Respiratory Physiology & Neurobiology 239 (2017) 55–63

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Dynamics of acid-base and hematological regulation in day 15 chicken embryos (*Gallus gallus domesticus*) exposed to graded hypercapnia and hypoxia

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ARTICLE INFO

Article history:
Received 19 November 2016
Received in revised form 22 January 2017
Accepted 6 February 2017
Available online 9 February 2017

Keywords:
Chicken embryos
Hypercapnia
Hypoxia
Acid-base disturbances
Hematology
Metabolic compensation

ABSTRACT

Most experiments examining acid-base regulation of chicken embryos have employed static, single time point measurements rather than dynamic, multiple time point measurements that might reveal additional components of developing acid-base regulation. Thus, we studied blood acid-base balance and hematology of day 15 chicken embryos under 24 h exposure to graded hypercapnia (1%–7% CO_2) accompanied by graded hypoxia (20% O_2 down to 13% O_2). Across all hypercapnic/hypoxic environments, respiratory acidosis occurred 2 h after exposure in proportion to the magnitude of hypercapnia. An additional metabolic alkalosis occurred in ≥16% O_2, and metabolic acidosis in ≤14% O_2. As exposure progressed, compensatory metabolic alkalosis occurred in all groups at 6 h, but partial metabolic compensation could not be preserved as hypoxia increased (≤18% O_2). Across all hypercapnic/hypoxic groups, hematocrit, mean corpuscular volume and red blood cell concentration significantly increased by 24 h, most likely due to hypoxia rather than hypercapnia. Overall, day 15 chicken embryos cannot maintain even partial compensation in their acid-base physiology after 24 h exposure to hypercapnic/hypoxic environments of ≥5% CO_2 + ≤15% O_2.

1. Introduction

Gas exchange in chicken embryos takes place by diffusion across the eggshell. Consequently, hypercapnia of various levels in an otherwise normoxic environment causes respiratory acidosis, with its extent depending upon blood buffer capacity during early (~1 h) exposure. However, day 15 (d15) embryos initiate metabolic compensation within ~2 h of exposure (Mueller et al., 2013, 2014; Burggren et al., 2015). Since embryos lack convective ventilation, and thus the capacity for respiratory compensation of respiratory acidosis, compensatory metabolic alkalosis proceeds along a Pco_2 isopleth during the next few hours (e.g., ~6 h). The magnitude of respiratory acidosis and compensatory metabolic alkalosis are proportional to imposed CO_2 concentrations ($[CO_2]$). As long as $[CO_2]$ is restricted to ≤10%, a metabolic compensation of ~45–50% develops independent of the actual magnitude of respiratory acidosis (Mueller et al., 2013, 2014). However, only a partial metabolic compensation progresses during the next 6 h–24 h of hypercapnic exposure. This compensation can only be maintained indefinitely if environmental O_2 concentrations ($[O_2]$) are at normoxic or hyperoxic levels (Dawes and Simkiss, 1971; Burggren et al., 2012, 2015; Mueller et al., 2013, 2014).

Moderate (15% O_2) to severe (10% O_2) hypoxia without hypercapnia creates metabolic acidosis without respiratory compensation (Burggren et al., 2012; Tazawa et al., 2012). The magnitude of metabolic acidosis is dependent on $[O_2]$. Accordingly, in environmental hypercapnia (5% CO_2) combined with moderate hypoxia (15% O_2), partial metabolic compensation for respiratory acidosis caused by hypercapnia cannot be preserved after ~6 h of hypercapnic exposure and returns to a non-compensated respiratory acidosis 24 h later (Burggren et al., 2012; Mueller et al., 2013). In hypercapnia (5% CO_2) combined with severe hypoxia (10% O_2), embryos can survive at least 2 h as pH decreases along the buffer line and thereafter along the Pco_2 isopleth. However, regulation ceases at a fatally low $[HCO_3^-]$ of ~10 mmol L^{-1} in d15 embryos (Tazawa et al., 2012). In severe hypoxia (10% O_2), it is likely that severe metabolic acidosis can't be compensated by metabolic alkalosis.

To summarize these findings, by d15 (during a time of rapid growth and development of physiological function), chicken

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http://dx.doi.org/10.1016/j.resp.2017.02.001
1569-9048/

embryos exposed to varied hypercapnia can respond to respiratory acidosis with a compensatory metabolic alkalosis. Acid-base regulation in response to hypoxia has also been studied in the presence or absence of hypercapnia (i.e., 5% CO_2). However, previous investigations into the limits to acid-base regulation, and thus the capacity of acid-base regulation in these altered gaseous environments, were separately studied hypercapnia with 20% O_2 or varied O_2 with 5% CO_2. Yet, possible synergies/interactions of these various conditions have not been identified. Consequently, the present study was conducted to elucidate the effects of a combination of varied, graded hypercapnia and varied, graded hypoxia in d15 chicken embryos. This is a novel approach that will allow us to learn the full capability of tolerance and reserve capacity of acid-base regulation in d15 chicken embryos. The levels of hypoxia and hypercapnia used include those that, while unlikely to be experienced by embryos in the wild, help elucidate potential reserve capacity of acid-base and hematological regulation. We hypothesized that d15 chicken embryos exposed to hypercapnic hypoxia (i.e., 5% CO_2 and 15% O_2, referred to as 5% CO_2 + 15% O_2) can still regulate acid-base disturbances produced in even more extreme hypercapnic and hypoxic environments. In an environment of 6% CO_2 + 14% O_2, or 7% CO_2 + 13% O_2, d15 embryos may suffer metabolic acidosis in addition to respiratory acidosis, but partially compensate pH due to compensatory metabolic alkalosis. However, we predicted subsequent metabolic acidosis caused by further severe hypoxia, e.g., <13% O_2, will increase to a level that cannot be compensated by metabolic alkalosis and thus will be lethal, revealing the limits of acid-base compensation.

Blood acid-base regulation in chicken embryos is closely correlated with, and perhaps even dependent upon, changes in hematocrit (Hct) and other hematological respiratory variables. For example, hypercapnia (5%) decreases Hct through a reduction in red blood cell concentration ([RBC]) after 24 h of exposure from d14 of development (Burggren et al., 2012). Further examination of the decrease in Hct in response to varied hypercapnia (1–10%), indicates that initially (at 2 h) Hct decreases through a decrease in mean corpuscular volume (MCV), followed by a subsequent decrease in [RBC] at 24 h (Mueller et al., 2013, 2014). Hypoxia also affects the hematology of chicken embryos, but in different ways from hypercapnia. For example, hypoxia (15% O_2) increases Hct with increases in MCV and [RBC] during or after 24 h of exposure (Burggren et al., 2012; Kohl et al., 2015), which is a different hematological response than produced by 24 h of hypercapnia. Combining hypercapnia and hypoxia (24 h of 5% CO_2 + 15% O_2) increases Hct because of a concomitant increase in MCV and subsequent increase in [RBC] at 24 h (Mueller et al., 2013; Kohl et al., 2015). In even more hypercapnic/hypoxic environments (e.g., 6% CO_2 + 14% O_2, or 7% CO_2 + 13% O_2), we hypothesized that d15 Lohmann White Leghorn embryos will similarly increase Hct during 24 h, in particular due to concomitant increase in MCV with subsequent increase in [RBC] at 24 h. Finally, since Hct increases in hypoxic environments, we have investigated how and when low [O_2] under 20% begins to increase Hct, and what the underlying mechanisms are for this change in d15 embryos.

