Hypercapnic thresholds for embryonic acid-base metabolic compensation and hematological
regulation during CO₂ challenges in layer and broiler chicken strains

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ABSTRACT

Time specific acid-base metabolic compensation and responses of hematological respiratory variables were measured in day 15 layer (Hyline) and broiler (Cornish Rock) chicken embryos during acute hypercapnic challenges (3, 6, 10 and 20% CO₂). Control acid-base status and hematology differed between two strains. Broiler embryos were relatively respiratory acidotic and had higher hematocrit (Hct) and hemoglobin concentration. The partial metabolic compensation for respiratory acidosis produced by $\leq 10\%$ CO₂ exposures occurred in proportion to CO₂ concentrations in both strains, but metabolic compensation for 20% CO₂ respiratory acidosis was depressed at 2, 6 and 24 h, particularly in broiler embryos. Exposure to $\leq 10\%$ CO₂ induced the same hematological responses across CO₂ concentrations; i.e., Hct and mean corpuscular volume (MCV) increased while RBC concentration remained unchanged. In response to 20% CO₂ exposure, Hct and MCV increased dramatically in both strains. Consequently, altered acid-base and hematology responses to 20% CO₂ exposure compared to $\leq 10\%$ CO₂ suggest that the hypercapnic threshold to compensation for acidosis and regulation of hematology is $>10\%$ CO₂.

Keywords: Acid-base balance, Chicken embryo, Hematological respiratory variables, Hypercapnic threshold, Metabolic compensation, Respiratory acidosis

1. Introduction

Gas exchange of an avian embryo developing inside its egg takes place by molecular gas diffusion through a porous eggshell between the environment and the blood in the chorioallantoic capillaries. Consequently, the diffusion of respiratory gases is governed by gas conductance of the eggshell. In chickens, the eggshell gas conductance is quite variable between eggs, causing large variations of P_{CO_2} in the air cell, and thus blood P_{CO_2} (Tazawa et al., 1983a; Visschedijk et al., 1985). In addition, some bird species lay eggs in burrow nests where embryos can experience a hypercapnic and hypoxic environment (White et al., 1978; Wickler and Marsh, 1981; Boggs et al., 1983). Therefore, in general, it is presumed that avian embryos should be able to demonstrate a high tolerance to hypercapnic environments. Indeed, exposures to 5-20% CO_2 has been used as an investigative tool in studies of reserve capacity of physiological functions of late chicken embryos (Dawes and Simkiss, 1971; Tazawa, 1981b; Andrewartha et al., 2011; Burggren et al., 2012; Tazawa et al., 2012; Mueller et al., 2013, 2014b). Unlike respiratory acidosis experienced in pulmonary breathers, the respiratory acidosis that bird embryos experience in altered CO_2 environments cannot be compensated by convective ventilation. Instead, metabolic compensation due to an increase in bicarbonate concentration ($[HCO_3^-]$) alone functions efficiently for embryos if they are exposed to $\leq 10\%$ CO_2 . Embryos of Lohmann White Leghorn chicken (hereafter referred to as Lohmann), subjected to environmental hypercapnia, were capable of regulating respiratory acidosis with partial metabolic compensation during exposures to $\leq 10\%$ CO_2 on days 14-15 (d14-15) of incubation (Mueller et al., 2014b). Partial metabolic compensation during 24 h exposure occurred in proportion to CO_2 concentration ($[CO_2]$), and thus percent compensation was the same irrespective of $[CO_2]$. Similarly, responses of hematological respiratory variables typically occurred independently of environmental $[CO_2] \leq 10\%$ (Mueller et al., 2014b). However, there were slight but significant differences in how red blood cell volume (mean corpuscular volume, MCV) and red blood cell concentration ($[RBC]$) contributed to the decrease in hematocrit (Hct) when embryos were exposed to 1 – 6% CO_2 compared to 10% CO_2 . Likewise, the change in mean

corpuscular hemoglobin concentration ([MCHb]) was different between 1 - 6% CO₂ and 10% CO₂ during early period after the onset of exposure. Hence, a major change in hematological regulation may occur as [CO₂] climbs above 6% and beyond.

Based on these previous findings, we hypothesized that in chicken embryos a threshold exists above 10% at which both metabolic compensation for hypercapnic respiratory acidosis and the associated response of hematological respiratory variables are modified. We examined acid-base and hematological responses to [CO₂] > 10% as well as ≤10% in an attempt to define this hypercapnic threshold. Importantly, we have also examined whether such acid-base regulation and hematological changes in response to CO₂ differed between different chicken strains. Besides the selected traits of fast growth or high egg-laying capacity, other important variables including growth and development, body temperature, oxygen consumption, heart rate and cardiovascular regulation differ between chicken strains (Janke et al., 2004; Ohta et al., 2004; Chwalibog et al., 2007; Yoneta et al., 2007; Everaert et al., 2008; Druyan, 2010; Ho et al., 2011; Crossley and Altimiras, 2012). Such differences between strains may account for variations in results reported in different studies. For instance, the reported time of onset of cholinergic chronotropic control of HR in embryos differs between studies (Höchel et al., 1998; Crossley and Altimiras, 2000; Crossly et al., 2003b; Aubert et al., 2004; Chiba et al., 2004; Yoneta et al., 2006) and may be due to the different strains used in each study (Crossley et al., 2003a, 2003b). In light of these potential physiological differences, we examined the time-specific responses of acid-base balance and hematological respiratory variables at d15 of both Hyline White Leghorn layer and Cornish Rock broiler embryos, each exposed to altered [CO₂] with 20% O₂.

2. Materials and methods

2.1. Egg incubation and exposure to hypercapnia

Eggs of the Hyline layer chicken (hereafter referred to as Hyline) were transported once a

week from a hatchery at Texas A&M University (College Station, Texas, USA) to the laboratory (University of North Texas, Denton, Texas) in April, May and June. Cornish Rock broiler eggs (hereafter referred to as broiler) were obtained weekly from a local hatchery in June and July. The eggs were lightly washed in water with a sponge to remove extraneous materials that may hinder gas exchange through the eggshell. On the day of incubation, eggs were weighed to 0.01 g on an electronic balance, large (>70 g) and small (<45 g) eggs were excluded and 30 eggs were numbered and set at 12:00 in an incubator (model 1502, G.Q.F. Manuf. Co.). Temperature and relative humidity of the incubator were kept at $37.5 \pm 0.1^\circ\text{C}$ and ~55%, respectively, and the eggs were turned automatically every 3 h.

