (20)**	264 ± 1(41)	7.4 ± 0.2 (20)	**43.4 ± 0.5 (20)**	**28.9 ± 0.3 (20)**
	15	C	57.11 ± 0.99 (28)	11.70 ± 0.36 (19)	1.0 ± 0.05 (19)	270 ± 2 (28)	9.4 ± 0.2 (19)	45.0 ± 0.5 (19)	34.8 ± 0.2 (19)
		E	57.48 ± 0.84 (29)	**10.71 ± 0.25 (20)**	**1.8 ± 0.13 (20)**	267 ± 2 (29)	9.5 ± 0.2 (20)	**42.4 ± 0.5 (20)**	**31.1 ± 0.2 (20)**
	17	C	58.30 ± 0.86 (37)	17.74 ± 0.47 (21)	1.0 ± 0.07 (21)	268 ± 2 (37)	10.5 ± 0.3 (21)	44.2 ± 0.6 (21)	34.2 ± 0.5(21)
		E	58.93 ± 0.87 (39)	16.90 ± 0.41 (21)	**2.1 ± 0.21 (21)**	269 ± 1 (39)	10.5 ± 0.3 (21)	**42.0 ± 0.5 (21)**	**30.9 ± 0.2(21)**
Hyperoxia (0%CO_2 40%O_2)	13	C	57.42 ± 0.76 (24)	6.51 ± 0.21 (20)	1.1 ± 0.04 (10)	271 ± 2 (24)	7.4 ± 0.1 (24)	48.9 ± 0.7 (24)	31.3 ± 0.2 (24)
		E	57.29 ± 0.88 (26)	6.36 ± 0.20 (21)	1.1 ± 0.07 (10)	267 ± 2 (26)	**6.8 ± 0.1 (26)**	48.9 ± 0.5 (26)	31.2 ± 0.2 (26)
	15	C	58.06 ± 0.78 (38)	12.21 ± 0.21 (28)	0.9 ± 0.07 (13)	269 ± 1 (38)	9.6 ± 0.2 (28)	46.9 ± 0.7 (28)	34.7 ± 0.2 (28)
		E	57.95 ± 0.64 (40)	12.58 ± 0.22 (27)	0.9 ± 0.10 (12)	270 ± 2 (40)	**9.0 ± 0.2 (27)**	47.7 ± 0.5 (27)	**36.5 ± 0.2 (27)**
	17	C	58.50 ± 0.66 (30)	18.93 ± 0.32 (30)	1.2 ± 0.10 (10)	268 ± 2(30)	10.8 ± 0.2 (30)	45.1 ± 0.6 (30)	34.6 ± 0.3 (30)
		E	59.31 ± 0.66 (30)	**20.09 ± 0.34 (30)**	1.3 ± 0.11 (10)	268 ± 2(30)	**9.9 ± 0.2 (30)**	45.6 ± 0.5 (30)	**37.1 ± 0.2 (30)**

Values are mean ± 1 S.E.M. (*N*). Bold values indicate a significant experimental (E) difference from control (C).

