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ORIGINAL PAPER

Dynamics of acid–base metabolic compensation and hematological regulation interactions in response to CO_2 challenges in embryos of the chicken (*Gallus gallus*)

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Abstract CO_2 exposure elicits multiple changes in the acid–base balance and hematology of avian embryos, but the time-specific, dose-dependent effects of graded increases in extrinsic CO_2 in a normoxic environment are poorly understood. Consequently, we exposed day 15 chicken embryos to 1, 3, 5, 6 or 10 % CO_2 in 20 % O_2. We hypothesized that both the magnitude of hypercapnic respiratory acidosis and the resultant metabolic compensation within 24 h of exposure to ≤10 % CO_2 are proportional to ambient CO_2 concentration ($[CO_2]$). We also predicted that regulation of hematological respiratory variables is graded according to $[CO_2]$. Time-course (2, 6 and 24 h) changes were determined for acid–base disturbances and hematological respiratory variables; hematocrit (Hct), red blood cell concentration ([RBC]), hemoglobin concentration, mean corpuscular volume (MCV) and other mean corpuscular indices. Both the decrease in uncompensated pH, which indicates uncompensated respiratory acidosis, and the compensatory pH increase, a sign of metabolic compensation, increased with $[CO_2]$. The partial metabolic compensation across all CO_2 gas mixtures was ~17, 46 and 53 % compensation at 2, 6 and 24 h, respectively. Hematological responses were nearly identical across the entire range of $[CO_2]$, with Hct decreasing across the time course of CO_2 exposure due to a decrease in MCV from 2 to 24 h and a decrease in [RBC] at 24 h. Even though hematological regulation was not graded, chicken embryos were able to compensate and survive exposure to ≤10 % CO_2.

Keywords Chicken embryo · Acid–base balance · Hematological respiratory variables · Hypercapnia · Metabolic compensation · Respiratory acidosis

Abbreviations

Hct	Hematocrit
[RBC]	Red blood cell concentration
MCV	Mean corpuscular volume
[Hb]	Hemoglobin concentration
MCH	Mean corpuscular hemoglobin
[MCHb]	Mean corpuscular hemoglobin concentration
$[CO_2]$	Carbon dioxide concentration
$[O_2]$	Oxygen concentration
P_aCO_2	Arterialized blood carbon dioxide partial pressure
pH_a	Arterialized blood pH
$[HCO_3^-]_a$	Arterialized blood bicarbonate concentration
Osm	Blood osmolality
ΔpH	Change in pH
$\Delta[HCO_3^-]$	Change in blood bicarbonate concentration
ΔHct	Percent change in hematocrit
Δ[RBC]	Percent change in red blood cell concentration
ΔMCV	Percent change in mean corpuscular volume

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Introduction

Gas exchange of avian embryos developing inside a porous eggshell cannot be regulated by lung ventilation, since gas exchange occurs solely by diffusion between the environmental air and the blood in the chorioallantoic capillaries (e.g., Piiper et al. 1980; Mueller et al. 2014). Since gas conductance of the eggshell is nearly constant (Bissonnette and Metcalfe 1978), any elevation of environmental CO_2 concentration ($[CO_2]$) directly increases air cell PCO_2 and consequently blood PCO_2 in the allantoic vein, typically called "arterialized blood PCO_2 (P_aCO_2)" (Piiper et al. 1980). Accordingly, experimental exposure of eggs to altered CO_2 environments inevitably causes respiratory acidosis in embryos that cannot be mitigated by respiratory compensation, but is partly mitigated by metabolic compensation alone (Dawes and Simkiss 1971; Tazawa 1982, 1986; Burggren et al. 2012; Mueller et al. 2013). While the presence of compensatory responses has been well established, whether such compensatory responses are dose-dependent, reflecting regulation of the response itself, is unknown.

In day 15 (d15) chicken embryos (*Gallus gallus*) exposed to environmental hypercapnia (5 % CO_2) with altered O_2 concentration ($[O_2]$) (15, 20, 40 or 90 % O_2), respiratory acidosis occurred within 2 h, but was partially compensated (~40–50 %) by metabolic alkalosis at 6 h. The subsequent responses varied with $[O_2]$ (Burggren et al. 2012; Mueller et al. 2013). In hypercapnic hypoxia (5 % CO_2, 15 % O_2), metabolic compensation was time-limited and deteriorated at 24 h of exposure, while embryos exposed to hypercapnic normoxia (5 % CO_2, 20 % O_2) or hypercapnic hyperoxia (5 % CO_2, 40 % or 90 % O_2) underwent continuous partial metabolic compensation for respiratory acidosis, evident even 24 h later. These different, time-dependent compensatory responses to exposure of 5 % CO_2 with altered $[O_2]$ indicate that the ability to metabolically compensate for acid–base disturbance requires environmental O_2 at normoxia (20 % O_2), and occurs irrespective of $[O_2]$ above 20 %.