On d13 of incubation, 30 eggs were candled to identify living embryos and an allantoic vein was marked for blood collection. On d14, viable eggs among 30 eggs were randomly divided into control eggs (not subjected to CO_2 exposure) and CO_2 -exposed eggs. All eggs were removed to a cardboard egg stand in a desk-top incubator (1588 Electr. Hova-Bator, G.Q.F. Manuf. Co.) maintained at 37.5°C . A 3.78-L gas exposure bag that could accommodate up to 16 eggs was placed within the desktop incubator. The gas exposure bag was ventilated with gas mixtures provided by a Wösthoff gas mixing pump (oHG, Bochum, Germany), as described previously (Burggren et al., 2012; Tazawa et al., 2012). Eggs were exposed to either 3, 6, 10 or 20% CO_2 with 20% O_2 , balanced by N_2 . According to the experimental schedule, gas-exposed eggs were either placed in the bag at 12:00 on d14 for 24 h exposure, or were exposed to the gas mixture for 2 or 6 h in the bag on d15 (2 or 6 h exposure). To investigate recovery from hypercapnic exposures, eggs exposed to one of the above gas mixtures for 24 h on d15 were returned from the gas exposure bag to the cardboard egg stand in the incubator, allowing recovery in air for 2 or 6 h. The above procedures were repeated during a period of 2-3 months to obtain sufficient data on embryos exposed to the four CO_2 concentrations (3, 6, 10 and 20%) at six time sequences (control (0 h), 2, 6 and 24 h exposure and 2 and 6 h recovery).

A subset of 20 fresh Hyline and broiler eggs was weighed, the yolk removed and the yolk weighed to 0.01 g. A different subset of 20 fresh eggs was boiled after egg mass was determined and

boiled yolk mass was measured to check that the determination of fresh yolk mass was accurate.

2.2. Blood collection and analysis

Blood could not be collected while the eggs were in the gas exposure bag, so we temporarily wrapped the egg in aluminum foil to preserve the blood gases immediately after removal from the exposure bag and during blood sampling which took less than 2 min (Burggren et al., 2012). Approximately 0.4 mL of allantoic vein blood was collected into a 1 mL heparinized plastic syringe. Blood was gently emptied into a 1.5 mL plastic vial and immediately analyzed for pH_a , $[\text{HCO}_3^-]_a$ (mmol L^{-1}) and P_aCO_2 (mmHg) with a blood gas system (ABL5, Radiometer Medical A/S, Denmark). Because the blood collected from the allantoic vein was arterialized by passage through the chorioallantoic capillaries (Piiper et al., 1980; Tazawa, 1980), measured variables represent arterial values (given by subscript a) corresponding to adult pulmonary venous blood. The relationship between pH_a and $[\text{HCO}_3^-]_a$ was depicted on a Davenport ($\text{pH}-[\text{HCO}_3^-]$) diagram as reported previously (Burggren et al., 2012; Mueller et al., 2014b). A buffer line was drawn on the Davenport diagram to indicate the buffer value of $-16 \text{ mmol L}^{-1} \text{ pH}^{-1}$ (Burggren et al., 2012).

The remaining blood was well stirred in the vial, and was then measured for $[\text{RBC}]$ (10^6 cells μL^{-1}) and hemoglobin concentration ($[\text{Hb}]$, g%) by a hematology analyzer (Coulter Analyzer A^c T, Beckman, USA), Hct (± 0.1 %) in duplication by a centrifuge (Readacrit Centrifuge, Becton Dickinson, USA) and osmolality (Osm, mmol kg^{-1}) by a vapor pressure osmometer (5520 Vapro, Wescor Inc., USA). Duplicate Hct measurements were averaged for each individual embryo. $[\text{RBC}]$ was determined by a Coulter Analyzer, and values corrected based on standard $[\text{RBC}]$ determinations using a hematometer (Tazawa et al., 2011). Mean corpuscular indices; MCV (μm^3), mean corpuscular hemoglobin (MCH, pg) and $[\text{MCHb}]$ (g%) were calculated from Hct, $[\text{RBC}]$ and $[\text{Hb}]$; i.e., $\text{MCV} = 10 \cdot \text{Hct}/[\text{RBC}]$, $\text{MCH} = 10 \cdot [\text{Hb}]/[\text{RBC}]$ and $[\text{MCHb}] = 100 \cdot [\text{Hb}]/\text{Hct}$. Lactate concentration ($[\text{La}^-]$, mmol L^{-1}) was determined by a Nova Lactate Plus Meter (Nova Biomedical, MA, USA).

After blood analyses, embryos were euthanized by cold exposure, removed from the shell, separated from the extra-embryonic membranes, blotted to remove excess fluid and weighed for body mass (BM) to 0.01 g on an electronic balance.

2.3. Statistical analysis.

All data were tested for normality and equal variance and parametric ANOVA or ANOVA on ranks were used where appropriate. Differences in mean values of egg mass, embryo BM, Osm and acid-base and hematological variables between controls for the two strains were examined by unpaired Student *t*-test. Differences in mean values of variables across gas exposure times and between different gas treatments were examined using a two-way ANOVA with all pairwise multiple comparisons by the Holm-Sidak test. Comparison of $[La^-]$ between different gas exposure times at each CO_2 was made by a one-way ANOVA with an unpaired Student *t*-test used for comparison between Hyline and broiler at each exposure time. The significance level was $P < 0.05$. All data were presented as mean $\pm$ 1 S.E.M.

3. Results

3.1. Fresh egg mass, yolk mass and embryonic body mass

Experiments were performed on 645 Hyline and 247 broiler embryos. Fresh egg mass was not significantly different between the two groups (Table 1). However, mean BM of d15 control embryos was significantly heavier in broiler than Hyline embryos. The ratio of BM to egg mass in control embryos was significantly larger in broiler (27.3 ± 0.4 %) compared to Hyline embryos (20.4 ± 0.2 %) ($P < 0.001$).

Mean mass of fresh and boiled yolk did not differ for either Hyline eggs (16.16 ± 0.17 g in fresh cf. 15.82 ± 0.25 g in boiled, $P = 0.273$) or broiler eggs (17.73 ± 0.31 g in fresh cf. 17.41 ± 0.32 g in boiled,

$P=0.474$). Total yolk mass was heavier in broiler eggs than Hyline (Table 1). Since fresh egg mass was not significantly different between broilers (58.98 ± 0.55 g, $N=40$) and Hyline eggs (59.27 ± 0.49 g, $N=40$) ($P=0.784$), the ratio of yolk mass to fresh egg mass was higher in broiler (29.8 ± 0.2 %) compared to Hyline eggs (27.0 ± 0.3 %) ($P<0.001$).

No mortality occurred during 20% CO_2 exposure in broiler embryos, but 3 of 44 Hyline embryos (~7%) died after 24 h of exposure to 20% CO_2 . In Hyline embryos, the effects of hypercapnia, treatment time and their interactions significantly affected BM (Table 2). At 24 h of exposure and 2 h of recovery, BM of embryos exposed to 20% CO_2 was lower than those exposed to 6% CO_2 (Table 3). In 10 and 20% CO_2 exposures, BM at 24 h of exposure and 2 h of recovery was significantly lower than that at 6 h of exposure. In broiler embryos, the effects of hypercapnia and treatment time on BM were also significant, but the interaction of these two factors was not significant (Table 2). Overall BM at all treatment times at 20% CO_2 was significantly lower than at 3% CO_2 (Table 4). Mean BM of embryos exposed to any level of hypercapnia was significantly higher at 6 h compared to at 24 h (~1.4 g) and 2 h of recovery (~1 g). Only BM at 24 h exposure was significantly reduced compared to control.