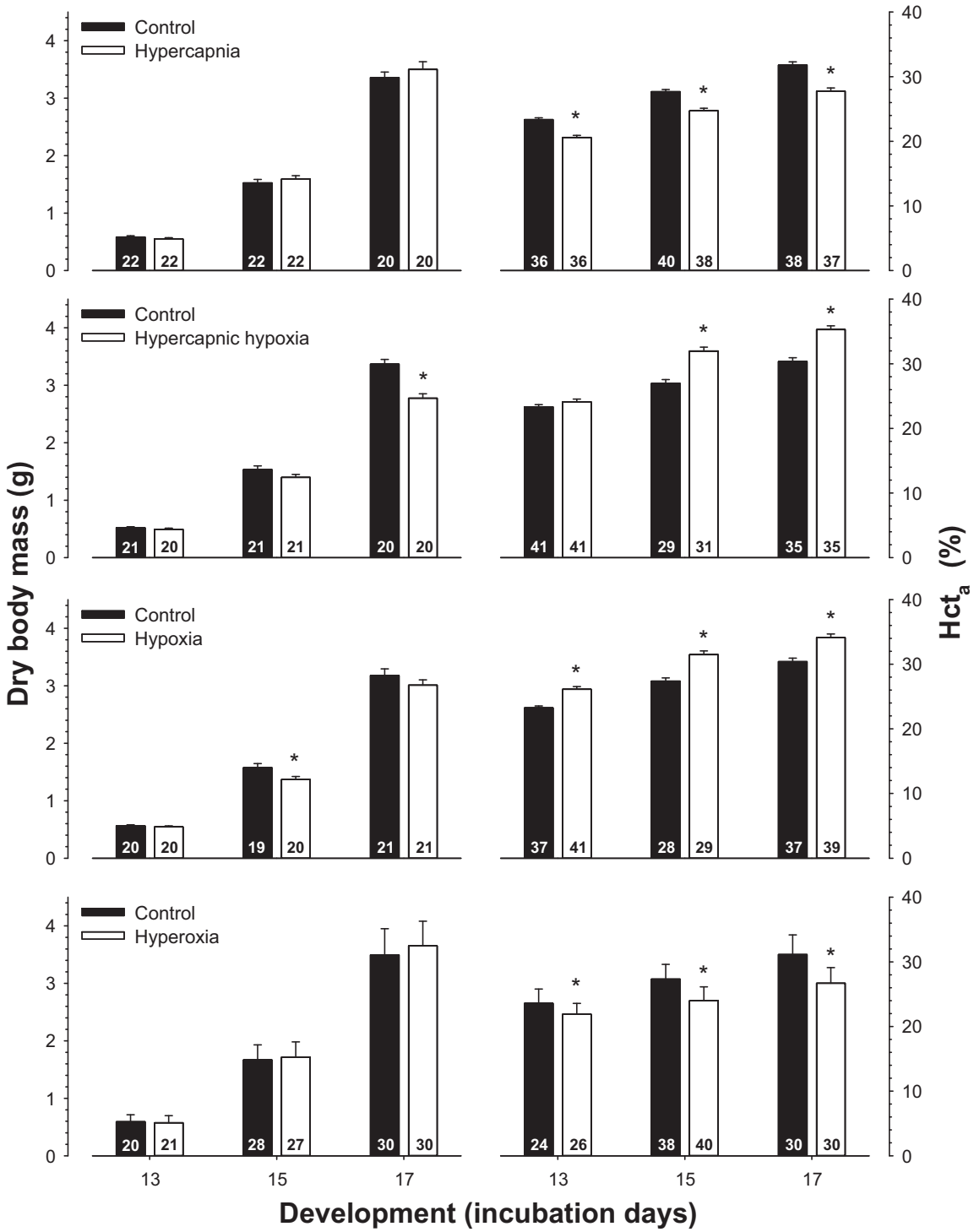

Fig. 2. Dry body mass and arterialized hematocrit (Hct_a) of embryos exposed to air (control) or altered [CO_2] and/or [O_2] for the last day of 13, 15 or 17 days of incubation. Mean values ± 1 S.E.M. are presented and *N* values are reported at the bottom of the bars.

values of ~17% and 14% in d15 and d17 embryos, respectively (Fig. 2). No concurrent changes in Osm_a 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 Pa_{CO_2} and decreased pH_a by 0.13 unit in all embryos (Fig. 4). The subsequent increase in [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 [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%.