Besides an understanding of acid–base responses to different $[O_2]$, the effect that $[CO_2]$ has in developmental acid–base regulation remains unclear. In chickens, variability of eggshell gas conductance is quite large among naturally laid eggs, resulting in large variations of air cell PCO_2 (e.g., 12–48 mmHg in d16 embryos) and P_aCO_2 (e.g., 30–55 mmHg in d16 embryos) (Tazawa et al. 1983; Visschedijk et al. 1985). Furthermore, some bird species lay eggs in burrow nests where their developing embryos are exposed to a simultaneously hypercapnic and hypoxic environment (White et al. 1978; Wickler and Marsh 1981; Boggs et al. 1983). Thus, during development avian embryos may potentially tolerate significant hypercapnia and continuously respond to exposure to 5–10 % CO_2. We hypothesize that respiratory acidosis produced by exposure to CO_2 will be compensated by metabolic alkalosis, with the extent of compensation being dose-dependent. That is, the larger the respiratory acidosis produced by CO_2, the greater the magnitude of metabolic compensation. Accordingly, we hypothesize that the extent (%) of partial metabolic compensation for respiratory acidosis induced by altered CO_2 is the same across all $[CO_2]$s of ≤10 %. Previous studies have also utilized high $[CO_2]$ to examine acid–base responses and cardiovascular function in embryonic chickens (e.g., 9 %: Dawes and Simkiss 1971; 21%: Tazawa 1981b). While these $[CO_2]$s may seem extreme, they allow us to fully examine the mechanisms of regulation of acid–base balance during development and help define the bounds of tolerance in these embryos.

Similar to acid–base balance responses, hematological respiratory variables respond to exposure to 5 % CO_2 with altered $[O_2]$ in different ways according to $[O_2]$ either below or above 20 % (Burggren et al. 2012; Mueller et al. 2013). In hypercapnic hypoxia (5 % CO_2, 15 % O_2), hematocrit (Hct) of developing embryos increases mainly due to an increase in red blood cell volume (i.e., mean corpuscular volume (MCV)). Again, once environmental O_2 exceeds 20 %, Hct drops due to decreases in red blood cell concentration ([RBC]) and MCV in d15 embryos. Collectively, these changes indicate that environmental O_2 is also an important factor governing responses of hematological respiratory variables during hypercapnia. However, because Hct does not respond to exposure to air, or normocapnic normoxia (0 % CO_2, 20 % O_2), CO_2 must also contribute to the decrease in Hct of embryos exposed to 20 % O_2. Therefore, we additionally hypothesize that RBCs are dehydrated (i.e., decrease in MCV) and sequestered (i.e., decrease in [RBC]) in response to exposure to 20 % O_2 in the presence of CO_2. Such alterations in RBC will likely be both time- and CO_2 dose-dependent, resulting in graded decreases in Hct during altered hypercapnic challenges of varying degrees. Accordingly, we investigate the time-specific responses of acid–base balance and hematological respiratory variables to exposure of d15 chicken embryos to 20 % O_2 with altered $[CO_2]$ over the range of 1–10 % CO_2.

Materials and methods

Egg incubation and exposure to gas mixtures

Fertile eggs of the domestic fowl (mainly Lohmann White Leghorn) were transported once a week from a hatchery at Texas A&M University (College Station, TX, USA) to the laboratory at the University of North Texas (Denton, TX, USA) in March and April. The eggs were lightly washed in water with a sponge to remove extraneous material and

weighed (±0.01 g) by an electronic balance after drying. Excessively large (>70 g) or small (<45 g) eggs were removed and the remaining eggs were numbered and set in an incubator (model 1502, G.Q.F. Manuf., GA, USA) at noon. Temperature and relative humidity of the incubator were kept at 37.5 ± 0.1 °C and ~55 %, respectively, and the eggs were turned automatically every 3 h.

On d13 of incubation, eggs were candled to confirm living embryos and to locate an allantoic vein. The eggshell over the vein was marked for blood collection performed on d15. On d14, eggs were randomly divided into "control" eggs and "gas-exposed" eggs, and transferred to a second desk-top incubator (1588 Electr. Hova-Bator, G.Q.F. Manuf., GA, USA) maintained at 37.5 °C. In the incubator, control eggs were placed on a cardboard egg stand and gas-exposed eggs were placed in a 3.78-L gas exposure bag ventilated with a gas mixture provided by a Wösthoff gas mixing pump (oHG, Bochum, Germany), as described previously (Burggren et al. 2012). Gas-exposed eggs were either placed in the bag at noon on d14 (24 h exposure) or exposed to the gas mixture for 2 or 6 h on d15 (2 or 6 h exposure).

Gas mixtures consisted of normoxia (20 % O_2) combined with one of 1, 3, 5, 6 or 10 % CO_2, balanced by N_2. Different eggs were also exposed to normocapnic normoxia (0 % CO_2, 20 % O_2) to obtain a baseline. The acid–base status and hematological variables were determined prior to varying CO_2 exposures (referred to as 0 h or control) and also at 2, 6 and 24 h of exposure.

Blood collection and analysis

Because blood could not be collected from eggs while in the gas exposure bag, an aluminum foil wrapping was used to preserve the blood gases during collection, as previously described (Burggren et al. 2012). Briefly, the egg was lightly wrapped in wrinkled aluminum foil immediately after removal from the gas exposure bag. A small ~1 cm window was torn in the foil over the site of the allantoic vein and a hole opened in the eggshell. Approximately 0.4 mL of blood was immediately collected into a 1 mL heparinized plastic syringe from the allantoic vein (see Tazawa 1980; Burggren et al. 2012). Blood was gently emptied into a 1.5 mL plastic vial and immediately analyzed for pH_a, $[HCO_3^-]_a$ (mmol L^{-1}) and P_aCO_2 (mmHg) by a blood gas system (ABL5, Radiometer Medical A/S, Denmark). Because the blood collected from the allantoic vein was arterialized by chorioallantoic capillaries (Piiper et al. 1980; Tazawa 1980), measured variables represent arterial values corresponding to adult pulmonary venous blood and were given the subscript "a". The relationship between pH_a and $[HCO_3^-]_a$ was depicted on a Davenport (pH–$[HCO_3^-]$) diagram previously constructed by plotting PCO_2 isopleths calculated from the Henderson-Hasselbalch equation using a CO_2 solubility factor of 0.0308 mmol L^{-1} $mmHg^{-1}$ and a serum carbonic acid pK' varying with pH (Severinghaus et al. 1956a, b; Burggren et al. 2012). A buffer line was drawn on the Davenport diagram to indicate the previously determined buffer value of −16 mmol L^{-1} pH^{-1} (Burggren et al. 2012).