3.2. Blood osmolality

Mean blood osmolality did not significantly differ between control populations of Hyline and broiler embryos (Table 1). In Hyline embryos, the effects of hypercapnia, treatment time and their interaction on Osm were significant (Table 2). Osm changed across the time course exposure at 10 and 20% CO_2 (Fig. 1A). At 20% CO_2 exposure, Osm at 2 and 6 h was significantly higher than control and Osm at 2 and 6 h of recovery was significantly lower than control. At 10% CO_2 exposure, Osm at 6 h of recovery was significantly lower than all other treatment times. In broiler embryos, only treatment time had a significant effect on Osm (Table 2). Consequently, mean Osm was significantly higher than control during 2, 6 and 24 h of hypercapnic exposure and returned to control level during recovery (Fig. 1B).

3.3. Dynamics of acid-base responses during arange of hypercapnic challenges

3.3.1. Control acid-base status

Control variables expressing acid-base balance (pH_a , $[\text{HCO}_3^-]_a$ and P_aCO_2) differed significantly between Hyline and broiler embryos (Table 1). pH_a was lower, and both $[\text{HCO}_3^-]_a$ and P_aCO_2 were higher, in the broiler embryos.

3.3.2. Hyline layer embryos

In response to 2 h exposure to 3, 6 and 10% CO_2 (Fig.2A, B, C, respectively), pH_a in Hyline embryos decreased from controls to 7.46 (Fig.2A), 7.40 (Fig.2B) and 7.32 (Fig.2C), respectively. During the same 2 h period, $[\text{HCO}_3^-]_a$ increased by ~4, 7 and 8 mmol L^{-1} , respectively. The resultant slopes of these acid-base changes (i.e., $\Delta[\text{HCO}_3^-]_a/\Delta\text{pH}_a$) after 2 h of CO_2 exposure were ~ -39, -35 and -28 $\text{mmol L}^{-1} \text{pH}^{-1}$, respectively. These values were larger than the slope of the buffer line (i.e., -16 $\text{mmol L}^{-1} \text{pH}^{-1}$), indicating that metabolic compensation had already occurred at just 2 h of exposure to $\leq 10\%$ CO_2 gas mixtures. This partially compensated respiratory acidosis $\leq 10\%$ CO_2 at 2h was expressed by the following linear regression equation;

$$[\text{HCO}_3^-] = -30.8 \times \text{pH} + 259.7 \quad (r=0.990, t=-13.840, P<0.001) \quad (\text{eq. 1})$$

which is represented by the long dashed line in Fig.2A, B, C. The hypercapnic respiratory acidosis was further compensated with additional time, and the regression equations for partial compensation $\leq 10\%$ CO_2 at 6 and 24 h were;

$$[\text{HCO}_3^-] = -90.1 \times \text{pH} + 709.5 \quad (r=0.988, t=-12.700, P<0.001) \quad (\text{eq. 2})$$

which is represented by the short dashed line in Fig.2A, B, C, and

$$[\text{HCO}_3^-] = -95.3 \times \text{pH} + 749.3 \quad (r=0.980, t=-9.804, P<0.001) \quad (\text{eq. 3}),$$

which is represented by the solid line in Fig.2A, B, C, respectively.

Exposure of embryos to 20% CO_2 decreased pH_a to 7.17 at 2 h and increased $[\text{HCO}_3^-]_a$ to ~36 mmol L^{-1} . This resulted in a slope of acid-base change of ~-19 $\text{mmol L}^{-1} \text{pH}^{-1}$ (thin arrowed solid

line, Fig.2D). Both pH_a and $[\text{HCO}_3^-]_a$ increased to 7.27 and $\sim 45 \text{ mmol L}^{-1}$ at 6 h and 7.33 and $\sim 48 \text{ mmol L}^{-1}$ at 24 h. During this period, P_{aCO_2} decreased significantly ($P < 0.001$) from 101 mmHg at 6 h to 92 mmHg at 24 h. The slope of a line connecting the control acid-base status to the partially compensated respiratory acidosis was ~ -51 and $-74 \text{ mmol L}^{-1} \text{ pH}^{-1}$ at 6 (broken line) and 24 h (solid line), respectively (Fig.2D).

When embryos were returned to air after 24 h of exposure to 3% CO_2 , P_{aCO_2} decreased to the control level by 2 h (Fig.2A). pH_a and $[\text{HCO}_3^-]_a$ returned to the control level by 6 h. Recovery from exposure to more severe hypercapnia (6, 10 and 20%) also took place during 6 h in air (Fig.2B, C and D, respectively). After 6 h of air exposure, acid-base status returned to control values, except in 20% CO_2 exposure where $[\text{HCO}_3^-]_a$ decreased to less than control values ($P < 0.001$).

3.3.3. Cornish Rock broiler embryos

Similar to Hyline embryos, at 2 h of exposure to $\leq 10\%$ CO_2 , hypercapnic respiratory acidosis was already partially compensated by metabolic alkalosis in broiler embryos (Fig.3A, B, C). This compensation was expressed by;

$$[\text{HCO}_3^-] = -30.6 \times \text{pH} + 258.9 \quad (r=0.990, t=-13.750, P<0.001) \quad (\text{eq. 4})$$

which is represented by the long dashed line in Fig.3A, B, C. Subsequently, the partial metabolic compensation further progressed during the following 4 h, resulting in the following regression equation;

$$[\text{HCO}_3^-] = -96.0 \times \text{pH} + 751.7 \quad (r=0.996, t=-23.289, P<0.001) \quad (\text{eq. 5})$$

which is represented by the short dashed line in Fig.3A, B, C.

During the period from 6 to 24 h, pH_a further significantly increased in 3 and 6% CO_2 ($P=0.048$ and $P=0.016$). However, the increase in 10% CO_2 was not significant ($P=0.361$). While $[\text{HCO}_3^-]_a$ remained unchanged at 6 and 24 h in $\leq 10\%$ CO_2 , P_{aCO_2} decreased in 3 and 6% CO_2 ($P=0.020$ and $P=0.003$) and remained unchanged in 10% CO_2 ($P=0.064$). Consequently, the regression equation for partial compensation across CO_2 treatments at 24 h was;

$$[\text{HCO}_3^-] = -100.7 \times \text{pH} + 786.8 \quad (r=0.999, t=-43.161, P<0.001) \quad (\text{eq. 6})$$

which is represented by the solid line in Fig. 3A, B, C.

Exposure to 20% CO₂ decreased pH_a to 7.15 and increased [HCO₃⁻]_a to 36 mmol L⁻¹ at 2 h (Fig. 3D), resulting in a slope of ~-14 mmol L⁻¹ pH⁻¹ (thin arrowed solid line in Fig. 3D). At 6 and 24 h, both pH_a and [HCO₃⁻]_a increased, but neither significantly differed between the two exposure times. Therefore, the slope of a line connecting the control acid-base status to the partially compensated respiratory acidosis was ~-43 mmol L⁻¹ pH⁻¹ at 6 and 24 h (thick solid line in Fig. 3D).

Partial recovery of acid-base disturbances occurred after 2 h exposure to air in all CO₂ treatments, and all treatments except 20% CO₂ exposure had returned to control levels after 6 h (Fig. 3). In 20% CO₂ exposure, [HCO₃⁻]_a decreased to less than the control (P<0.001).