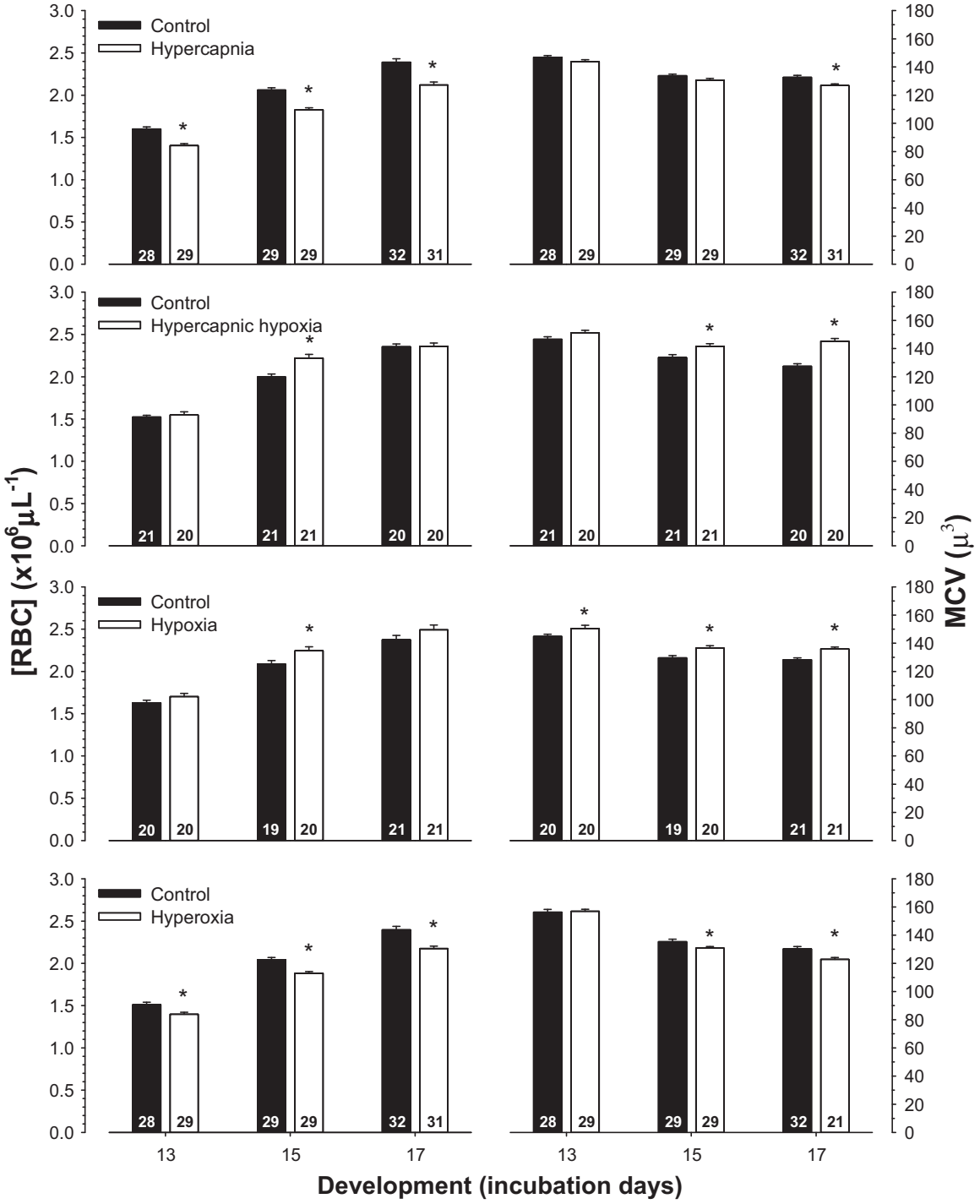

Fig. 3. [RBC] and MCV of embryos exposed to air (control) or altered [CO_2] and/or [O_2] for the last day of 13, 15 or 17 days of incubation. Mean values ± 1 S.E.M. are presented and *N* values are reported at the bottom of the bars.