The remaining blood was well stirred in the vial and measured for [RBC] (10^6 cells μL^{-1}) and hemoglobin concentration ([Hb]) (g %) using a hematology analyzer (Beckman Coulter Analyzer A^c T10, USA). Additionally, osmolality (Osm, mmol kg^{-1}) was measured with a vapor pressure osmometer (5520 Vapro, Wescor Inc. USA) and Hct (±0.1 %) measured on duplication samples by a centrifuge (Readacrit Centrifuge, Becton–Dickinson, USA). Two determinations were averaged for a value of Hct in individual embryos. Red blood cell concentration determined by the Coulter Analyzer was modified using an expression previously derived from a relationship with [RBC] determined by a hematometer (Tazawa et al. 2011). Mean corpuscular indices (MCV) (μm^3), mean corpuscular hemoglobin (MCH) (pg) and mean corpuscular hemoglobin concentration ([MCHb]) (g %) were calculated from Hct, [RBC] and [Hb].

Statistical analysis

All data were tested for normality and equal variance and parametric ANOVA or ANOVA on ranks were used where appropriate. Differences in egg mass, embryo body mass, Osm and hematological variables across gas exposure times or at each exposure time of all gas exposure procedures were examined using a one-way ANOVA. All pairwise multiple comparisons by the Tukey test or Dunn's method were done in SigmaStat 3.5 (Systat Software Inc., Chicago, IL, USA). ANCOVA was used to compare regression slopes in JMP 10 (SAS Institute Inc., Cary, NC, USA) and a Bonferroni correction was applied to P values to account for the pairwise slope comparisons. The significant level was $P < 0.05$. All data were presented as mean ± 1 SEM.

Results

Fresh egg mass, body mass and osmolality

Measurements were carried out on a total of 428 chicken embryos across six CO_2 treatments, including d15 embryos examined with 5 % CO_2 obtained previously (Mueller et al. 2013); 0 % ($N = 65$), 1 % ($N = 81$), 3 % ($N = 59$), 5 % ($N = 94$), 6 % ($N = 52$) and 10 % ($N = 77$). All embryos survived exposure to the various [CO_2]. Mean fresh egg

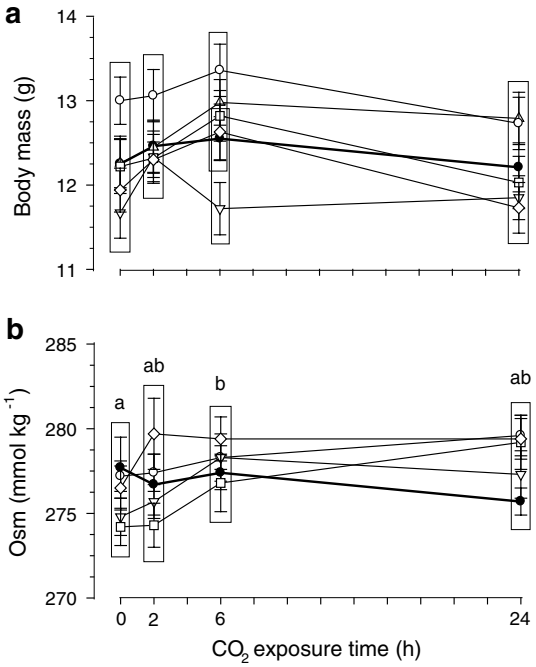

Fig. 1 Time-course changes in **a** wet body mass and **b** osmolality (Osm) of embryos during 24 h of exposure to varying CO_2. *Heavy solid lines* connect **a** mean body mass and **b** mean Osm at individual times of 0 % CO_2 (i.e., air) exposures. Mean values ± 1 SEM are plotted. Means not significantly different from each other are grouped within the same *box*. *Uncapitalized letters* in panel **b** indicate significant differences between exposure times for all treatments combined. Note that mean body mass is not significantly different between individual exposure times. CO_2 concentrations; 0 % (*closed circle*), 1 % (*upward open triangle*), 3 % (*open circle*), 5 % (*downward open triangle*), 6 % (*open square*) and 10 % (*open diamond*)

mass was 59.89 ± 0.19 g and the differences between all 24 sub-groups (six $[CO_2]$ treatments, four exposure times) were not significant ($P = 0.192$). Mean body mass was 12.37 ± 0.06 g, but there were significant differences between the 24 sub-groups ($P < 0.001$). At each exposure time, including 0 h or control, the differences of body mass between CO_2 treatments were not significant, with the single exception of 6 h of exposure where the mean body mass of embryos exposed to 1 and 3 % CO_2 differed from embryos exposed to 5 % CO_2 (Fig. 1a). Mean body mass of control embryos and at 2, 6 and 24 h of exposure was not significantly different (Fig. 1a), indicating that mean body mass was not changed during the 24 h exposure to varying $[CO_2]$.

Mean whole blood Osm was 277 ± 0.3 mmol kg^{-1} ($N = 428$). Mean Osm values of all 24 sub-groups were not significantly different ($P = 0.059$) and Osm did not differ with $[CO_2]$ at any exposure time (Fig. 1b). However, mean blood Osm at 6 h of exposure was significantly higher than mean control Osm (Fig. 1b).