3.3.4. Lactate concentration

Control [La⁻] was the same in Hyline and broiler embryos, except prior to the 6% CO₂ treatment (Fig. 4). [La⁻] of Hyline embryos decreased during 6% CO₂ exposure so that the [La⁻] of the two groups were no longer significantly different (Fig. 4B). [La⁻] of both groups remained unchanged across 3% CO₂ exposure and recovery (Fig. 4A), but decreased significantly during 10 and 20% CO₂ exposures (Fig. 4C, D). In 20% CO₂, [La⁻] increased significantly during early period of recovery in air (2 h), with a subsequent decrease after 6 h (Fig. 4D). During recovery, changes in [La⁻] were larger in Hyline than broiler.

3.4. Dynamics of hematological responses during a range of hypercapnic challenges

3.4.1. Control hematological variables

All measured control values of hematological respiratory variables significantly differed between the two chicken strains, with the sole exception of MCH (Table 1). Hct, [RBC], MCV and [Hb] were higher in broiler compared to Hyline embryos, but [MCHb] was significantly lower in broiler than Hyline.

3.4.2. *Hylinelayerembryos*

The effects of hypercapnia, treatment time, and their interaction on Hct in Hyline embryos were all significant (Table 2). While Hct at 20% CO₂ exposure was significantly higher than $\leq 10\%$ CO₂ exposures at 6 h, Hct response to $\leq 10\%$ CO₂ exposures did not significantly differ at any treatment time (Fig. 5A). Mean Hct of 3, 6 and 10% CO₂ combined was maximal at 2 h. The percent increase was $\sim 7\%$, calculated as ΔHct (i.e., $\Delta\text{Hct} = 100 \times (\text{Hct}_{\text{ex}} - \text{Hct}_{\text{c}})/\text{Hct}_{\text{c}}$, where Hct_{c} and Hct_{ex} are mean control Hct and mean Hct at a time of exposure to CO₂). Hct subsequently decreased, but was still higher than control, at 6 h ($\sim 5\%$). Hct then returned to control level at 24 h exposure ($\sim 3\%$) and at 2 and 6 h of recovery in air. In response to 20% CO₂ exposure, Hct significantly increased at 2 h ($\sim 10\%$) and further increased at 6 h ($\sim 17\%$), with a subsequent decrease back to control level at 24 h exposure ($\sim 5\%$) and during 2 and 6 h of recovery in air.

Hypercapnia and treatment time significantly affected [RBC], but the interaction of these two factors was not significant (Table 2). Mean [RBC] across all treatment times at 20% CO₂ was significantly lower than $\leq 10\%$ CO₂ (Fig. 5B). Mean [RBC] of all [CO₂] combined did not vary at any treatment time from control, however, there was a significant difference between 2 and 6 h of exposure and between 24 h and 2 h of recovery.

The effects of hypercapnia, treatment time and their interaction on MCV were significant (Table 2). MCV of 3, 6 and 10% CO₂ treatments was maximal at 2 h with a subsequent decrease, but was still significantly high at 6 and 24 h compared to the control (Fig. 5C). MCV in 20% CO₂ significantly increased at 2 h ($\Delta\text{MCV} = \sim 13\%$) and further increased at 6 h ($\sim 19\%$). A subsequent decrease, but to levels still above control, occurred at 24 h ($\sim 8\%$). MCV returned to control level at 2 h of recovery following exposure to all [CO₂].

Hypercapnia and treatment time significantly affected [Hb], but their interaction was not significant (Table 2). Mean [Hb] across all treatment times in 20% CO₂ was significantly lower than 3, 6 and 10% CO₂ (Fig. 5D). Mean [Hb] of all [CO₂] combined remained unchanged from control with only a significant difference between 2 and 24 h of exposure and 2 h of recovery. The effect of hypercapnia on MCH was significant, but the effects of treatment time and the interaction between

hypercapnia and treatment time were insignificant (Table 2). Mean MCH across all treatment times in 20% CO₂ was significantly lower than 3, 6 and 10% CO₂, and MCH averaged across all [CO₂] remained unchanged throughout exposure and recovery (Figure 5E).

The effects of hypercapnia, treatment time and their interaction on [MCHb] were significant (Table 2). [MCHb] decreased in response to all CO₂ treatments, but the decrease was larger in 20% CO₂ compared to 3, 6 and 10% CO₂ (Fig. 5F). The decrease in [MCHb] in 3% CO₂ was smaller compared with 6 and 10% CO₂. In response to 20% CO₂, [MCHb] significantly decreased at 2 h (Δ [MCHb] = ~-14%) and further decreased at 6 h (-18%). Subsequently, it increased at 24 h (~-9%), but levels were still low, only reaching control level during recovery in air.

3.4.3. *Cornish Rock broiler embryos*

The effects of hypercapnia and treatment time on Hct and [RBC] in broiler embryos were significant, but their interaction was not (Table 2). Mean Hct across all treatment times was significantly different between 20 and 10% CO₂ exposures (Fig. 6A). Mean Hct for all CO₂ treatments combined increased significantly at 2 h (Δ Hct = ~6%) and further at 6 h (~12%), maintaining a high level until 24 h (8%). Hct returned to the control level at 2 and 6 h of recovery in air. Mean [RBC] across all treatment times was significantly higher at 3% CO₂ compared to 6, 10 and 20% CO₂ (Fig. 6B). Mean [RBC] of all CO₂ treatments combined remained unchanged compared with the control throughout exposure, with only a slight increase at 2 h of recovery.

Mean corpuscular volume was significantly affected by hypercapnia, treatment time and their interaction (Table 2). MCV responded differently to 20% CO₂ compared to the other CO₂ treatments at 6 and 24 h (Fig. 6C). MCV at 3, 6 and 10% CO₂ did not differ throughout exposure. Hence, mean MCV of the three treatments combined was maximal at 2 h with a subsequent decrease at 6 and 24 h. In 20% CO₂, MCV increased at 2 (Δ MCV = ~11%), 6 (~15%) and 24 h (~18%), with a subsequent decrease during recovery in air.

The effects of hypercapnia and treatment time on [Hb] were significant, but their interaction was not (Table 2). Mean [Hb] across all treatment times at 3% CO₂ was significantly higher than 6,

10 and 20% CO₂ (Fig. D). Mean [Hb] of all CO₂ treatments combined remained unchanged compared with the control throughout exposure, with only a slight increase at 2 h of recovery. MCH was not significantly affected by hypercapnia, treatment time, or their interaction, but all three effects significantly altered [MCHb] (Table 2, Fig. 6E). [MCHb] decreased in response to all CO₂ treatments, but the decrease was greater at 20% CO₂ compared to 3, 6 and 10% CO₂ (Fig. 6F). In response to $\leq 10\%$ CO₂, the significant decrease of mean [MCHb] at 2 h (Δ [MCHb] = -8%) persisted at 6 (-8%) and 24 h (~-5%). In 20% CO₂, [MCHb] significantly decreased at 2 h (~-12%), decreased further at 6 h (~-16%), and then remained unchanged at 24 h. [MCHb] returned to control level during recovery in air.