3.3.4. Hypoxia

A reduction in both wet and dry body mass was only observed in d15 embryos exposed to hypoxia (0%CO_2, 15%O_2) for 1 day (Fig. 2, Table 2). Hct significantly increased in all embryos exposed to hypoxia with no concurrent alterations in Osm_a 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_a decreased with ΔpH 0.04, 0.08 and 0.07 units in d13, d15 and d17 embryos, respectively. There was no significant change in Pa_{CO_2}. Consequently, [HCO_3^-]$_a$ decreased significantly in d15 and d17 embryos, indicating metabolic acidosis (Fig. 4).

3.3.5. Hyperoxia

Hyperoxia (0%CO_2, 40%O_2) increased wet body mass of d17 embryos only (Table 2), but did not alter dry body mass at any

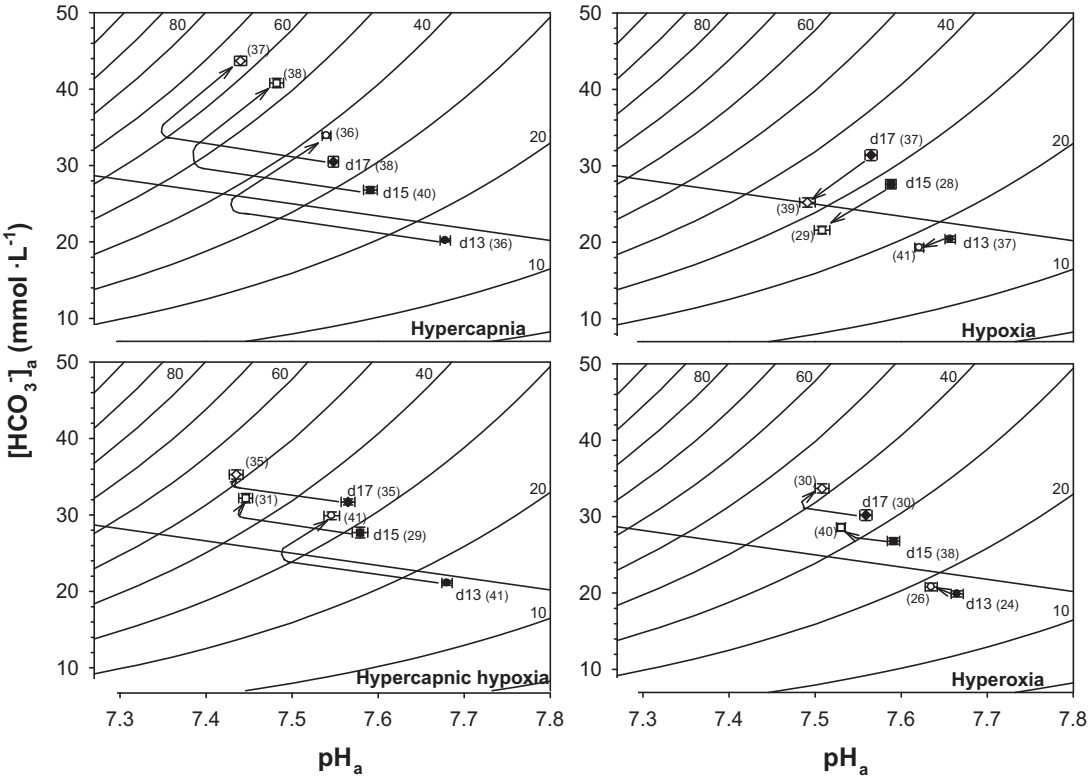

Fig. 4. Davenport diagram illustrating acid–base balance (pH_a and $[HCO_3^-]_a$) of arterialized blood of chicken embryos exposed to air (control) or altered $[CO_2]$ and/or $[O_2]$ for the last day of 13, 15 or 17 days of incubation. The solid arrows indicate changes in blood acid–base status from control. Numbered P_{CO_2} are isopleths, in mmHg. The solid regression is an arbitrary buffer line with the determined mean slope of −16 mmol L^{-1} pH $unit^{-1}$ across the three developmental days. Mean values ± 1 S.E.M. are presented and N values are reported in parentheses.