Dynamics of acid–base changes during hypercapnic challenges

The control status of acid–base balance, expressed as pH_a, $[HCO_3^-]_a$ and P_aCO_2, of the control populations of all CO_2 treatment series combined (1, 3, 5, 6 and 10 % CO_2) was 7.563 ± 0.004, 25.6 ± 0.3 mmol L^{-1}, and 28.7 ± 0.6 mmHg ($N = 92$) (Fig. 2). Exposure to 1 % CO_2 (1 % CO_2, 20 % O_2) significantly decreased pH_a to 7.47 ($P < 0.001$, $N = 20$) and increased $[HCO_3^-]_a$ to 27 mmol L^{-1} after 2 h. This created a slope of ~−17 mmol L^{-1} pH^{-1} for changes in acid–base status (i.e., $\Delta[HCO_3^-]_a/\Delta pH_a$). After 24 h, pH_a increased to 7.49 and $[HCO_3^-]_a$ increased to 30 mmol L^{-1} ($P < 0.001$, $N = 21$). An increase of CO_2 to 3 % (3 % CO_2, 20 % O_2) decreased pH_a to 7.41 ($P < 0.001$, $N = 15$) and increased $[HCO_3^-]_a$ to 31 mmol L^{-1} after 2 h, resulting in a slope of ~−31 mmol L^{-1} pH^{-1}. Subsequently, 24 h later both pH_a and $[HCO_3^-]_a$ increased to 7.49 ($P < 0.001$, $N = 15$) and 35 mmol L^{-1} ($P < 0.001$), respectively. The slope further increased to ~−113 mmol L^{-1} pH^{-1}. These changes in pH_a and $[HCO_3^-]_a$ indicate that the respiratory acidosis caused by CO_2 was partially compensated by $[HCO_3^-]_a$, exceeding the value estimated from the buffer capacity (−16 mmol L^{-1} pH^{-1}).

As $[CO_2]$ and respiratory acidosis increased, partial metabolic compensation was increasingly augmented over time, i.e., 17 ± 3, 42 ± 6 and 49 ± 5 % for 2, 6 and 24 h of exposure, respectively (Table 1). For example, exposure to 10 % CO_2 decreased control pH_a of 7.56 to 7.30 at 2 h with a subsequent increase to 7.39 at 6 h and 7.41 at 24 h,

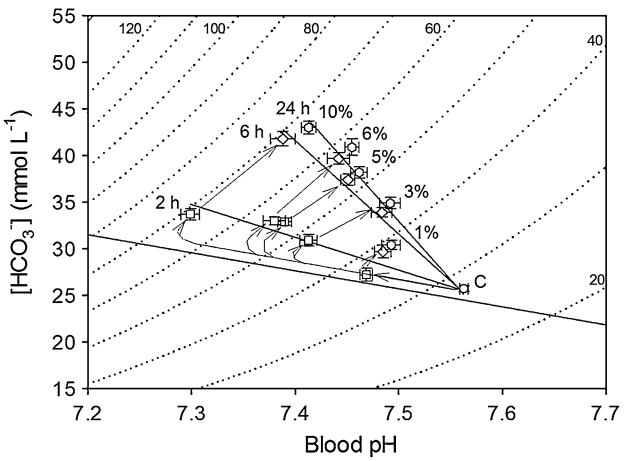

Fig. 2 Dynamics of acid–base regulation of embryos in response to 1, 3, 5, 6 and 10 % CO_2 exposures at 2, 6 and 24 h. The control acid–base status in individual CO_2 treatments was not different and thus the combined control status is shown *C*. Acid–base status at 2, 6 and 24 h of exposures to 1, 3, 5, 6 and 10 % CO_2 are connected by *individual regression lines*. The *diagonal solid line* is a buffer line indicating visually buffer capacity

Table 1 Arterialized blood PCO_2, control pH, uncompensated pH and compensated pH at 2, 6 and 24 h of hypercapnic exposure

$[CO_2]$ (%)	P_aCO_2	(A)	Uncompensated		2 h			6 h			24 h		
			(B)	(C)	pH_a	(D)	(E) (%)	pH_a	(D)	(E) (%)	pH_a	(D)	(E) (%)
1	39	7.56	7.46	0.10	7.47	0.01	10	7.48	0.02	20	7.49	0.03	30
3	47	7.56	7.38	0.18	7.41	0.03	17	7.48	0.09	50	7.49	0.10	56
5	55	7.57	7.34	0.23	7.39	0.05	22	7.45	0.11	48	7.46	0.12	52
6	58	7.56	7.32	0.24	7.38	0.06	25	7.44	0.12	50	7.46	0.14	58
10	69	7.56	7.26	0.30	7.30	0.04	13	7.39	0.13	43	7.41	0.15	50

The percent metabolic compensation at each time point is also shown

[CO_2] CO_2 concentration of hypercapnic gas mixture, P_aCO_2 mean PCO_2 of arterialized blood during 24 h hypercapnic exposure, *A* control pH, *B* uncompensated pH of hypercapnic respiratory acidosis, *C* uncompensated ΔpH (uncompensated change in pH from control pH = *A* – *B*), pH_a arterialized blood pH during respiratory acidosis, *D* compensated ΔpH (|compensated change in pH from uncompensated pH| = $|B - pH_a|$), *E* percent metabolic compensation (100 × compensated ΔpH/uncompensated ΔpH = 100 × *D*/*C*)