4. Discussion

4.1. Egg mass, body mass and blood osmolality

Broiler embryos on day 15 were ~30% heavier than Hyline embryos, as well as heavier than Lohmann embryos used in a previous study (Mueller et al., 2014b), despite no differences in fresh egg mass (Table 1). The larger embryos of broilers, a result of the selected trait of faster growth, may be attributed in part to the larger yolk mass (Table 1). The greater yolk mass contains a larger lipid fraction, and therefore greater energy for growth (Sotherland and Rahn, 1987; Dzialowski and Sotherland, 2004; Mueller et al., 2014a).

Exposure to all [CO₂] combined reduced the body mass of broiler embryos after 24 h and exposure to 20% CO₂ reduced BM after 24 h in Hyline embryos (Tables 3,4). Since the natural increase in BM of White Leghorn is ~2.5 g during the 24 h period from d14 to d15 (e.g., Romanoff, 1967), the currently observed decreases in BM over a 24 h period (~-1.4g in Hyline and ~-0.9 g in broiler) may be a delay in growth related to CO₂ exposures. However, the mass of ~0.7 – 0.9 g was regained during 6 h of recovery and ~0.5-0.6 g was gained during the first 6 h of CO₂ exposure in both strains, suggesting BM changes during hypercapnia may not be caused by growth differences. Instead, we speculate that changes in BM may be the result of alteration in water balance

between intra- and extra-embryonic spaces during CO₂ exposure. However, the slight increase in Osm in both strains during CO₂ exposure was not related to changes in BM. Therefore, if water balance is altered, it is not detected in blood osmolality alone. Further study on water balance during CO₂ exposure is needed.

4.2. Dynamics of acid-base changes

4.2.1. Control acid-base status

Control acid-base status of broiler embryos was relatively respiratory acidotic compared with Hyline (Table 1) of the present study, and with Lohmann embryos from a previous study (Mueller et al., 2014b). Air cell CO₂ partial pressure, which P_aCO₂ approximates, is proportional to metabolic rate and inversely proportional to eggshell gas conductance (Rahn et al., 1974; Mueller et al., 2014a). Heat production in Lohmann embryos is about 26-30% lower than that of broiler (Ross) during d12 to d20 of incubation (Janke et al., 2004) and the eggshell gas conductance is ~10% higher in White Leghorn embryos than broiler embryos during d10-d14 of incubation (Yoneta et al., 2006). These differences in both variables between the two strains could contribute to P_aCO₂ being higher in broilers than layer embryos, as more CO₂ was likely to be dissolved in the blood of broiler embryos, creating a higher [HCO₃⁻] compared to layer embryos. The different control acid-base status between the two strains also influenced the acid-base time course after hypercapnic exposure, with broiler embryos being more acidotic compared with Hyline embryos, as will now be discussed.

4.2.2. Partial compensation for respiratory acidosis induced by ≤10% CO₂ exposures

Metabolic compensation at any CO₂ treatment at any given exposure time can be estimated from linear regressions that express partially compensated respiratory acidosis across the different CO₂ treatments at different times, together with the Henderson-Hasselbalch equation for a P_{CO₂} isopleth and buffer line. For example, in Hyline embryos exposed to 3% CO₂ (Fig. 2A), the intersection of the P_{CO₂} isopleth equation ($\text{pH} = 6.095 + \log ([\text{HCO}_3^-] / (0.0308 \times 43))$) and partial compensation

regression equation at 2h (eq. 1) was at a pH of 7.452. The uncompensated pH determined by an intersection of 43 mmHg isopleth and buffer line passing through control acid-base status (i.e., $[\text{HCO}_3^-] = -16.0 \text{ pH} + 147.0$, control pH, 7.594) is a pH of 7.424. Accordingly, pH is compensated by 0.028 units, while uncompensated change in pH is 0.17 units. Thus, the percent partial compensation is ~16%. Similarly, partial compensation for 3% CO_2 -respiratory acidosis at 6 and 24 h is ~45 and 48%, respectively. For 6% CO_2 -respiratory acidosis (Fig.2B), uncompensated pH is 7.357 and partial compensation was ~16, 44 and 46% at 2, 6 and 24 h, respectively. For 10% CO_2 -respiratory acidosis (Fig.2C), uncompensated pH is 7.267 and partial compensation was ~15, 43 and 45%, respectively. As a result, Hyline embryos show an average compensation for 3, 6 and 10% CO_2 -respiratory acidosis of ~16, 44 and 46% at 2, 6 and 24 h, respectively.

Similarly, the averaged compensation in broiler embryos exposed to 3 – 10% CO_2 (Fig.3A, B, C) was ~14, 45 and 46% at 2, 6 and 24 h, respectively. In Lohmann embryos previously examined, the partial metabolic compensation was ~17, 46 and 53% at 2, 6 and 24 h, respectively (Mueller et al., 2014b). Consequently, acid-base perturbation due to exposure to $\leq 10\%$ CO_2 progresses in proportion to increased $[\text{CO}_2]$. Importantly, the metabolic compensation occurs to the same degree for the same length of exposure period, irrespective of $[\text{CO}_2]$. These acid-base changes occur in a very similar manner in embryos of all three chicken strains.

4.2.3. Partial compensation for respiratory acidosis induced by 20% CO_2 exposure

The similar magnitude of metabolic compensation measured over the $\leq 10\%$ CO_2 range does not occur with an additional increase in acid-base challenge induced by 20% CO_2 exposure (Figs.2D and 3D). The slopes describing metabolic compensation at 2, 6 and 24 h of 20% CO_2 exposure were much smaller compared to the regression lines produced by 3, 6 and 10% CO_2 in both Hyline and broiler embryos. For instance, at 24 h the slope was ~-74 and -43 $\text{mmol L}^{-1} \text{pH}^{-1}$ for Hyline and broiler embryos, compared to the slopes of ~-95 and -101 $\text{mmol L}^{-1} \text{pH}^{-1}$ for Hyline and broiler embryos exposed to 3, 6 and 10% CO_2 . Thus, metabolic compensation in 20% CO_2 is much reduced compared to exposures to $\leq 10\%$ CO_2 .

In Hyline embryos exposed to 20% CO₂, partial compensation was calculated to be ~4, 27 and 40% at 2, 6 and 24 h, respectively (Fig.2D). The compensation of 40% at 24 h included compensation due to respiratory alkalosis produced by the decrease of Pco₂ from 101 to 92 mmHg. Since the respiratory compensation originated from the decrease in blood Pco₂, it indicates a possible impairment of metabolism, illustrated by a drop in CO₂ production after 24 h exposure to 20% CO₂. The death of a small number of Hyline embryos after 24 h exposure to 20% CO₂ further indicates the lowered tolerance of these embryos to severely hypercapnic environments. However, broiler embryos responded to 20% CO₂ exposure differently, with no partial compensation at 2 h, and little progression of partial compensation at 6 and 24 h (Fig.3D). The partial metabolic compensation for respiratory acidosis at 6 and 24 h was ~20%, without respiratory alkalosis. It can be inferred that metabolism was not impaired, but the ability to metabolically compensate was insufficient compared to Hyline embryos in 20% CO₂. These different responses of Hyline and broiler embryos at 20% CO₂ occur despite very similar metabolic compensation at ≤ 10% CO₂ between two strains. Put differently, at ≥ 10% CO₂ different chicken strains show strain-specific patterns and capabilities of acid-base regulation. Exposure to 20% CO₂ had a greater detrimental impact on broiler embryos compared to Hyline, suggesting their acid-base regulation may be impaired at levels closer to 10% than 20% CO₂. Future research is required to determine if the hypercapnic threshold that results in a change in acid-base regulation - which this study suggests to lie between 10 and 20% CO₂ - is different between strains.