age (Fig. 2). Hct decreased (ΔHct = 7%, 11% and 14% in d13, d15 and d17 embryos, respectively) without changes in Osm_a or $[La^-]$ (Fig. 2, Table 2). [RBC] decreased by 7%, 8% and 9% in d13, d15 and d17 embryos, respectively. MCV decreased by 3% and 5% in d15 and d17 embryos and remained unaltered in d13 embryos (Fig. 3). [Hb] decreased by ~6–8% in all embryos with MCH remaining unchanged, whereas [MCHb] increased in d15 and d17 embryos (Table 2).

pH_a decreased with ΔpH 0.03, 0.06 and 0.05 units in d13, d15 and d17 embryos, respectively. This was accompanied by an increase in $[HCO_3^-]$ which was similar to the value predicted from the buffer value in d13 and d15 embryos and slightly larger than predicted in d17 embryos (Fig. 4). Concurrently, Pa_{CO_2} increased by 2, 7 and 9 mmHg, respectively. Thus, hyperoxia induced a respiratory acidosis in all embryos which was uncompensated in d13 and d15 embryos and partially compensated by metabolic alkalosis in d17 embryos (Fig. 4).

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 Pco_2 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–$[HCO_3^-]$ diagram ("Davenport diagram"), which requires quantification of both an embryo buffer line and P_{CO_2} 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^{-1} for embryos from d14 to d18 (Tazawa et al., 1983). Hemoglogin, 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%CO_2, 20%O_2) increased Pa_{CO_2} and decreased pH_a 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 CO_2 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%O_2/80%SF_6 atmosphere (Tazawa et al., 1981) and in embryos exposed to a range of [CO_2] (4, 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%CO_2) 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%CO_2, 15%O_2) 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 O_2 level is required for metabolic compensation to occur. Potentially, lower $\dot{M}_{O_2}$ (*i.e.*, less O_2 leaving the circulation) and an overall higher allantoic Pa_{O_2} (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 HCO_3^- 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 [HCO_3^-] 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 HCO_3^- 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 HCO_3^- (within 4–6 h, H. Tazawa, unpublished data) makes eggshell re-absorption an unlikely candidate, in our view. More likely, the elevated P_{CO_2} results in increased hydration of CO_2 in the blood. This will increase the [H^+] (which is excreted from the blood) and [HCO_3^-] which accumulates in the blood. The chorioallantoic membrane (CAM) actively transports Na^+ from the allantoic fluid to the blood (Stewart and Terepka, 1969; Boutilier 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 $\dot{M}_{O_2}$ cannot be maintained for active transport).

Potentially, down-regulated $\dot{M}_{O_2}$ (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 HCO_3^- 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, $\dot{M}_{O_2}$ is reduced without anaerobic energy compensation (Bjønnes et al., 1987; Mortola and Besterman, 2007). However, once a lower threshold $\dot{M}_{O_2}$ is reached, embryos turn to anaerobic glycolysis and the blood lactate concentration increases (Grabowski, 1961, 1966; Bjønnes et al., 1987). Although d11 and d16 embryos do not incur an O_2 debt during 40 min hypoxic exposure (Mortola and Besterman, 2007), longer exposure (such as the 1 day in this study) resulted in $\dot{M}_{O_2}$ reaching the lower threshold and the induction of anaerobic respiration causing an accumulation of lactate and ensuing decrease in pH_a. However, the increase in [La^-] of ~1 mmol L^{-1} only accounts for ~1/6th of the decrease in [HCO_3^-] (~6 mmol L^{-1}) in the present study (Table 2). Hypoxic exposure (1 day) in d13 embryos did not result in metabolic acidosis (Fig. 4; Tazawa, 1986) likely due to the lower $\dot{M}_{O_2}$ requirements of younger embryos resulting in a lower (unreached in this study) threshold $\dot{M}_{O_2}$ 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). $\dot{M}_{O_2}$ (and consequently $\dot{M}_{CO_2}$) is increased when embryos are exposed to hyperoxia (Piiper et al., 1980; Visschedijk et al., 1980; Høiby et al., 1983; Stock et al., 1985; Tazawa et al., 1992). Increased CO_2 is accumulated in the blood, due to a fixed gas conductance of the eggshell, and hydrated to create H^+ and HCO_3^- 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 $\dot{M}_{O_2}$ 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 $\dot{M}_{O_2}$ 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 O_2 and CO_2