along with an increase of mean P_aCO_2 to 69 mmHg (Fig. 2; Table 1). The uncompensated pH of 7.26, which is predicted to occur as a result of exposure to 10 % CO_2, is estimated from an intersection of the buffer line and the PCO_2 isopleth of 69 mmHg. Here, the buffer line passing through control acid–base status, C, is $[HCO_3^-] = -16\,pH + 146.6$ and the PCO_2 isopleth of 69 mmHg is $pH = 6.095 + \log([HCO_3^-]/0.0308 \times PCO_2)$. Consequently, the uncompensated change in pH from control pH_a (i.e., uncompensated ΔpH) was 0.30 unit and the compensated change in pH_a from uncompensated pH (i.e., compensated ΔpH) was 0.04 unit at 2 h. If compensation is expressed by a percent ratio of compensated ΔpH to uncompensated ΔpH (i.e., 100 × compensated ΔpH/uncompensated ΔpH) (Tazawa et al., 1981), pH_a was compensated by ~13 % at 2 h due to metabolic alkalosis. The metabolic compensation was similarly calculated as ~43 and 50 % at 6 and 24 h, respectively (Table 1). For the remaining CO_2 treatments (i.e., 1, 3, 5 and 6 % CO_2), the uncompensated decrease in pH (i.e., uncompensated pH and uncompensated ΔpH) and the partial metabolic compensation (pH_a, compensated ΔpH and percent compensation) with time (2, 6 and 24 h) are summarized in Table 1.

The partially compensated respiratory acidosis at individual exposure times (2, 6 and 24 h) after exposure to five CO_2 gas mixtures (1, 3, 5, 6 and 10 %) was expressed by significant linear regressions (Fig. 2);

$$\left[HCO_3^-\right] = -35.2 \times pH + 291.8 \quad (r = 0.939,\ t = 5.455,\ P < 0.001) \text{ at 2 h} \tag{1}$$

$$\left[HCO_3^-\right] = -100.2 \times pH + 783.2 \quad (r = 0.948,\ t = 5.944,\ P < 0.001) \text{ at 6 h} \tag{2}$$

$$\left[HCO_3^-\right] = -124.4 \times pH + 966.4 \quad (r = 0.954,\ t = 6.361,\ P < 0.001) \text{ at 24 h} \tag{3}$$

The slope at 2 h was significantly low compared to 6 h ($P = 0.013$) and 24 h ($P = 0.004$), which did not differ from each other ($P = 1$).

Dynamics of hematological changes during hypercapnic challenges

Mean control values of hematological respiratory variables determined on 110 embryos were as follows; Hct: 27.7 ± 0.2 %, [RBC]: 2.09 ± 0.02 × 10^6 μL^{-1}, MCV: 133.1 ± 0.4 μm^3, [Hb]: 8.9 ± 0.06 g %, MCH: 42.9 ± 0.2 pg and [MCHb]: 32.3 ± 0.1 g %.

The Hct of embryos exposed to varying $[CO_2]$ (1, 3, 5, 6 and 10 %) did not differ among treatments at any exposure time (Fig. 3a). Hematocrit also did not differ significantly ($P = 0.085$) from the baseline (0 % CO_2) in control embryos or at 2 h ($P = 0.055$) and 6 h ($P = 0.052$). However, by 24 h, mean Hct significantly decreased at all $[CO_2]$s ($P < 0.001$). Although the decrease from the control was not significant at 2 h, it was significant at 6 h and 24 h, with a significant difference between 6 and 24 h. The percent decrease in Hct is calculated as $\Delta Hct = 100 \times (Hct_{ex} - Hct_c)/Hct_c$, where Hct_c and Hct_{ex} are mean control Hct and mean Hct at a time of exposure to 1–10 % CO_2. Percent change in Hct was ~−5 % at 6 h and ~−14 % at 24 h.

Mean [RBC] in the control and at 2 h differed among the five CO_2 treatments ($P = 0.002$ and 0.008, respectively), but mean [RBC] at 6 and 24 h was not different among treatments (Fig. 3b). The mean value of the five CO_2 treatments combined remained unchanged until 6 h, but significantly decreased at 24 h ($P < 0.001$). The percent decrease, expressed by Δ[RBC] calculated as $\Delta[RBC] = 100 \times ([RBC]_{ex} - [RBC]_c)/[RBC]_c$, was ~−11 % at 24 h.

Control MCV was not significantly different among CO_2 treatments (Fig. 3c). Mean MCV in the 1–6 % CO_2 treatments was not different at any time of exposure and significantly decreased ($P < 0.001$) from the control at 2,