4.3.4. *Hysteresis of acid-base regulation*

When Hyline and broiler embryos were returned to air after 20% CO₂ exposure, the respiratory alkalosis resulting from a decrease in Pco₂ was partially compensated by a decrease in [HCO₃⁻] (Figs.2D and 3D), accompanied by an increase in [La⁻] (Fig.4D). Assuming no metabolic compensation occurred at 2 h of recovery, pH predicted from the buffer value was 7.80 against a measured pH_a of 7.76. Thus, the compensated change in pH was 0.04 units in Hyline embryos. As the uncompensated change in pH from the 24 h state to 2 h recovery was 0.47 pH units, the

respiratory alkalosis was partially compensated by ~9%. With continued recovery, $[\text{HCO}_3^-]$ further decreased, with no change in P_{CO_2} , to a level even lower than the control. Consequently, during early periods of both exposure and recovery to and from altered $[\text{CO}_2]$, respiratory changes in P_{CO_2} occurred much faster than metabolic changes in $[\text{HCO}_3^-]$, producing a hysteresis loop of acid-base responses (Figs. 2 and 3). The hysteretic response of acid-base regulation also occurred in embryos exposed to severe extrinsic hypercapnic hypoxia (5% CO_2 , 10% O_2) or in embryos submerged in water (producing severe intrinsic hypercapnic hypoxia), where changes in $[\text{HCO}_3^-]$ were matched by those in $[\text{La}^-]$ (Tazawa et al., 2012; Andrewartha et al., 2014).

After 6 h of recovery in air from 20% CO_2 exposure, $[\text{HCO}_3^-]_{\text{a}}$ decreased to less than control values in both strains (Figs. 2D and 3D), implying “over-compensation”. During recovery in air, we can assume that the capacity of a tissue or an organ responsible for $[\text{HCO}_3^-]$ regulation (presumably the chorioallantoic membrane) to remove HCO_3^- from the blood is the same as the capacity to transfer HCO_3^- into the blood during maximal partial metabolic compensation (~45%) in response to respiratory acidosis. However, partial metabolic compensation for 20% CO_2 -respiratory acidosis was only ~40 and 20% at 24 h in Hyline and broiler embryos. Thus, $[\text{HCO}_3^-]_{\text{a}}$ was over-compensated. This is likely to occur even when the transfer of HCO_3^- (presumably from the allantoic fluid) to blood to compensate for respiratory acidosis is restricted by 20% CO_2 exposure. Such over-compensation of $[\text{HCO}_3^-]_{\text{a}}$ during recovery also occurred in eggs half-submerged into water for 24 h (Mueller, Tazawa and Burggren, unpublished data). Hence, over-compensation appears to occur irrespective of status (or degree) of metabolic compensation for respiratory acidosis produced by external or internal hypercapnia accompanied by varied $[\text{O}_2]$. This overcompensation phenomenon may reflect a lack of maturity of the overall acid-base regulatory systems of chicken embryos.

4.2.5. Lactate concentration

Control $[\text{La}^-]$ in chicken embryos is relatively low, ranging from 0.5 – 1.5 mmol L^{-1} in a normoxic environment (Freeman, 1965; Tazawa et al., 1983b, 2012; Tazawa, 1986; Bjønnes et al.,

1987; Høiby et al., 1987, Burggren et al., 2012; present study). However, when O₂ supply to the tissues is deficient, [La⁻] increases markedly due to anaerobic glycolysis. This induces metabolic acidosis in embryos suffering severe extrinsic or intrinsic hypoxia (Tazawa et al., 2012; Andrewartha et al., 2014). The metabolic acidosis attributed to an increase in [La⁻], i.e., lactic acidosis, is also produced by hypocapnia in adult chickens that are artificially hyperventilated or exposed to heat stress (Frankel, 1965; Koelkebeck and Odom, 1994). Addition of hypercapnia to heat-stressed chickens lowered [La⁻] to pre-exposure levels. The effect of hypercapnia on suppression of lactate production was also clearly evident in embryos. Hypercapnia (e.g., 5% CO₂) significantly lowered blood [La⁻] at 24 h even in mild hypoxia (15%) and normoxia (Burggren et al., 2012). In the present experiment, [La⁻] decreased even after 2 h of hypercapnia (6, 10 and 20% CO₂), and remained low during 24 h exposure (Fig.4). However, the magnitude of decrease was far less than the increases in [HCO₃⁻] during hypercapnic exposures. Consequently, the changes in [La⁻] did not contribute to compensatory changes in [HCO₃⁻] during hypercapnic respiratory acidosis. This is in contrast to [La⁻] governing hypoxic metabolic acidosis in embryos exposed to severe hypoxia with recovery in air (Tazawa et al., 2012; Andrewartha et al., 2014). While La⁻ did not contribute to compensation for hypercapnic respiratory acidosis in the present study, whether La⁻ contributes to compensation for hypocapnic respiratory alkalosis (compensatory lactic acidosis) warrants further study in chicken embryos, given the ease with which hypocapnic respiratory alkalosis can be induced (Tazawa et al., 1981; Tazawa, 1981, 1982).

4.3. Dynamics of hematological changes

4.3.1. Hematology under control conditions.

Control hematological variables differed significantly between the two strains. Most noticeable for broiler embryos was the higher Hct, a result of both a high [RBC] and MCV, and higher [Hb] (Table 1). While there are slight differences in hematology of the Hyline embryos compared to previously published data on the Lohmann strain (Mueller et al., 2014b), these differences are not as

great as seen between broiler and the two White Leghorn strains. It is likely that selection for fast growth in the broiler has potentially also selected for higher Hct and [Hb]. These hematological changes presumably increase oxygen delivery by the blood, helping to support higher metabolism and faster growth. This is in accordance with the higher oxygen consumption of Ross broiler embryos compared to Lohmann White Leghorn (Janke et al., 2004).

4.3.2. Different hematological regulation between $\leq 10\%$ and 20% CO_2 exposures.

The increase, or no change, in Hct in response to exposure to 3, 6 and 10% CO_2 in Hyline and broiler embryos sharply contrasts with the decrease in Hct measured in Lohmann embryos (Mueller et al., 2014b). Hct responded most strongly to 20% CO_2 (Figs. 5A, 6A). Because [RBC] showed no response to altered CO_2 levels (Figs. 5B and 6B), the increase in Hct was due to the increase in MCV, which differed between 20% CO_2 and the mean responses to $\leq 10\%$ CO_2 (Figs. 5C and 6C).