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%CO_2 produced the same acid–base status (*i.e.*, comparable degree of respiratory alkalosis) between all O_2 groups irrespective of [O_2], the ΔHct of blood equilibrated to 0%O_2 differed significantly from ΔHct equilibrated to >10%O_2. Similarly, equilibration to 5%CO_2 resulted in the same acid–base status between all O_2 groups irrespectively of [O_2], but the ΔHct of blood equilibrated to 0%O_2 was larger than the ΔHct of blood equilibrated to >10%O_2. Consequently, Hct_{eq} was not influenced by pH and [HCO_3^-] *in vitro*. Nonetheless, [O_2] did play an important role. Hct_{eq} was greatest after anoxic equilibration (0%O_2) irrespective of the presence or absence of CO_2 (Fig. 1). There was no change in Hct when blood was equilibrated to any gas mixture containing O_2 (10–100%O_2) in the absence of CO_2 (0%CO_2). Equilibration with 5%CO_2 mitigated the decreases in MCV, resulting in a smaller ΔHct which was similarly unchanged by increases in >10%O_2 (Fig. 1). Thus, the RBCs likely hydrate in anoxia and dehydrate in the presence of O_2. These alterations occurred without changes in blood osmolality. In anoxia, RBC hydration was augmented by the addition of CO_2 and, conversely, the dehydration of the RBCs was mitigated by the addition of CO_2. These *in vitro* results suggest that it may be possible for Hct to increase *in vivo* through increased MCV in embryos with low Pa_{O_2}. Additionally, this could account for a constant low Hct (due to decreased MCV) across a wide range of high Pa_{O_2}. Indeed, Hct increased *in vivo* through increased MCV in hypoxic environments irrespective of [CO_2]. MCV decreased in d15 and d17 embryos in hyperoxia, however, likely due to dehydration in the presence of O_2.

When interpreting these results, it is important to bear in mind that an arterialized P_{CO_2} = 28 mmHg (~4%) is normocapnic for the blood of d15 embryos (Table 1). Thus, equilibration with 5%CO_2 is not expected to alter MCV, and hence Hct, significantly from *in vivo* arterialized values, as observed. Altering P_{CO_2}, in this case below

normocapnia *in vivo* values (*i.e.* 0%CO_2) resulted in changes in Hct through MCV. Thus, the Hct changes during altered P_{CO_2} (*i.e.*, hypercapnia) *in vivo* are potentially the result of MCV. Further, alterations in P_{O_2} above normoxia did not elicit changes in MCV (and Hct) at 5%CO_2 (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 CO_2, 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, O_2 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 embryo age. Chronic exposure to 15%O_2 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%O_2) 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 O_2 requirements (Burggren et al., 2000, 2004).