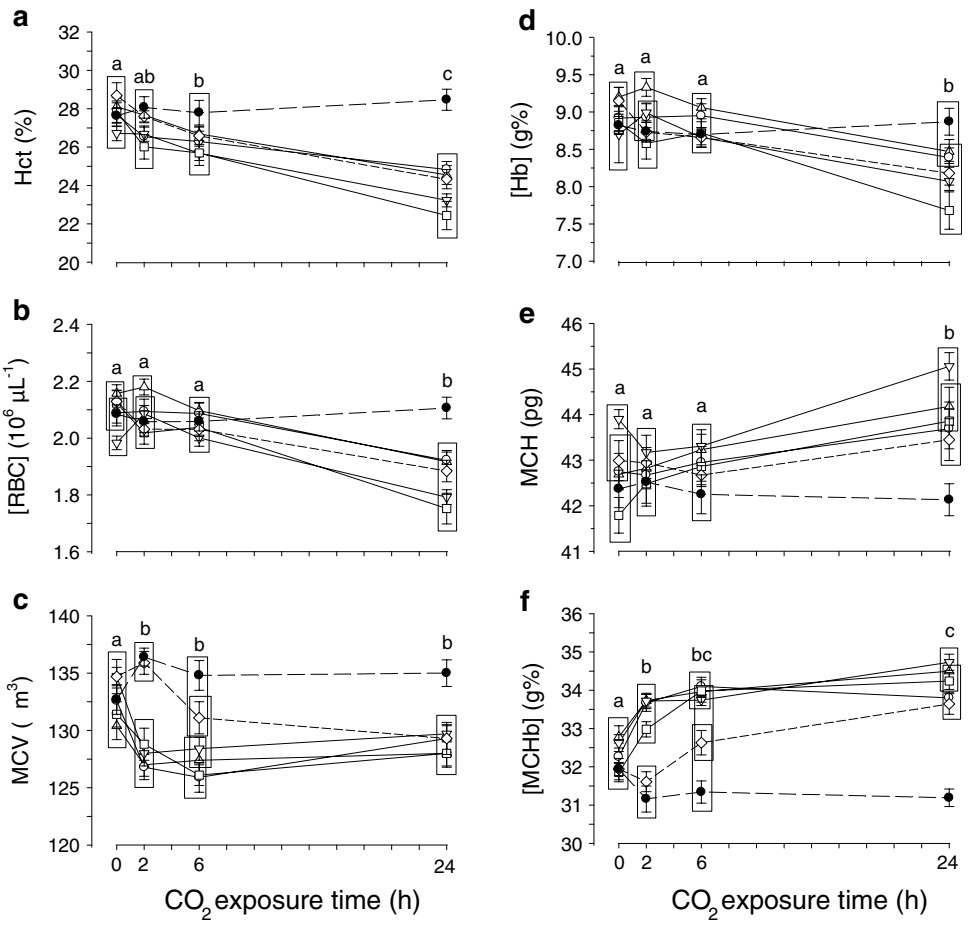

Fig. 3 Time-course changes in **a** hematocrit (Hct) **b** red blood cell concentration ([RBC]) **c** mean corpuscular volume (MCV) **d** hemoglobin concentration ([Hb]) **e** mean corpuscular hemoglobin (MCH) and **f** mean corpuscular hemoglobin concentration ([MCHb]) in embryos exposed to variable CO_2 levels. Mean values ± 1 SEM are plotted. Means not significantly different from each other are grouped within the *same box*. *Uncapitalized letters* in *each panel* indicate significant differences between exposure times for all treatments combined. CO_2 concentrations; 0 % (*closed circle*), 1 % (*upward open triangle*), 3 % (*open circle*), 5 % (*downward open triangle*), 6 % (*open square*) and 10 % (*open diamond*). The *lines with long and short dashes* connect responses to 0 and 10 % CO_2 exposures, respectively

6 and 24 h of exposure. Percent change in mean corpuscular volume (ΔMCV) (i.e., $\Delta MCV = 100 \times (MCV_{ex} - MCV_c)/MCV_c$) of the 1–6 % CO_2 treatments combined at 2 and 6 h was ~−4 %. Mean corpuscular volume in 10 % CO_2 remained unchanged at 2 h and then decreased significantly afterwards ($P < 0.001$). At 24 h, MCV did not differ among the CO_2 treatments, and mean MCV was significantly lower ($P < 0.001$) compared to baseline MCV, i.e., the decrease expressed by ΔMCV was ~−3 %.

Mean [Hb] at all [CO_2]s was not significantly different in control or 6 h, but differed at 2 and 24 h (Fig. 3d). Although there were subtle differences among groups, mean [Hb] of 1–10 % CO_2 treatments combined at 24 h was significantly lower ($P < 0.001$) from mean [Hb] in control and at 2 and 6 h.

Mean MCH in control ($P < 0.001$) and at 24 h ($P < 0.001$) was different among the five 1–10 % CO_2 treatments (Fig. 3e). However, comparison of mean MCH between exposure times for all CO_2 treatments combined was significantly higher ($P < 0.001$) at 24 h compared with control, 2 and 6 h.

Mean control [MCHb] was not different between all 0–10 % CO_2 treatments (Fig. 3f). Mean [MCHb] in 1–6 % CO_2 was also not different at 2, 6 and 24 h, but [MCHb] in 10 % CO_2 remained unchanged from the baseline at 2 h with a subsequent increase afterwards. Consequently, mean [MCHb] of 1–6 % CO_2 treatments combined, excluding 10 % CO_2, increased significantly ($P < 0.001$) from the control during 2, 6 and 24 h of exposure.

Discussion

This study of compensatory acid–base and hematological dynamics in response to CO_2 exposure was undertaken based on previous studies in chicken embryos suggesting that there might be major time-dependent influences of [O_2] on metabolic compensation for hypercapnic (5 % CO_2) respiratory acidosis and the potential effect of [CO_2] (Burggren et al. 2012; Mueller et al. 2013). Consequently, we have elucidated the time-specific, dose-dependent effects of graded increases in extrinsic CO_2 in a normoxic environment on acid–base disturbances with metabolic

compensation and hematological respiratory variables over a 24 h exposure.

Body mass and osmolality

Exposure to CO_2 at any concentration had no influence on embryo body mass, which agrees with a previous study showing no effect of a 24 h exposure to different [O_2] with 5 % CO_2 on body mass (Mueller et al. 2013). Although mean blood Osm increased at 6 h compared with the control, the changes in Osm are not related to either body mass (Fig. 1) nor the significant changes in Hct and other hematological respiratory variables (Fig. 3).