2. Materials and methods

2.1. Incubation of eggs and exposure to gas mixtures

All experiments were conducted at the University of North Texas (Denton, TX, USA) under approval by the UNT Institutional Animal Care and Use Committee. Fertile eggs of the domestic chicken (Lohmann White Leghorn) were obtained weekly from a hatchery at Texas A&M University (College Station, Texas, USA) and shipped to the laboratory at UNT. Eggs were lightly cleaned in running water with a sponge to remove extraneous materials and then weighed to 0.01 g by an electronic balance after drying. Eggs were numbered and set in an incubator (model 1502, G.Q.F. Manuf., GA, USA) at noon according to an incubation schedule. Temperature and relative humidity of the incubator was 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 locate an allantoic vein and the eggshell over the vein was marked for blood collection to be made on d15. Eggs were randomly divided into "control" eggs and "gas-exposed" eggs, and transferred to a second desk-top incubator (model 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 (26.8 cm x 29.7 cm) gas exposure bag that could accommodate up to 16 eggs. The bag was ventilated throughout the exposure period at a rate of ~600 mL min^{-1} with a gas mixture provided by a Wösthoff gas mixing pump (oHG, Bochum, Germany). It was previously shown that there was no difference in wet or dry body mass or in blood gas or hematological variables between control embryos and experimental embryos exposed to air ventilated for 24 h in the bag, verifying effectiveness of gas exposure bag system (Burggren et al., 2012). Gas-exposed eggs were placed in the bag at noon on d14 for 24 h exposure or exposed to the gas mixture for 2 h or 6 h on d15 (2 h or 6 h exposure).

Eight experimental groups, each with a corresponding control incubated in air (21% O_2, 0% CO_2), were used in these experiments. The gas mixtures, which were all balanced by N_2, for the experimental groups were as follows;

- [1] 1% CO_2 + 20% O_2,
- [2] 1% CO_2 + 19% O_2,
- [3] 2% CO_2 + 18% O_2,
- [4] 3% CO_2 + 17% O_2,
- [5] 4% CO_2 + 16% O_2,
- [6] 5% CO_2 + 15% O_2,
- [7] 6% CO_2 + 14% O_2 and
- [8] 7% CO_2 + 13% O_2.

2.2. Blood collection and analysis

On d15 of incubation, arterialized blood was collected from the allantoic vein. Blood could not be collected from eggs while they were in the gas exposed bag, so an aluminum foil wrapping method was used to preserve *in vivo* blood gases during the brief period of blood collection, as previously described (Burggren et al., 2012). Immediately after removal from the gas exposed bag, the egg was lightly wrapped in an aluminum foil, a small ~1 cm window was torn in the foil over the site of the vein and a 6–8 mm diameter region removed in the eggshell. The underlying vein was gently lifted by forceps through the hole and ~0.4 mL of blood immediately collected into a 1 mL heparinized plastic syringe (see Tazawa and Mochizuki, 1977; Burggren et al., 2012). Blood sampling was typically completed within ~1 min after egg removal from the gas exposure bag.

Blood was gently emptied into a 1.5 mL plastic vial and ~120 μL of blood was immediately aspirated into a blood gas analyzer ($ABL^{TH}5$, Radiometer Medical A/S, Copenhagen, Denmark) for determination of pH, Pco_2 and [HCO_3^-]. The relationship between pH and [HCO_3^-] 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 for the Davenport diagram was previously determined from a buffer value of −16 mmol L^{-1} $mmHg^{-1}$ (Burggren et al., 2012).

Table 1
Eight groups of CO_2, O_2 gas mixtures, fresh egg mass (g), body mass (g) of embryos examined before exposure (0 h, control) and at 2, 6 and 24 h of exposure with statistical analyses. Uppercase bold letters indicate a significant difference between treatments within a time point, lowercase bold letters indicate significant differences across time points within a treatment. In exposure to [7] 6% CO_2, 14% O_2 and [8] 7% CO_2, 13% O_2, a few or all embryos died during 6–24 h of exposure and the number of living embryos which were analyzed for acid-base and hematology was indicated in the numerator [gp]; group.

[gp] CO_2, O_2	Egg mass (N)	Body mass (N)				P
		0 h	2 h	6 h	24 h	
Combined control groups	59.12 ± 0.34 (129)	12.41 ± 0.11 (129)	–	–	–	–
[1] 1%, 20%	60.31 ± 0.42 (81)	12.26 ± 0.32 (20)	12.45 ± 0.31 (20)	12.98 ± 0.27 (20)	12.79^{A} ± 0.31 (21)	0.351
[2] 1%, 19%	59.35 ± 0.54 (51)	13.04 ± 0.34 (10)	12.25 ± 0.50 (13)	12.41 ± 0.32 (14)	12.10^{AB} ± 0.18 (14)	0.329
[3] 2%, 18%	59.95 ± 0.55 (48)	11.94 ± 0.30 (11)	12.41 ± 0.25 (12)	12.61 ± 0.23 (11)	11.88^{ABC} ± 0.18 (14)	0.100
[4] 3%, 17%	58.76 ± 0.52 (55)	12.60^{a} ± 0.27 (13)	12.52^{a} ± 0.31 (14)	12.80^{a} ± 0.23 (14)	$11.28^{BC,b}$ ± 0.38 (14)	**0.003**
[5] 4%, 16%	59.12 ± 0.44 (74)	12.45^{a} ± 0.35 (17)	12.81^{a} ± 0.26 (19)	13.02^{a} ± 0.32 (19)	$11.67^{ABC,b}$ ± 0.26 (19)	**0.010**
[6] 5%, 15%	58.73 ± 0.39 (99)	12.58^{a} ± 0.27 (27)	13.02^{a} ± 0.33 (19)	12.23^{a} ± 0.28 (23)	$11.11^{BC,b}$ ± 0.23 (30)	**<0.001**
[7] 6%, 14%	59.55 ± 0.47 (74)	12.43^{a} ± 0.26 (19)	12.37^{a} ± 0.49 (12)	13.67^{a} ± 0.37 (12/13)	$10.72^{C,b}$ ± 0.21 (19/30)	**<0.001**
[8] 7%, 13%	60.32 ± 0.51 (62)	11.85^{a} ± 0.23 (12)	11.68^{a} ± 0.22 (15)	12.23^{a} ± 0.26 (12/25)	– (0/10)	0.292
P	0.076	0.364	0.193	0.051	**<0.001**	