Respiratory acidosis occurred in all embryos exposed to all experimental gas mixtures excepting 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; Nikinmaa, 1992; Hoffmann et al., 2009 for review). The general trend of decreased pH_a 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 1.88×10^6 μL^{-1} (Fig. 3). In addition, the MCV of advanced embryos (d15 and 17) decreased. In d15 embryos, for example, MCV decreased from 135μ^3 to 131μ^3 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 O_2 levels did not elicit changes in MCV (and Hct) at 5%CO_2 (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_{O_2}, P_{CO_2} and pH (see Nikinmaa, 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 O_2 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 O_2 carrying capacity when RBCs are sequestered, *i.e.*, a match of potential to supply with demand. Using the mean MCV of 133μ^3 (MCV decreased from 135μ^3 in control to 131μ^3 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 (ΔHct = ~13–15%) when d12–16 embryos were exposed to hypercapnia (5%CO_2, 20%O_2) for 1 day (Fig. 2). Similar to hyperoxia, a decrease in [RBC] (Δ[RBC] = ~11%) was predominantly responsible with a small contribution of MCV only in d17 embryos (ΔMCV = 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 $\dot{M}_{O_2}$ 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, 2009 for review). In neonatal mammals, this reduction in $\dot{M}_{O_2}$ 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 $\dot{M}_{O_2}$ 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 mmHg; ~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 *in ovo* hypoxia exposure produced no difference in d21 (externally pipping) embryo and hatchling 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 $\dot{M}_{O_2}$ and that the supply of O_2 was sufficient to meet demand ($\dot{M}_{O_2}$). 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 for each) and no difference between control and experimental egg mass existed at either d0 (57.02 ± 1.07 g for control *cf.* 56.85 ± 1.39 g for experimental, t = 0.010, P = 0.922) or d14 (55.53 ± 1.04 g for control *cf.* 52.88 ± 1.26 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 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 ~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.

Variable	Control	Ventilated at 600 mL min^{-1}	P
Water loss from egg d14–15 (g)	0.46 ± 0.02	0.59 ± 0.04	0.005
Embryo wet mass (g)	13.43 ± 0.46	14.07 ± 0.35	0.278
Embryo dry mass (g)	1.99 ± 0.11	2.19 ± 0.10	0.215
[La^-] (mmol L^{-1})	0.9 ± 0.1	1.0 ± 0.1	0.343
Osm (mmol kg^{-1})	259 ± 1	260 ± 1	0.838
pH_a	7.541 ± 0.007	7.555 ± 0.011	0.185
$[HCO_3^-]_a$	28.6 ± 1.1	28.9 ± 0.7	0.815
P_aco_2	33.6 ± 1.5	32.6 ± 1.6	0.652
Hct (%)	29.5 ± 1.1	29.1 ± 0.6	0.744
[RBC] (x10^6 μL)	2.09 ± 0.06	2.13 ± 0.04	0.574
MCV (μ^3)	141 ± 3	137 ± 2	0.150
[Hb] (g%)	9.4 ± 0.2	9.5 ± 0.2	0.779
MCH (pg)	45.0 ± 0.5	44.5 ± 0.3	0.413
[MCHb] (g%)	32.0 ± 0.5	32.6 ± 0.3	0.305

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%CO_2, 20%O_2) 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 (P_{CO_2}, pH, [HCO_3^-] and P_{O_2}), 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 P_{CO_2} (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 [HCO_3^-] (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 P_{O_2} and increased P_{CO_2} with unchanged [HCO_3^-], Hct and Osm compared with both control and aluminum wrapped embryos (Fig. 5). CO_2 is highly soluble and relatively large amounts are stored within the gas spaces of eggs. Elimination of CO_2 stored in the gas-filled spaces in dead d19 embryos exposed to pure N_2 lasted for ~10 min before the elimination of CO_2 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, O_2 was depleted from the blood and CO_2 accumulated increasing P_{CO_2} by ~11 mmHg (Fig. 5). This highlights the greater efficiency of gas exchange due to blood perfusion of the CAM in contrast to CO_2 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 O_2 was depleted and less CO_2 accumulated in the blood. Consequently, blood pH was maintained at a level similar to control (Fig. 5).

Hypercapnic exposure (5%CO_2, 20%O_2) 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.