Values for [Hb] and MCH in both Hyline and broiler did not change during CO_2 exposures (Figs. 5D, E, and 6D, E), in contrast to the decrease in [Hb] and increase in MCH at 24 h in Lohmann embryos (Mueller et al., 2014b). As a result, changes in [MCHb] in Hyline and broiler inversely mirrored the changes in MCV (Figs. 5F and 6F). MCV and [MCHb] responded in almost the same manner and same magnitude to $\leq 10\%$ CO_2 , which differed from the response to 20% CO_2 in both strains. These results partially support our previous prediction that exposure to $>10\%$ CO_2 hydrates RBCs and alters the hematological responses from that seen in embryos exposed to 1 - 6% CO_2 (Mueller et al., 2014b). However, RBC hydration occurred even during exposure to $\leq 10\%$ CO_2 , and the response in 10% CO_2 was not different from that in 3 and 6% CO_2 . In a previous *in vitro* experiment, RBCs were dehydrated when the blood was equilibrated with air and then showed graded hydration with addition of 3 and 6% CO_2 in 20% O_2 (Andrewartha et al., 2012). In Hyline and broiler embryos, RBCs were hydrated as in the *in vitro* experiment, but equally hydrated in $\leq 10\%$ CO_2 environments and prominently hydrated in 20% CO_2 , with clear differences between the two strains. The prominent hydration (i.e., increase in MCV accompanied by unchanged [RBC]), measured in Hyline and broiler embryos also occurred after just 10 min, and continued to

increase thereafter, during 2 h exposure to severe extrinsic hypoxia (10% O₂) with or without 5% CO₂ in Lohmann (Tazawa et al., 2012) and Hyline embryos (Kohl, Crossley, Tazawa and Burggren, unpublished data). Responses of MCV and [RBC] to hypercapnia and hypoxia vary among strains and even within the same strain, and thus study of the regulation of these variables must be approached with care.

5. Conclusions

Both Hyline White Leghorn layer and Cornish Rock broiler chicken strains used in this study showed an alteration in acid-base and hematological regulation when hypercapnic exposure exceeded 10% CO₂. In $\leq 10\%$ CO₂, acid-base regulation occurred in proportion to [CO₂], and hematological responses were the same irrespective of [CO₂]. However, this trend did not persist at 20% CO₂, indicating the existence of a hypercapnic threshold for metabolic compensation and hematological regulation above 10% CO₂. Whether this threshold is common in all avian embryos, or whether those species that may be more likely to experience a hypercapnic environment have a higher threshold, is the subject of future study. Control acid-base status and acid-base responses to CO₂ show clear strain differences between broiler, Hyline and previously studied Lohmann layer embryos. However, strain-specific differences did not occur for hematological responses to CO₂. Despite different control Hct and [Hb] levels, the hematological responses to CO₂ exposure were quite similar between broiler and Hyline embryos, to the point where the similarities were greater than between the Hyline embryos and responses previously published for another White Leghorn strain, Lohmann (Mueller et al., 2014b). Therefore, while there are certainly strain effects, there may be other factors, such as maternal and environmental effects (Burggren et al., 1994; Dzialowski and Sotherland, 2004; Ho et al., 2011; Burggren, 2014) that contribute to the differences in responses of hematological variables and acid-base balance between strains, or even within the same strains.

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Figure legends

Fig. 1. Changes in blood osmolality (Osm) of day 15 embryos during exposures to varying CO₂ in (A) Hylinelayer and (B) Cornish Rock broiler. Symbols with vertical bars represent mean±S.E.M. and are as follows; 3% (open circle), 6% (open square), 10% (open diamond) and 20% (open hexagon) CO₂. The sample size for individual mean values in Hyline and broiler embryos is the same as shown in Table 3 and Table 4, respectively. Means at each time that are not significantly different are grouped within boxes in (A). The set of letters in (A) denote differences between exposure times for 10 and 20% CO₂ groups. The set of bold letters in (B) indicate differences between exposure times for all groups combined.

Fig.2. Dynamics of acid-base regulation of Hylinelayer embryos exposed to (A) 3%, (B) 6%, (C) 10% and (D) 20% CO₂ at 0 (Control), 2, 6 and 24 h and during recovery in air at 2 (R2h) and 6 h (R6h). The long and short dashed lines and solid line in panels (A), (B) and (C) are regression lines connecting the acid-base responses at 2, 6 and 24 h to 3, 6 and 10% CO₂ exposures. The short dashed line and solid line in panel (D) connect the acid-base status at 6 and 24 h with the control state, respectively. The buffer line was depicted to indicate buffer capacity (-16 mmol L⁻¹ pH⁻¹).

Fig.3. Dynamics of acid-base regulation of Cornish Rock broiler embryos exposed to (A) 3%, (B) 6%, (C) 10% and (D) 20% CO₂ at 0 (Control), 2, 6 and 24 h and during recovery in air at 2 (R2h) and 6 h (R6h). Other details as in Fig.2.

Fig.4. Changes in blood lactate concentration ([La⁻]) of Hyline layer (open circles) and Cornish Rock broiler (crossed closed circles) embryos during (A) 3%, (B) 6%, (C) 10% and (D) 20% CO₂ exposures and recovery in air. Other presentation details as described in Fig. 1.

Fig.5. Time-course changes in (A) hematocrit (Hct), (B) red blood cell concentration ([RBC]), (C) mean corpuscular volume (MCV), (D) hemoglobin concentration ([Hb]), (E) mean corpuscular hemoglobin (MCH) and (F) mean corpuscular hemoglobin concentration ([MCHb]) in Hyline layer embryos exposed to 3% (open circle), 6% (open square), 10% (open diamond) and 20% (closed hexagon) CO₂. The set of letters in (A, C, F) denote differences between exposure times for 20% CO₂ group. The set of bold letters in (B, D) indicate differences between exposure times for all groups combined.

Fig.6. Time-course changes in (A) hematocrit (Hct), (B) red blood cell concentration ([RBC]), (C) mean corpuscular volume (MCV), (D) hemoglobin concentration ([Hb]), (E) mean corpuscular hemoglobin (MCH) and (F) mean corpuscular hemoglobin concentration ([MCHb]) in Cornish Rock broiler embryos exposed to 3% (open circle), 6% (open square), 10% (open diamond) and 20% (closed hexagon) CO₂. The set of letters in (C, F) denote differences between exposure times for 20% CO₂ group. The set of bold letters in (B, D) indicate differences between exposure times for all groups combined.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Table 1. Comparison of control (no CO₂ exposure) variables in arterialized blood of day 15 Hyline layer and Cornish Rock broiler embryos. Control acid-base variables include pH (pH_a), bicarbonate concentration ([HCO₃⁻]_a) and carbon dioxide partial pressure (P_aCO₂). Control hematological variables include hematocrit (Hct), red blood cell concentration ([RBC]), mean corpuscular volume (MCV), hemoglobin concentration ([Hb]), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration ([MCHb]). Data presented as mean ± 1 S.E.M. (N). P < 0.05 indicate significant differences between the two strains.