The remaining blood was stirred in the vial and assayed for [RBC] (10^6 cells μL^{-1}) and hemoglobin concentration ([Hb], g%) using a hematology analyzer (Beckman Coulter Analyzer A^{c}T10, USA). Hct (±0.1%) was measured on duplicate samples using a hematocrit centrifuge (Readacrit Centrifuge, Becton-Dickinson, USA). Two determinations were averaged for a value of Hct in individual embryos. [RBC] determined by the Coulter Analyzer was modified using an expression previously derived from a relationship with [RBC] determined by a hemocytometer (Tazawa et al., 2011). Mean corpuscular volume (μm^3), mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration ([MCHb], g%) were calculated from Hct, [RBC] and [Hb], i.e., MCV = 10 x Hct/[RBC], MCH = 10 x [Hb]/[RBC] and [MCHb] = 100 x [Hb]/Hct.

Osmolality (Osm, mmol kg^{-1}) can be a key determinant of red blood cell volume, and thus hematocrit. Consequently, Osm was measured with a vapor pressure osmometer (5520 Vapro, Wescor Inc., USA).

After blood collection, embryos were euthanized by putting the eggs in a plastic bag ventilated by N_2 continuously, the yolk and extra-embryonic membranes removed and the embryo's wet body mass determined to 0.01 g with an electronic balance.

2.3. Statistical analysis

All data were tested for normality and equal variance and parametric ANOVA or ANOVA on ranks was used where appropriate. Differences in egg mass, body mass of embryos, Osm and hematological variables across gas exposure times (0, 2, 6 and 24 h) or at each exposure time of all gas exposure procedures (8 groups) were examined by a one-way ANOVA with post hoc (pair-wise) multiple comparisons by the Tukey test or Dunn's method. Differences in acid-base variables across gas exposure times were examined by a one-way ANOVA. A significance level of $P < 0.05$ was adopted for all statistical tests. All data were presented as mean ± 1 SEM.

3. Results

3.1. Fresh egg mass and embryo mass

3.1.1. Egg mass

Mean egg masses between the eight exposure groups were not significantly different (P = 0.076) (Table 1). Similarly, the mean egg masses between four exposure sub-groups within eight individual groups were not significantly different.

3.1.2. Embryo mass

Embryo body mass was affected in some but not all groups by hypercapnic/hypoxic exposure (Table 1). Essentially, the longer an exposure persisted, and the more severe the combination of hypoxia and hypercapnia, the greater the reduction in body mass.

3.2. Blood osmolality

Mean blood osmolality of all embryos was 278 ± 0.3 mmol kg^{-1} (N = 509). Mean Osm did not significantly differ (P = 0.670) between the control (0 h), 2, 6 and 24 h exposure sub-groups in all eight altered CO_2—O_2 groups.

3.3. Changes in acid-base balance in response to graded hypercapnic and hypoxic exposures

3.3.1. Control acid-base status

As expected, there was no significant difference in the acid-base characteristics of the eight control groups exposed to air (21% O_2, 0% CO_2), with a pH of 7.565 ± 0.003 (P = 0.550), a [HCO_3^-] of 25.5 ± 0.2 mmol L^{-1} (P = 0.764) and a P_{CO2} of 28.4 ± 0.4 mmHg (P = 0.829) (N = 129). The number of determinations in individual gas mixtures is shown in Table 1.

3.3.2. Acid-base changes in 1% CO_2 with normoxia

Exposure to 1% CO_2 in normoxia (20% O_2) significantly decreased pH to 7.47 ($P < 0.001$) with slight increase in [HCO_3^-] to 27 mmol L^{-1} (P = 0.355) after 2 h (Fig. 1A). This created a slope ($\Delta[HCO_3^-]/\Delta pH$) of ~−17 mmol L^{-1} pH^{-1} for changes in acid-base status, which was almost identical to the buffer capacity (−16 mmol L^{-1} pH^{-1}). As time progressed from 6 to 24 h in this condition, pH increased to 7.48 and 7.49 and [HCO_3^-] increased to 30 and 31 mmol L^{-1} at 6 and 24 h, respectively, along a mean Pco_2 isopleth of ~40 mmHg. Both of these variables were significant at 24 h compared with 2 h (P = 0.030 and P = 0.004 for pH and [HCO_3^-], respectively). Accordingly, respiratory acidosis produced by exposure to 1% CO_2 + 20% O_2 was compensated after 6 and 24 h of exposure, as follows. First, uncompensated pH of respiratory acidosis was predicted to be 7.437 from intersection