Acid–base dynamics

Time-specific, dose-dependent metabolic compensation for hypercapnic respiratory acidosis

Insufficient tissue O_2 caused by moderate (14 or 15 % O_2) or severe (10 % O_2) extrinsic hypoxia without CO_2 (normocapnic hypoxia) induces an uncompensated metabolic acidosis after a few hours up to 24 h (Tazawa 1986; Burggren et al. 2012; Tazawa et al. 2012). Exposure to 5 % CO_2 causes respiratory acidosis subjected to metabolic compensation that varies with O_2 concentration (Mueller et al. 2013). The hypercapnic respiratory acidosis in 20 % O_2 or higher was partially compensated by ~40–50 % at 24 h, and complete compensation did not occur even if exposure was continued for a few more developmental days (Dawes and Simkiss 1971; Mueller et al. 2013). Consequently, we predicted that the dynamics of metabolic compensation for hypercapnic respiratory acidosis could be elucidated by hypercapnic exposure over the time points of 2, 6 and 24 h.

The addition of only 1 % CO_2 in normoxia produced hypercapnic respiratory acidosis in d15 embryos (Fig. 2; Table 1). However, metabolic compensation was small compared with higher [CO_2] exposures. Although the time-specific metabolic compensation varied considerably among the five CO_2 gas mixtures (Table 1), the partially compensated respiratory acidosis was expressed by significant linear regressions at each exposure time, i.e., Eqs. 1, 2 and 3 for compensation at 2, 6 and 24 h, respectively (Fig. 2). Using these equations, together with the Henderson-Hasselbalch equation for a PCO_2 isopleth and buffer line equation, we can estimate arithmetically partial metabolic compensation at 2, 6 and 24 h of exposure to any CO_2 gas mixtures (Fig. 4). For instance, pH at 2 h of exposure to 10 % CO_2 gas mixture (corresponding to pH_a), calculated at an intersection of the 2 h-regression line (Eq. 1) and 69 mmHg-PCO_2 isopleth, is 7.307 (while pH_a measured was 7.30, Table 1). Because uncompensated pH is calculated as 7.255 at the intersection of the buffer line and the 69 mmHg-PCO_2 isopleth, uncompensated change in pH, i.e., uncompensated ΔpH, is 0.308 unit (while mean control pH is 7.563) and compensated ΔpH is 0.052 unit (Fig. 4). Consequently, percent partial metabolic compensation (100 × compensated ΔpH/uncompensated ΔpH) is calculated as ~17 % (while compensation estimated from measured pH_a was ~13 %, Table 1). Likewise, uncompensated pH originated from exposure to 1, 3, 5 and 6 % CO_2 results in 7.455, 7.390, 7.336 and 7.316, respectively. The differences of these values of uncompensated pH from the control pH_a (7.563), i.e., uncompensated ΔpH, are 0.108, 0.173, 0.227 and 0.247 units, increasing with [CO_2] as predicted (Fig. 4). The magnitude of compensated respiratory acidosis (compensated ΔpH) at 2, 6 and 24 h of exposure to 1, 3, 5, 6 and 10 % is also proportional to [CO_2] (Fig. 4). Consequently, the percent metabolic compensation is ~17, 46 and 53 % for 2, 6 and 24 h exposures to five CO_2 gas mixtures.

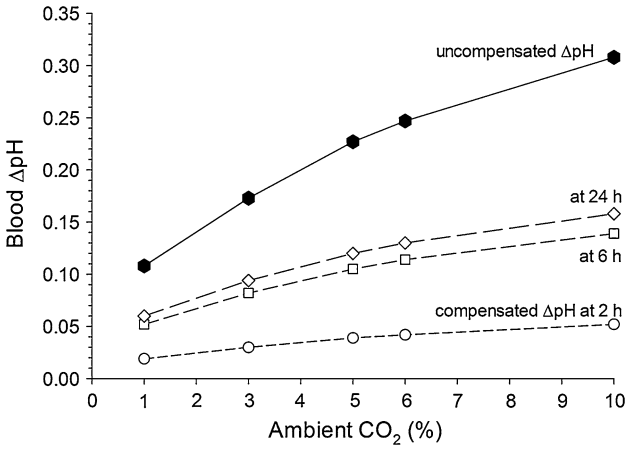

Fig. 4 The uncompensated respiratory acidosis (i.e., uncompensated ΔpH) caused by exposure to ambient hypercapnia (1, 3, 5, 6 and 10 % CO_2) and the partial metabolic compensation (i.e., compensated ΔpH) at 2, 6 and 24 h of hypercapnic exposures. The percent metabolic compensation is the ratio of compensated ΔpH to uncompensated ΔpH times 100; i.e., ~17, 46 and 53 % at 2, 6 and 24 h, respectively

Significance of time-course investigation

In addition to investigations into the time course of respiratory acidosis in chicken embryos using CO_2 exposure, acid–base compensation has also been studied by lowering eggshell gas conductance, by either reducing the gas diffusion area of eggshell using gas-impermeable egg wrapping, or by exposing the eggs to SF_6 atmosphere with an accompanying PO_2 decrease (Tazawa et al. 1971, 1981; Tazawa 1981a, 1982, 1986). This type of respiratory acidosis may be referred to as 'low-conductance respiratory acidosis' versus 'hypercapnic respiratory acidosis' produced

by exposure to hypercapnia. In low-conductance respiratory acidosis, blood pH_a begins to decrease in 10 min or less, concurrent with an increase in $[HCO_3^-]_a$. This respiratory acidosis is compensated within 1 h, with resultant rapid metabolic compensation, which stalls 3–4 h later (Tazawa 1981a, 1982; Tazawa et al. 1981). Likewise, metabolic compensation for acid–base disturbances produced by intravenous infusion of $NaHCO_3$ or NH_4Cl takes place within a similar time course (Tazawa 1982, 1986). For instance, infusion of 0.02 mL of 5 M NH_4Cl into the allantoic vein decreased control pH_a, along with PCO_2 isopleth, within 10 min, with a subsequent increase in pH_a 1–6 h later. Similarly, metabolic alkalosis produced by infusion of 0.15 mL of 1 M $NaHCO_3$ was completely reversed after 6 h of infusion. Thus, time-course changes in metabolic compensation for hypercapnic respiratory acidosis likely occur similarly. That is, the acid–base status turns to uncompensated respiratory acidosis within ~30 min of exposure to hypercapnia and metabolic compensation occurs until 24 h, ending with partial compensation. Although in the current study we did not determine acid–base status within 2 h of perturbations, we evaluated early acid–base disturbances from the buffer line and PCO_2 isopleths as uncompensated respiratory acidosis.