	Hyline White Leghorn	Cornish Rock broiler	P
Fresh egg mass (g)	58.93 ± 0.25 (645)	59.47 ± 0.29 (247)	0.485
Body mass (g)	12.02 ± 0.12 (142)	15.94 ± 0.22 (41)	<0.001
Yolk mass (g)	15.99 ± 0.16 (40)	17.57 ± 0.22 (40)	<0.001
Osmolality (mmol kg ⁻¹)	272 ± 0.4 (142)	273 ± 0.6 (41)	0.244
<i>Acid-base status:</i>			
pH _a	7.60 ± 0.004	7.53 ± 0.005	<0.001
[HCO ₃ ⁻] _a	26±0.2	29±0.4	<0.001
P _a CO ₂	27±0.3	35±0.9	<0.001
<i>Hematological variables:</i>			
Hct (%)	26.9 ± 0.2	29.3 ± 0.3	<0.001
[RBC] (10 ⁶ µL ⁻¹)	2.03 ± 0.01	2.11 ± 0.02	0.010
MCV (µm ³)	133 ± 0.6	139 ± 0.9	<0.001
[Hb] (g%)	9.2 ± 0.08	9.7 ± 0.11	0.004
MCH (pg)	45.4 ± 0.3	45.9 ± 0.2	0.242
[MCHb] (g%)	34.3 ± 0.2	32.9 ± 0.2	<0.001

Number of control eggs examined for acid-base status and hematological variables in two strains is the same as that of eggs used for control body mass measurement.

Table 2. Results from a two-way ANOVA and Holm-Sidak test for the effects of different levels of hypercapnia (CO₂), different times of exposure and recovery (Treatment time) and the interaction between hypercapnia and treatment time (Interaction) on body mass (BM), osmolality (Osm) and hematological respiratory variables (Hct, [RBC], MCV, [Hb], MCH and [MCHb]) in day 15 Hyline White Leghorn layer and Cornish Rock broiler embryos. P < 0.05 indicates a significant effect.

	Hyline White Leghorn layer			Cornish Rock broiler		
	CO ₂	Treatment time	Interaction	CO ₂	Treatment time	Interaction
BM	0.004	<0.001	0.004	0.032	<0.001	0.455
Osm	<0.001	<0.001	<0.001	0.057	<0.001	0.419
Hct	<0.001	<0.001	<0.001	0.006	<0.001	0.165
[RBC]	<0.001	0.001	0.463	0.022	<0.001	0.100
MCV	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
[Hb]	<0.001	0.004	0.836	0.015	<0.001	0.133
MCH	<0.001	0.080	0.537	0.768	0.140	0.354
[MCHb]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 3. Body mass (g) of day 15 layer (Hyline White Leghorn) embryos exposed to varied (3, 6, 10 and 20%) CO₂ during 24 h followed by 6 h recovery in air. Embryos which were not exposed to CO₂ were measured for body mass as control (C). Body mass was measured at 2, 6 and 24 h of CO₂ exposure and at 2 and 6 h during recovery in air after 24 h of CO₂ exposure (R2 h, R6 h). Data presented as mean $\pm$ S.E.M. (N). Different capital letters (A, B) indicate significant differences between CO₂ treatments within the 24h and R2h time points. Different lowercase letters (a, b, c) indicate significant differences between exposure and recovery time points within 10 and 20% CO₂ exposures.

CO ₂ (%)	C	CO ₂ exposure			Recovery		Total
		2 h	6 h	24 h	R2 h	R6 h	
3	11.97 $\pm$ 0.27 (23)	12.17 $\pm$ 0.30 (19)	12.31 $\pm$ 0.28 (21)	11.90 ^A $\pm$ 0.27 (23)	12.14 ^{AB} $\pm$ 0.34 (15)	12.15 $\pm$ 0.34 (15)	12.10 $\pm$ 0.11 (116)
6	11.92 $\pm$ 0.20 (44)	11.93 $\pm$ 0.23 (33)	12.48 $\pm$ 0.23 (32)	11.80 ^A $\pm$ 0.25 (28)	12.54 ^A $\pm$ 0.31 (17)	12.37 $\pm$ 0.31 (17)	12.11 $\pm$ 0.10 (171)
10	11.65 ^{ab} $\pm$ 0.26 (25)	11.76 ^{ab} $\pm$ 0.28 (21)	12.46 ^a $\pm$ 0.27 (24)	11.29 ^{b, AB} $\pm$ 0.25 (27)	11.15 ^{b, B} $\pm$ 0.32 (16)	11.60 ^{ab} $\pm$ 0.32 (16)	11.67 $\pm$ 0.12 (129)
20	12.31 ^{ab} $\pm$ 0.18 (50)	12.32 ^{ab} $\pm$ 0.20 (43)	12.87 ^a $\pm$ 0.20 (44)	10.88 ^{c, B} $\pm$ 0.20 (41)	11.34 ^{c, B} $\pm$ 0.26 (25)	11.57 ^{bc} $\pm$ 0.25 (26)	11.97 $\pm$ 0.10 (229)
Total	12.02 $\pm$ 0.12 (142)	12.08 $\pm$ 0.13 (116)	12.58 $\pm$ 0.12 (121)	11.39 $\pm$ 0.11 (119)	11.74 $\pm$ 0.15 (73)	11.88 $\pm$ 0.15 (74)	

Table 4. Body mass (g) of day 15 broiler (Cornish Rock) embryos exposed to varied (3, 6, 10 and 20%) CO₂ during 24 h followed by 6 h recovery in air. Embryos which were not exposed to CO₂ were measured for body mass as control (C). Body mass was measured at 2, 6 and 24 h of CO₂ exposure and at 2 and 6 h during recovery after 24 h of CO₂ exposure (R2 h, R6 h). Data presented as mean $\pm$ S.E.M. (N). Different capital letters (A, B) indicate significant differences between CO₂ treatments. Different lowercase letters (a, b) indicate significant differences between exposure and recovery time points.

CO ₂ (%)	C	CO ₂ exposure			Recovery		Total
		2 h	6 h	24 h	R2 h	R6 h	
3	15.76 ± 0.31 (8)	16.73 ± 0.38 (8)	16.34 ± 0.28 (8)	15.06 ± 0.47 (8)	16.07 ± 0.47 (8)	16.81 ± 0.51 (8)	16.13 ^A ± 0.19 (48)
6	15.89 ± 0.71 (10)	16.18 ± 0.47 (10)	16.26 ± 0.40 (10)	14.93 ± 0.31 (10)	15.39 ± 0.37 (10)	16.01 ± 0.48 (9)	15.77 ^{AB} ± 0.20 (50)
10	15.77 ± 0.40 (11)	15.92 ± 0.25 (11)	16.78 ± 0.41 (12)	15.48 ± 0.29 (12)	15.63 ± 0.28 (11)	16.49 ± 0.46 (10)	16.01 ^{AB} ± 0.15 (67)
20	16.26 ± 0.29 (12)	15.68 ± 0.29 (12)	16.32 ± 0.24 (12)	14.85 ± 0.36 (13)	14.84 ± 0.61 (12)	14.94 ± 0.28 (12)	15.47 ^B ± 0.17 (73)
Total	15.94 ^{ab} ± 0.22 (41)	16.07 ^{ab} ± 0.18 (41)	16.44 ^a ± 0.17 (42)	15.08 ^c ± 0.20 (43)	15.42 ^b ± 0.23 (41)	15.97 ^{ab} ± 0.23 (39)	