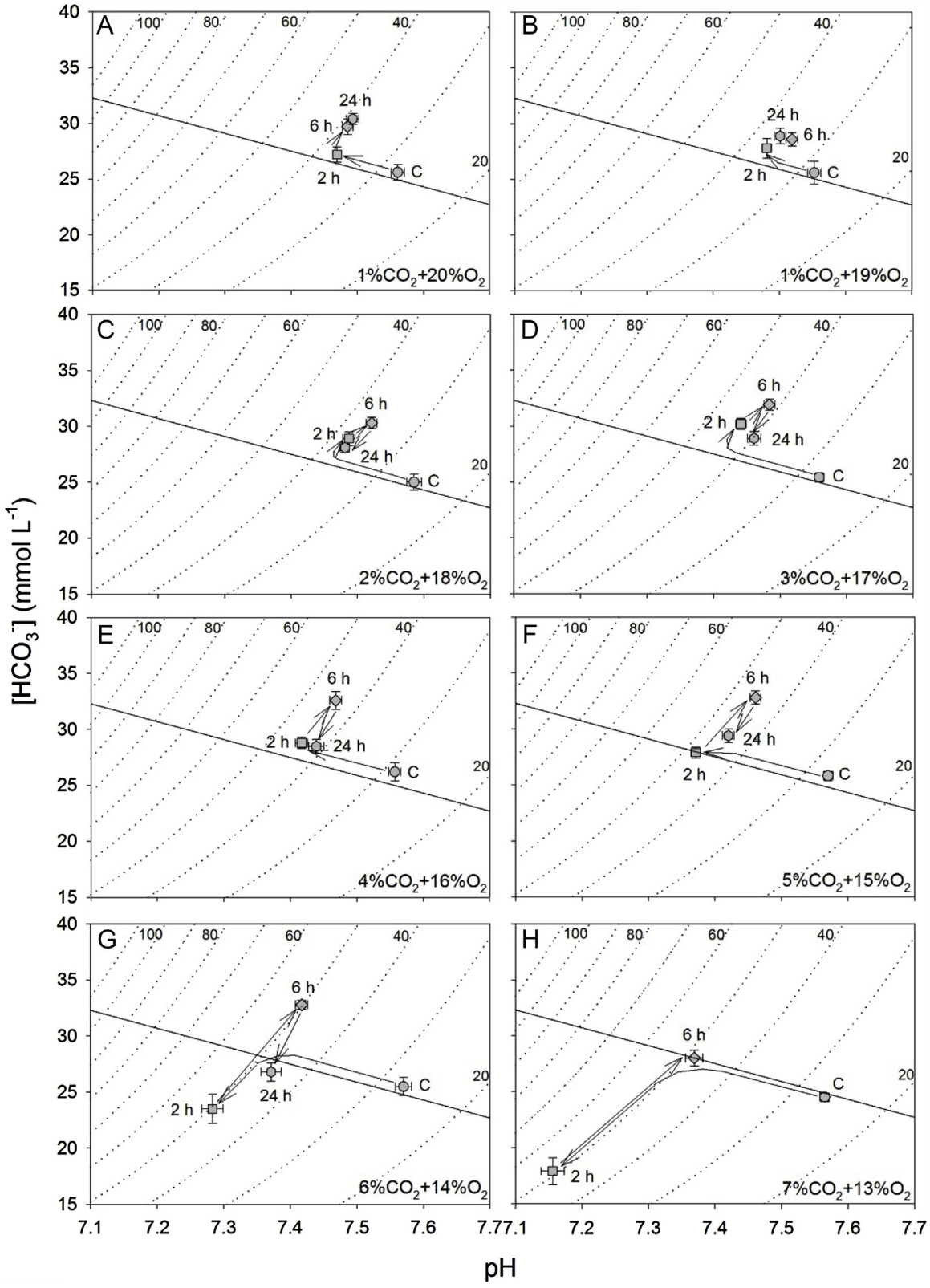

Fig. 1. Acid-base regulation in day 15 chicken embryos in response to 24 h exposure to the indicated hypercapnic hypoxic gas mixtures.

of the buffer line passing through control acid-base values, i.e., $[HCO_3^-] = -16\ pH + 146.6$, and Pco_2 isopleth, i.e., $pH = 6.09 + \log([HCO_3^-]/(0.0308 \times Pco_2))$. Uncompensated change in pH from control pH (i.e., uncompensated ΔpH) and compensated change in pH from uncompensated pH (i.e., compensated ΔpH) were 0.124 and 0.056 at 24 h, respectively. Thus, compensation expressed by a percent ratio of compensated ΔpH to uncompensated ΔpH (after Tazawa et al., 1981) was ~45% compensation. Similarly, partial metabolic compensation at 6 h was ~39%.

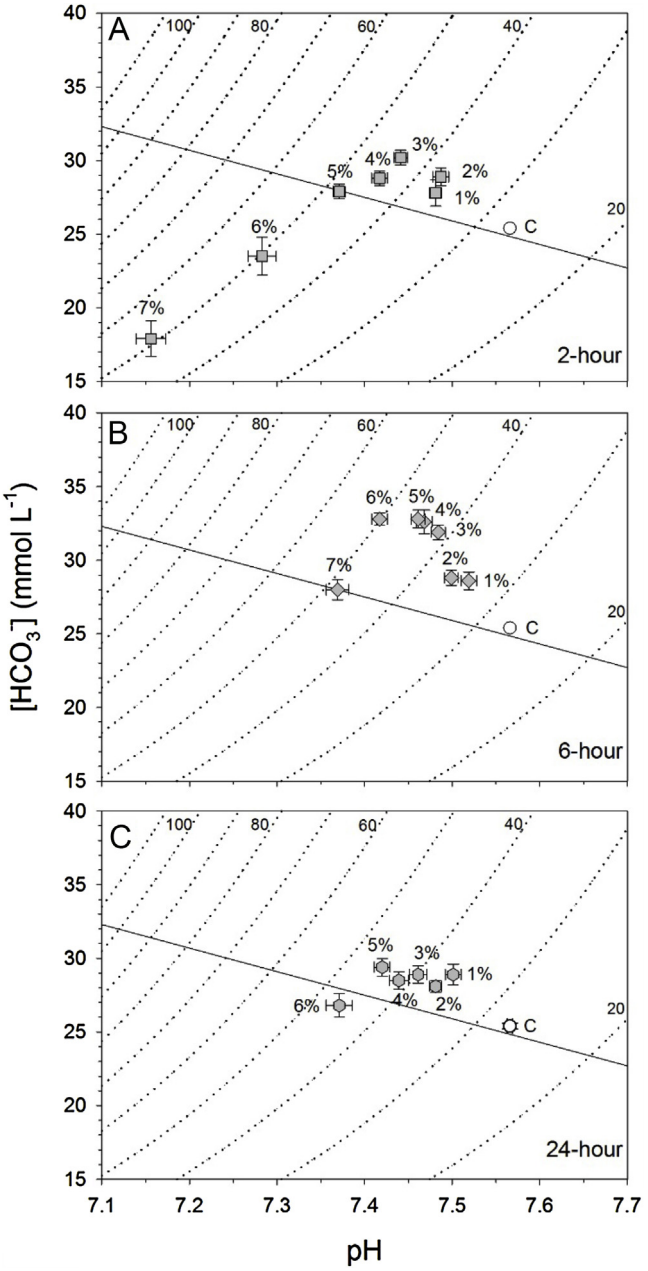

Fig. 2. Acid-base status at (A) 2 h, (B) 6 h and (C) 24 h of exposure to the indicated hypercapnic hypoxic gas mixtures. 1%; 1% CO_2 + 19% O_2, 2%; 2% CO_2 + 18% O_2, 3%; 3% CO_2 + 17% O_2, 4%; 4% CO_2 + 16% O_2, 5%; 5% CO_2 + 15% O_2, 6%; 6% CO_2 + 14% O_2 and 7%; 7% CO_2 + 13% O_2 with C; air.

3.3.3. Acid-base changes in graded increase in [CO_2] with graded decrease in [O_2]

The responses of eight experimental exposures to various combinations of CO_2 and O_2, will now be considered in turn.

Exposure to 1% CO_2 + 19% O_2 significantly decreased ($P < 0.001$) pH from the control (7.553) at 2, 6 and 24 h (Fig. 1B). Concurrently, [HCO_3^-] at 24 h was significantly higher than the control ($P = 0.041$), but [HCO_3^-] at 2 and 6 h were not different from control, each other, or 24 h. At 24 h there was an acid-base status of pH = 7.501, [HCO_3^-] = 28.9 mmol L^{-1} and Pco_2 = 37.4 mmHg. The partial metabolic compensation was calculated as follows. The uncompensated pH of respiratory acidosis was predicted to be 7.461 as pH at the intersection of buffer line and Pco_2 isopleth. Uncompensated ΔpH and compensated ΔpH were 0.092 (7.553–7.461) and 0.040 (7.501–7.461), respectively, yielding ~43% compensation. Similarly, partial metabolic compensation was calculated as ~24 and ~53% at 2 and 6 h, respectively.