The results of the present study, in which an initial acidosis is followed by subsequent metabolic compensation, vary from previous studies examining chronic hypercapnia in chicken embryos. When chicken embryos were exposed to chronic hypercapnia (4 % CO_2) during the second half of incubation, blood $[HCO_3^-]_a$ and pH_a were consistently higher than control embryos (Everaert et al. 2008). This produced metabolic alkalosis without hypercapnic respiratory acidosis. In another report on continuing exposure of broiler and layer embryos to 4 % CO_2, blood P_aCO_2, pH_a and $[HCO_3^-]_a$ increased in both strains (Everaert et al. 2011a, b). Although there was a difference in increased $[HCO_3^-]_a$ between broiler and layer embryos, the data likely suggest that hypercapnic respiratory acidosis developed, but was then eliminated via metabolic alkalosis. Moreover, it has been reported that continuous exposure of chicken embryos to 1.5 % CO_2 during the first 10 days of incubation increased blood P_aCO_2 with a concomitant increase in $[HCO_3^-]_a$ and without a change in pH_a in d10 and d11 embryos (Bruggeman et al. 2007). Accordingly, they suggested that increased $[HCO_3^-]_a$ buffered the higher PCO_2 in CO_2-incubated embryos to stabilize pH_a. That is, hypercapnic (1.5 % CO_2) respiratory acidosis was fully compensated by metabolic alkalosis, even 24–48 h after termination of 1.5 % CO_2-incubation. Collectively, these different studies suggest that the acid–base responses to CO_2 are complicated and that an experiment elucidating the time course of acid–base status during perturbations and also recovery in air is required.

Hematological dynamics

The hematological changes in response to hypercapnic exposure reported in the present study are very similar to those previously reported by Burggren et al. (2012) and Mueller et al. (2013). Mean Hct gradually decreased with exposure time (Fig. 3a). Because [RBC] remained unchanged during 6 h of exposure (Fig. 3b), the decrease in Hct can be attributed to the decrease in MCV in the 1–6 % CO_2 exposures (Fig. 3c). The decrease in MCV in the 10 % CO_2 exposure was slower than the other treatments, but contributed partially to the decrease in Hct at 24 h. [Hb] responded to altered CO_2 similar to [RBC] (Fig. 3d). Because MCH remained unchanged during 6 h of exposure, the changes in [MCHb] reflected inversely the changes in MCV (Fig. 3f cf. Fig. 3c). The changes in MCV and [MCHb] in response to altered $[CO_2]$ were different between 1–6 % CO_2 exposures and 10 % CO_2 treatment during early (2 and 6 h) exposure. Besides these responses of MCV and [MCHb], the responses of other variables were the same across all $[CO_2]$s from 1 to 10 %. This leads us to reject our original hypothesis that hematological variables would show graded changes according to the degree of altered $[CO_2]$. In a previous in vitro experiment, which helped develop our hypothesis, RBCs were dehydrated when the blood was equilibrated with air at 37 °C (i.e., ΔHct became negative) and then subjected to graded hydration (positive changes in ΔHct) with addition of 3–6 % CO_2 in normoxia in d15 embryos (Andrewartha et al. 2011). However, dehydration of RBCs in vivo occurs equally independently of $[CO_2]$ at 24 h of exposure, except during 2–6 h when RBCs are dehydrated in 1–6 % CO_2 exposure but remains unchanged in 10 % CO_2. Further study is required to examine if exposure to >10 % CO_2 may induce hydration of RBCs and alter the responses of hematological respiratory variables from that measured in 1–6 % CO_2.

Summary

We hypothesized that exposure to 5–10 % CO_2 would not be an unrealistic imposition on chicken embryos and thus disturbances of acid–base balance and hematological respiratory variables caused by exposure to altered $[CO_2]$ of <10 % would be regulated in proportion to $[CO_2]$ at any time of a 24 h exposure. We discovered that the uncompensated hypercapnic respiratory acidosis occurred in proportion to $[CO_2]$ and was compensated partially in proportion to $[CO_2]$ at 2 h, resulting in a similar percent partial metabolic compensation (~17 %) across varying $[CO_2]$. The metabolic compensation progressed during the next 4 h (~46 %) and increased slightly more at 24 h (~53 %),

suggesting termination of the compensation process well short of complete compensation. The regulation of Hct, [RBC], [Hb] and MCH during 24 h exposure occurred identically across varying [CO_2]. However, the response of MCV and [MCHb] to 10 % CO_2 exposure differed from 1 to 6 % CO_2 exposures. This suggests that an imposition of ≥10 % CO_2 on chicken embryos impairs or alters the responses of hematological respiratory variables and probably also acid–base balance. Examination of acid–base and hematological responses and dynamics to [CO_2] ≥10 % will help define the hypercapnic threshold at which chicken embryos can successfully compensate. Additionally, exposing embryos of burrowing bird species to high [CO_2] will allow us to examine if acid–base and hematological responses to hypercapnia differ depending upon the natural incubation environment.

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