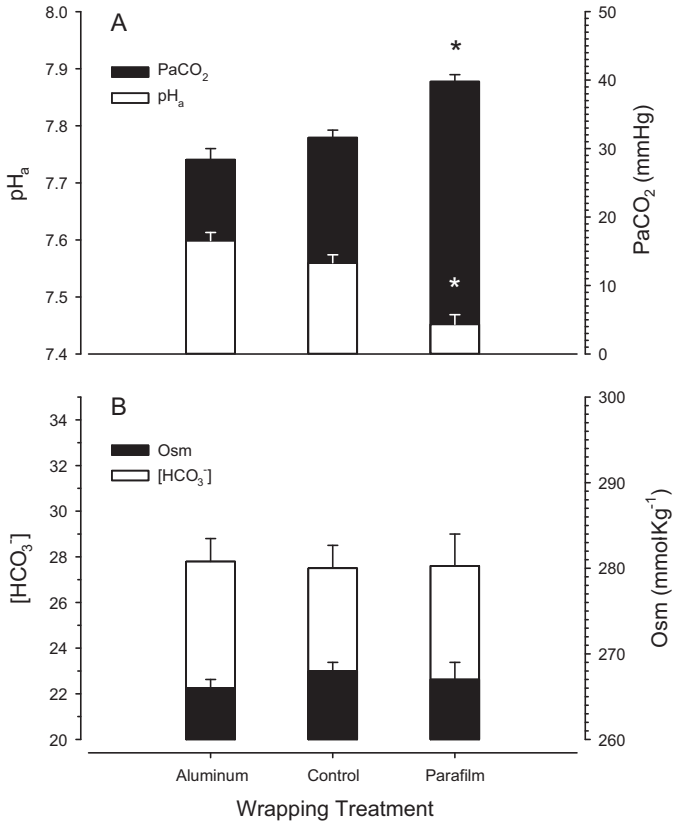

Fig. 5. Effect of eggshell asphyxiation with aluminum foil and parafilm on arterialized blood (A) pH_a and Pa_{CO_2} and (B) $[HCO_3^-]_a$ and Osm_a in d15 chicken embryos. Mean values ± 1 S.E.M. are presented ($N = 13$).

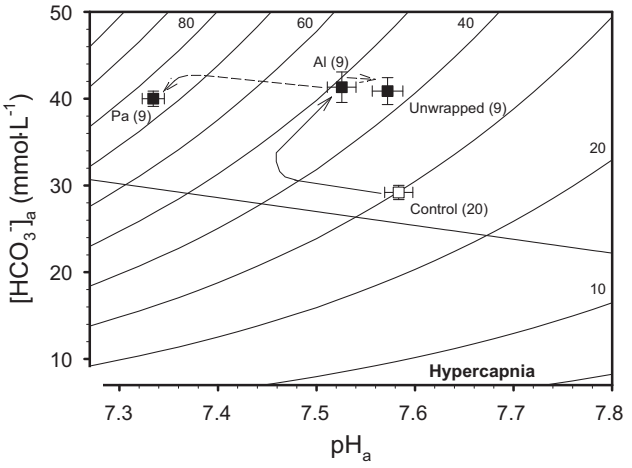

Fig. 6. Davenport diagram of arterialized blood acid–base balance (pH_a and $[HCO_3^-]_a$) of d15 chicken embryos exposed to air (control) and embryos wrapped with either parafilm (Pa) or aluminum foil (Al) for arterialized blood sampling after exposure to hypercapnia (5%CO_2). The solid arrow indicates the alteration in blood acid–base status for aluminum-wrapped eggs (most representative of hypercapnic exposure). The dashed arrows indicate the further alterations that occur after hypercapnic exposure if eggs are wrapped in parafilm (Pa) or unwrapped. Numbered P_{CO_2} isopleths are in mmHg. The solid regression is an arbitrary buffer line with the determined mean slope of -16 mmol L^{-1} pH $unit^{-1}$ across the three developmental days. Mean values ± 1 S.E.M. are presented and *N* values are reported in parentheses.

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 $[CO_2]$ and $[O_2]$.

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