In 2% CO_2 + 18% O_2 (Fig. 1C), blood pH decreased significantly from the control at 2, 6 and 24 h ($P < 0.001$). Concurrently, [HCO_3^-] increased significantly from the control at 2 h ($P < 0.001$), but 2 h did not differ from 6 and 24 h. The partial metabolic compensation was estimated to be ~30, ~49 and ~21% at 2, 6 and 24 h, respectively.

Similarly, in 3% CO_2 + 17% O_2 (Fig. 1D), pH significantly decreased from the control at 2 h ($P < 0.001$), significantly increased from 2 h to 6 h ($P = 0.003$) and then returned to a level similar to 2 h at 24 h ($P = 0.332$ for difference between 2 and 24 h). [HCO_3^-] increased significantly from the control at 2, 6 and 24 h ($P < 0.001$). The estimation of partial metabolic compensation resulted in ~29, ~49 and ~27% at 2, 6 and 24 h, respectively.

In 4% CO_2 + 16% O_2 (Fig. 1E), pH decreased from the control at 2 h ($P < 0.001$) along the buffer line. Concurrently, a significant increase in [HCO_3^-] ($P = 0.048$) occurred. pH then increased at 6 h ($P = 0.002$), along with a significant increase in [HCO_3^-] ($P = 0.001$). Both subsequently returned to a level similar to 2 h at 24 h. The estimation of partial metabolic compensation was ~11, ~43 and ~14% at 2, 6 and 24 h, respectively.

In 5% CO_2 + 15% O_2 (Fig. 1F), a decrease in pH from the control at 2 h occurred ($P < 0.001$), with no significant increase in [HCO_3^-] ($P > 0.05$), resulted in a slope (~−11 mmol L^{-1} pH^{-1}) smaller than buffer value. Since uncompensated pH was estimated to be 0.372, partial metabolic compensation was ~−1% at 2 h. Subsequently, pH increased accompanying a significant increase in [HCO_3^-] at 6 h ($P < 0.001$) and decreased again with significant decrease in [HCO_3^-] at 24 h ($P < 0.05$), resulting in partial metabolic compensation of ~39 and ~15% at 6 and 24 h, respectively.

In 6% CO_2 + 14% O_2 (Fig. 1G), pH decreased from the control at 2 h ($P < 0.001$), with no significant change in [HCO_3^-] ($P > 0.05$). Since [HCO_3^-] did not increase, pH at 2 h was much lower than uncompensated pH predicted (i.e., 7.356). Accordingly, compensated ΔpH was −0.073 and thus partial metabolic compensation was calculated as ~-34%, indicating that metabolic acidosis progressed without metabolic compensation. Subsequently, metabolic compensation occurred; pH increased significantly along Pco_2 isopleth (~51 mmHg) with significant increase in [HCO_3^-] at 6 h (i.e., 7.417) ($P < 0.05$) and partial metabolic compensation was ~31%. At 24 h of exposure, pH decreased again below the predicted uncompensated pH, indicating metabolic acidosis progressing. One among 13 embryos examined in this gas mixture died at 6 h and further 11 out of 30 embryos could not tolerate 24 h of exposure (Table 1).

In 7% CO_2 + 13% O_2 (Fig. 1H), pH at 2 h (7.156) decreased far below the predicted uncompensated pH (7.334), with a significant decrease in [HCO_3^-] ($P < 0.05$), resulting in partial metabolic compensation of ~−77%, indicative of metabolic acidosis without metabolic compensation at 2 h. With further exposure, pH increased along Pco_2 isopleth (~50 mmHg) accompanied by an increase in [HCO_3^-], resulting in a partial metabolic compensation of 8% at 6 h. However, 13 among 25 embryos and all 10 embryos exposed to this gas mixture died at 6 and 24 h, respectively (Table 1).

To summarize these changes, across the entire range of graded increases in [CO_2] accompanied with graded decreases in [O_2], respiratory acidosis occurred first during early period of exposures, e.g., 2 h, and its magnitude was in proportion to magnitudes of individual hypercapnia. Embryos responded with subsequent additional metabolic changes, i.e., metabolic alkalosis when exposed to ≥16% O_2, or metabolic acidosis when exposed to ≤14% O_2 (Fig. 2A). With an additional 4 h, compensatory metabolic alkalosis occurred in all groups and increased pH from the status at 2 h (Fig. 2B). However, at 24 h of exposure, partial metabolic compensation could not

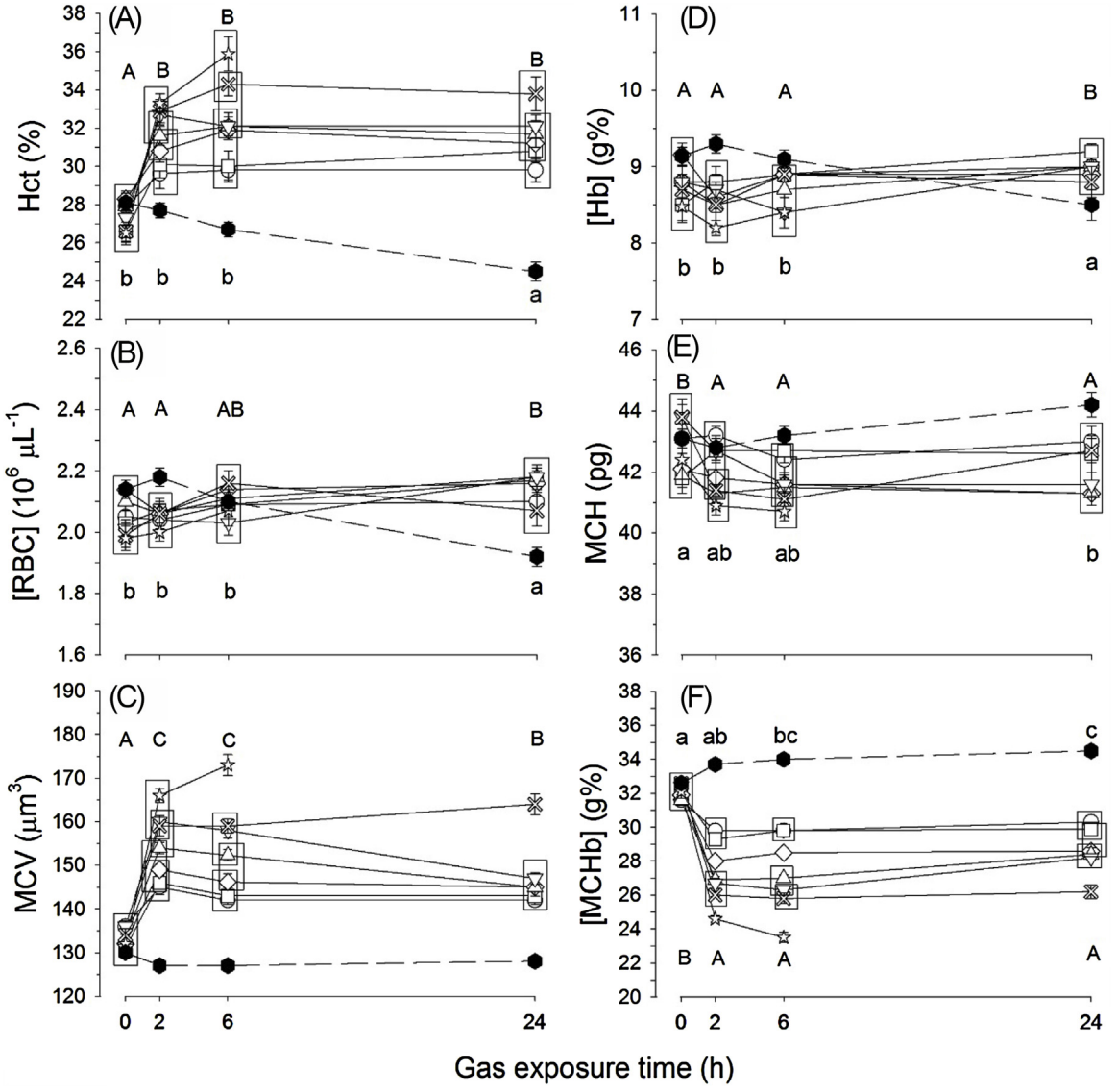

Fig. 3. Time specific changes in (A) hematocrit (Hct), (B) red blood cell concentration ([RBC]), (C) mean corpuscular volume (MCV), (D) hemoglobin concentration ([Hb]), (E) mean corpuscular hemoglobin (MCH) and (F) mean corpuscular hemoglobin concentration ([MCHb]) in day 15 chicken embryos exposed to the indicated gas mixtures. Means not significantly different from each other are grouped within the same box. The different uppercase letters indicate significant difference between gas exposure times for varied hypercapnia and hypoxia, ≥1% CO_2 + ≤19% O_2, exposures. The different lowercase letters indicate significant difference between gas exposure times for 1% CO_2 + 20% O_2. Closed hexagon; 1% CO_2 + 20% O_2, Circle; 1% CO_2 + 19% O_2, Square; 2% CO_2 + 18% O_2, Diamond; 3% CO_2 + 17% O_2, Upward triangle; 4% CO_2 + 16% O_2, Downward triangle; 5% CO_2 + 15% O_2, Cross; 6% CO_2 + 14% O_2 and Star; 7% CO_2 + 13% O_2.

be preserved (Fig. 2C), indicating the temporal limits to acid-base stress that can be accommodated.

3.4. Hematological changes in response to graded hypercapnic and hypoxic exposures

3.4.1. Control hematology values

There was no significant difference in the hematology of the eight control groups exposed to air (21% O_2, 0% CO_2). For a N = 129 of the combined control groups, Hct was 27.4 ± 0.2% (P = 0.108), [RBC] was 2.05 ± 0.01 × 10^6 µL^{-1} (P = 0.079), MCV was 133 ± 1 µm^3 (P = 0.165), [Hb] was 8.8 ± 0.1 g% (P = 0.082), MCH was 42.9 ± 0.2 pg (P = 0.053) and [MCHb] was 31.2 ± 0.2 g% (P = 0.061).

3.4.2. Hematological changes in 1% CO_2 with normoxia

Mean Hct of embryos significantly decreased with increasing exposure time through to 24 h (P < 0.001, Fig. 3A). Reduced Hct was likely the result of a significant fall in [RBC] from 0 h to 24 h (P < 0.001, Fig. 3B). At the same time, there was no significant decrease in MCV (P = 0.131, Fig. 3C). Mean [Hb] decreased from 0 h to 24 h (P < 0.001, Fig. 3D). MCH increased significantly from 0 h to 24 h (P = 0.002, Fig. 3E). The increase in [MCHb] from 0 h to 24 h was also significant (P < 0.001, Fig. 3F). Thus, even mild hypercapnia influenced hematology.

3.4.3. Hematological changes in graded increase in [CO_2] with graded decrease in [O_2]

Exposure of embryos to graded hypercapnia and hypoxia significantly increased Hct from control values at all exposure times, with significant differences between some gas mixtures at each exposure time. The combined Hct of embryos across all exposure times tended to increase with increases in [CO_2] and decreases in [O_2]. For instance, the Hct of embryos exposed to 1% CO_2 + 20% O_2 (i.e., 26.7 ± 0.3%) was significantly lower compared with embryos exposed to all other hypoxic gas mixtures (P < 0.001). Additionally, the Hct of embryos exposed to 1% CO_2 + 19% O_2 (29.4 ± 0.3%)

was significantly lower than that exposed to 6% CO_2 + 14% O_2 (31.5 ± 0.6%) or 7% CO_2 + 13% O_2. (32.1 ± 0.7%).

Mean [RBC], [Hb] and MCH were not significantly different among gas treatments at any exposure time. However, in all treatments these variables at 24 h were either significantly higher ([RBC], [Hb]) or lower (MCH) at 24 h ($P < 0.001$) compared to pre-exposure levels.

Exposure of embryos to graded hypercapnia and hypoxia significantly increased MCV from the control at all exposure times with significant differences between some gas mixtures ($P < 0.001$). The combined MCV across all exposure times tended to increase with an increase in [CO_2] and decrease in [O_2]. For instance, MCV of embryos exposed to 1% CO_2 + 20% O_2 (i.e., 128 ± 1 μm^3) was significantly lower compared with all other hypoxic gas mixtures ($P < 0.001$). Additionally, MCV of embryos exposed to ≥5% CO_2 + ≤15% O_2 (e.g., 149 ± 1 μm^3 for 5% CO_2 + 15% O_2) was significantly higher than that of embryos exposed to ≤3% CO_2 + ≤17% O_2 (e.g., 143 ± 1 μm^3 for 3% CO_2 + 17% O_2).

Combined [MCHb] across all exposure times tended to decrease with an increase in [CO_2] and decrease in [O_2]. For instance, [MCHb] of embryos exposed to 1% CO_2 + 20% O_2 (i.e., 33.7 ± 0.1 g%) was significantly higher compared with all other hypoxic gas mixtures ($P < 0.001$). Additionally, [MCHb] of embryos exposed to ≥4% CO_2 + ≤16% O_2 (e.g., 28.4 ± 0.3 g% for 4% CO_2 + 16% O_2) was significantly smaller than that of embryos exposed to ≤2% CO_2 + ≥18% O_2. (e.g., 30.2 ± 0.2 g% for 2% CO_2 + 18% O_2).

4. Discussion

4.1. Embryo body mass

Chicken embryo body mass increases from ~9.9 g on d14 to 12.5 g on d15, i.e., ~2.6 g (Romanoff, 1967). Accordingly, average body mass likely increases ~0.11 g per h during the development in the current experiments. The 4 h time difference between 2 and 6 h exposures started simultaneously on d15, would likely produce a ~0.44 g increase in body mass. Exposure for 24 h was started on d14 and blood collection was performed on d15 together with control embryos (0 h) and 2–6 h exposure embryos so that body mass differences between the four sub-groups (0, 2, 6 and 24 h) would be minimized within ~0.44 g. Mean body mass of embryos exposed to the first three gas mixtures ([1], [2] and [3]), consisting of moderate changes in hypercapnia and hypoxia, was not different between the four sub-groups (Table 1). However, body mass was significantly decreased after 24 h of exposure to more severe levels of hypercapnia and hypoxia, i.e., ≥3% CO_2 + ≤17% O_2, with larger decreases at more extreme [CO_2] and [O_2], indicating dehydration that might occur after 6 h of exposure to gas mixtures of ≥3% CO_2 + ≤17% O_2.

Significantly smaller body mass was also reported in d15 chicken embryos exposed to 5% CO_2 + 15% O_2 for 24 h (Burggren et al., 2012; Mueller et al., 2013). This was also the case for d15 embryos submerged half into water with air cell down for 24 h, which might have undergone hypercapnia accompanied with hypoxia (Branum et al., 2016). On the one hand, body mass of d15 chicken embryos remained unchanged after 24 h of exposure to pure hypercapnia (5% CO_2 or 1–10% CO_2 with 20% O_2) (Burggren et al., 2012; Mueller et al., 2014) or hypercapnia with hyperoxia (5% CO_2 with 40% or 90% O_2) (Mueller et al., 2013). On the other hand, body mass decreased in response to 24 h exposure to pure hypoxia (15% O_2) (Burggren et al., 2012). Accordingly, hypoxia ≤17% O_2, likely causes dehydration of body mass in chicken embryos exposed for 24 h. In chickens, hypoxic incubation (14–15% O_2) decreases body mass of late embryos (Stock and Metcalfe, 1987; Burton and Palmer, 1992; Dzialowski et al., 2002; Miller et al., 2002; Rouwet et al., 2002; Azzam and Mortola, 2007; Azzam et al., 2007; Copeland and Dzialowski, 2008). After hatching, body mass of hatchlings from hypoxic eggs was not different from control chicks hatched from normoxic eggs (Dzialowski et al., 2002; Azzam et al., 2007; Szdzuy and Mortola, 2007; Ferner and Mortola, 2009; Mortola, 2011). Hypometabolism resulting from hypoxic incubation may reduce growth rate, causing reduced body mass. Another possibility is that moderate sustained hypoxia during incubation likely affected water regulation resulting in dehydration as shown in the present short-term (24 h) hypoxic exposure. A more detailed study with acute and sustained hypoxic effects on embryo dehydration and its mechanism in chicken embryos is warranted.

4.2. Blood osmolality

Mean blood Osm in d15 chicken embryos previously determined ranged from 264 to 277 mmol kg^{-1} (Burggren et al., 2012, 2015; Tazawa et al., 2012; Mueller et al., 2013, 2014; Andrewartha et al., 2014; Branum et al., 2016). As many physiological variables in chicken embryos have phenotypic developmental plasticity induced by preincubation egg storage, the overall Osm of embryos in eggs stored at 22 °C (266 ± 0.1 mmol kg^{-1}, N = 329) is significantly higher than that in eggs stored at 15 °C (264 ± 0.2, N = 394) (Branum et al., 2016). Osm of embryos stored at 22 °C increased at 2 and 6 h during 24 h-half submersion with air cell down, but Osm of the latter (stored at 15 °C) failed to respond despite that they were siblings (Branum et al., 2016). Although Osm significantly increased in response to severe extrinsic hypoxia (10% O_2 with or without 5% CO_2) (Tazawa et al., 2012) and complete submersion in water (Andrewartha et al., 2014), Osm remained unchanged at 24 h of moderately altered O_2 and CO_2 environments (Burggren et al., 2012; Mueller et al., 2013, 2014). MCV increased during severe extrinsic hypoxia and complete submersion in water and also increased during moderate hypoxia and partial submersion. Accordingly, relation between Osm and MCV has not been always consistent. The present results also failed to show a significant relation.

4.3. Dynamics of acid-base regulation during graded hypercapnic and hypoxic challenges

The present study shows that the combination of varied, graded hypercapnia and hypoxia in d15 chicken embryos creates unique acid-base responses that represent combinations of the individual effects of hypercapnia or hypoxia. Exposure to hypercapnia (i.e., 5% CO_2) accompanied with hypoxia (i.e., 15% O_2) during 24 h produces compensatory metabolic alkalosis originating from hypercapnia that is partially offset by metabolic acidosis induced by hypoxia. This finding matches that found in previous studies examining hypercapnia and hypoxia in isolation, in which pure hypercapnia induces respiratory acidosis, which is partially compensated by metabolic alkalosis at 24 h of exposure, and pure hypoxia induces metabolic acidosis during 24 h. For example, the graded increase in extrinsic CO_2 (1–10% CO_2) in normoxia has been shown to produce a hypercapnic respiratory acidosis with a graded increase in magnitude during early (2 h) exposure. Only a small metabolic alkalosis, increasing with time, occurs during the next 6–24 h (Mueller et al., 2014; Burggren et al., 2015). The magnitude of this compensatory metabolic alkalosis increases in proportion to [CO_2], resulting in an identical partial metabolic compensation across all CO_2 gas mixtures. In hypoxia, metabolic acidosis occurs along the Pco_2 isopleth with magnitudes depending upon the degree of [O_2] (Burggren et al., 2012; Tazawa et al., 2012).

This study, which uses combinations of graded hypercapnia (1% up to 7% CO_2) and hypoxia (19% down to 13% O_2), further demonstrates that metabolic acidosis occurred in addition to respiratory acidosis during early exposure (2 h) with severity of hypoxia

(14–13% O_2), but was still compensated by metabolic alkalosis at 6 h but not at 24 h. This supports previous findings that partial metabolic compensation for respiratory acidosis cannot be preserved at 24 h if hypoxia is included (Fig. 4 in Burggren et al., 2012). In fact, the investigation of the time course of acid-base regulation during 24 h exposure of d15 embryos to hypercapnic hypoxia (5% CO_2 + 15% O_2) showed that the respiratory acidosis at 2 h, induced by hypercapnia, was partially compensated by metabolic alkalosis at 6 h but could not be preserved at 24 h (Fig. 1 in Mueller et al., 2013). Presumably, lactic acid was produced in response to severe hypoxia as previously shown (Tazawa et al., 2012), while respiratory acidosis occurred in response to hypercapnia during early exposure and thereafter replaced by bicarbonate ion transferring from the allantoic fluid through the chorioallantoic membrane (CAM). It seems like that the preservation of [HCO_3^-] in blood through the CAM might need O_2. In embryos exposed to hypercapnia along with normoxia or hyperoxia, partial metabolic compensation was preserved at 24 h, indicating that preservation of metabolic compensation needs ≥20% O_2 (Mueller et al., 2013). Additionally, the current study indicates the preservation of metabolic compensation apparently occurs ≥19% O_2.

To summarize our acid-base findings for chicken embryos, the combination of hypercapnia and hypoxia produced a unique response in which compensatory metabolic alkalosis produced in response to hypercapnia was partially cancelled by metabolic acidosis induced by severe hypoxia. During early (2 h) exposure the respiratory acidosis occurred in proportion to the level of hypercapnia. Subsequent compensatory metabolic alkalosis began to occur in embryos exposed to moderate hypoxia (≥16% O_2), or metabolic acidosis occurred in embryos exposed to severe hypoxia (≤14% O_2) (Fig. 2A). During the next 6 h, compensatory metabolic alkalosis augmented or occurred even in severe hypoxia (Fig. 2B). However, insufficient supply of O_2 (≤18% O_2) could not preserve the partial metabolic compensation at 24 h (Fig. 2C).

4.4. Hematology dynamics during graded hypercapnic and hypoxic challenges

Hct, [RBC] and MCV all increased at 24 h across the entire range of graded increases in [CO_2] and decreases in [O_2] studied. The increase in Hct occurred with as little as 1% CO_2 + 19% O_2 and the effect became larger with a greater degree of hypercapnia and hypoxia. This is in contrast to the decrease in Hct in 1% CO_2 + 20% O_2. The present findings demonstrate that the presence of hypoxia in hypercapnic environments reverses hematological responses to hypercapnic exposure in isolation. In hypercapnic environments, hematological responses during 24 h-exposure to 5% CO_2 or graded hypercapnia (≤10% CO_2) with normoxia (20% O_2) in d15 embryos is nearly identical across all CO_2 concentrations (Burggren et al., 2012; Mueller et al., 2013, 2014), with the exception of responses of MCV and [MCHb] to 10% CO_2 (Mueller et al., 2014). In the present hypercapnic exposures, Hct decreased across the time course of CO_2 exposure due to a decrease in MCV and [RBC]. [Hb] responded to altered [CO_2] similarly to [RBC], while the changes in [MCHb] reflected inversely those in MCV because of MCH remaining unchanged during 6 h of exposure (Mueller et al., 2014). These hematological responses to hypercapnic exposures occur not only in normoxic (20% O_2) environment but also in hyperoxia (40% or 90% O_2) (Mueller et al., 2013), and resemble those currently determined in 1% CO_2 + 20% O_2 (Fig. 3).

Since Hct decreases in either pure hypercapnia or hypercapnia combined with hyperoxia, it is likely that the increases currently found in Hct, [RBC], MCV and [Hb] are the result of hypoxia. Consequently, in d15 Lohmann White Leghorn embryos undergoing 24 h-exposure to altered CO_2 and O_2 gas environments, Hct decreases in response to varied hypercapnia in normoxia and hyperoxia. This decrease occurred from a combined result of a decrease in MCV occurring during 24 h and a decrease in [RBC] at 24 h (Burggren et al., 2012; Mueller et al., 2013, 2014; present study). In contrast, Hct increases in response to varied hypoxia (19%–13% O_2), irrespective of presence or absence of CO_2, with an increase in MCV occurring during 24 h and increase in [RBC] at 24 h (Burggren et al., 2012; Mueller et al., 2013; present study). D15 chicken embryos can survive 10% O_2 with or without hypercapnia (5% CO_2) for at least 2 h (Tazawa et al., 2012; Kohl et al., 2015). Hct increases during 2 h of severe hypoxic (10% O_2) exposure with increase in MCV, but [RBC] remains unchanged during 2 h; that is, the identical hematological responses occur during 2 h exposures in 19%–13% O_2 and 10% O_2. Although hematological responses to 12 and 11% O_2 during 2 h exposures were not included in the present experiments, it is likely that the identical responses occur throughout hypoxia from 19% to even severe 10% O_2 at 2 h of exposure.

5. Conclusions

This study is the first to comprehensively assess the effects of combined hypercapnia and hypoxia on acid-base and hematological regulation in d15 chicken embryos. We hypothesized that d15 embryos exposed to hypercapnic hypoxia would be able to regulate acid-base balance to quite extreme stressor levels. Indeed, compensatory metabolic alkalosis produced in response to hypercapnia is partially cancelled by metabolic acidosis induced by hypoxia. Intrinsic progressive hypercapnia with progressive hypoxia occurs in embryos whose gas exchange through the eggshell is partially or completely blocked (Andrewartha et al., 2014). The trajectories of acid-base regulation in these embryos are likely mimicked by those in the embryos exposed to extrinsic graded increases in hypercapnia and hypoxia for a period of <2 h when respiratory acidosis occurs, but metabolic compensatory alkalosis has not yet occurred (Fig. 2A and 7 in Andrewartha et al., 2014). Subsequent compensatory responses could not be maintained until 24 h. Thus, our prediction that subsequent metabolic acidosis with severe hypoxia (<14%) would cause mortality was validated. These results indicate embryos can only successfully respond to hypercapnic and hypoxic environments of ≤5% CO_2 + ≥15% O_2 for 24 h.

Additionally, we demonstrated that Hct increased under all [CO_2] and [O_2] exposure combinations below 20% O_2, and that the underlying mechanism was an early (after 2 h exposure) increase in MCV followed by a later (24 h exposure) increase in [RBC]. This increase in Hct has also been reported in d15 embryos partially submerged with air cell in air or completely submerged in water (Andrewartha et al., 2014) or submerged half with air cell down in water (Branum et al., 2016). It is worth noting that hematological regulatory processes are not always identical in other strains of chickens. For example, in hypercapnic normoxia environments or submersion, d15 Cornish Rock broiler embryos respond with a sharp increase in MCV and no response in [RBC] (Burggren et al., 2015; Branum et al., 2016). Such strain effects and maternal and environmental effects have previously been noted (Burggren et al., 1994, 2015; Dzialowski and Sotherland, 2004; Ho et al., 2011; Burggren, 2014), but whether the response in Lohmann White Leghorn embryos is common to other strains should to be demonstrated in a comparative study.

Acknowledgement

Support for this study was provided by NSF operating grant IOS-1025823 to Warren W. Burggren.

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