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Acute regulation of hematocrit and blood acid–base balance during severe hypoxic challenges in late chicken embryos (*Gallus gallus*)

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ABSTRACT

Acid–base and hematocrit (Hct) responses of vertebrate embryos to severe hypoxia are as yet unknown, but may reveal the maturation process of physiological regulatory mechanisms. The present study elucidated how acute, severe hypoxia (10% O_2, with and without 5% CO_2) affects Hct and blood acid–base balance in late prenatal (days 11–19) chicken embryos. The time-course of the resulting Hct changes and blood acid–base disturbances was examined in detail in day 15 (d15) embryos to further understand the magnitude and time-components of these physiological changes. We hypothesized that Hct of developing embryos increases during severe hypoxia (10% O_2) and hypercapnic hypoxia (5%CO_2, 10%O_2), due to increased mean corpuscular volume (MCV) and red blood cell concentration ([RBC]). We additionally hypothesized that 10% O_2 would induce anaerobic glycolysis and the attendant increase in lactate concentration ([La^-]) would create a severe metabolic acidosis. Hct increased in all embryos (d11–d19) during severe hypoxia (2 h) but, with the exception of d19 embryos, the increase was due to increased MCV and was therefore unlikely related to O_2 transport. The time-course of the d15 embryonic Hct response to hypoxic or hypercapnic hypoxic exposure was very rapid with MCV increasing within 30 min. Severe metabolic acidosis occurred in all developing embryos (d11–d19) during 2 h hypoxic exposure. Additionally, respiratory acidosis was induced in d15 embryos during hypercapnic hypoxia, with acid–base status recovering within 120 min in air. Throughout hypoxic exposure and recovery, changes in [HCO_3^-] were matched by those in [La^-], indicating that anaerobic glycolysis is a key factor determining the metabolic alterations and overall acid–base status. Further, the blood gas and Hct values recovered in air and unchanged embryo mass suggest that the hypoxia-induced disturbances were only transient and may not affect long-term survival.

1. Introduction

Hematocrit (Hct) is a function of mean corpuscular volume (MCV), red blood cell concentration ([RBC]) and plasma volume (PV). Increasing [RBC], and thus Hct, during hypoxia to maintain adequate tissue O_2 delivery would appear to be a logical response. Yet, previous studies have demonstrated that the Hct increase during 1 day of moderate hypoxia in developing embryos is largely created through changes in MCV and is therefore unlikely related to O_2 transport (e.g., Ackerman, 1970; Burggren et al., 2012). In addition, contribution of PV to changes in Hct, i.e., $\Delta Hct = \Delta[RBC] + \Delta MCV + \Delta PV$, was little (Burggren et al., 2012). Hypoxia-induced disturbances of blood acid–base balance can alter Hct through changes in MCV, which is under the influence of a multitude of factors. These include potassium-chloride co-transport, taurine transport and sodium-dependent beta-amino acid transport systems modulated via changes in osmolality (Osm), P_{O_2}, P_{CO_2} and pH (for reviews see Cossins and Gibson, 1997; Nikinmaa, 1992 and Hoffmann et al., 2009).

The avian embryo allows acid–base responses to hypoxia and hypercapnic hypoxia to be studied in a relatively simple vertebrate system that lacks mechanical ventilation (gas exchange is via diffusion only) and possesses a relatively underdeveloped renal system (e.g., metanephric development continues after hatching – Romanoff, 1960; Carretero et al., 1995). Accordingly, the embryo cannot employ respiratory compensation and potentially renal compensation to combat acid–base imbalances. Uncompensated metabolic acidosis results when day 15 (d15) and d17 embryos are exposed to moderate hypoxia (15% O_2) for 1 day (Burggren et al., 2012). In contrast, uncompensated respiratory acidosis results from additionally exposing embryos to hypercapnia (i.e., hypercapnic hypoxia: 5%CO_2, 15%O_2). In both cases, an increase in Hct occurred due to an increase in both MCV and [RBC] in d15 embryos (Burggren et al., 2012). These data indicate that low O_2 concentration is a strong driver for Hct regulation in the presence or absence of CO_2. However, Hct changes during alterations of ambient O_2 or CO_2

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should be interpreted within a blood acid–base balance framework because of the complex interactions between the acid–base and hematological respiratory systems. Further, because both 'normal' blood acid–base balance and Hct and their responses during perturbations (such as hypoxia) change as maturation progresses (e.g., Tazawa, 1980; Tazawa et al., 1992, 2011; Burggren et al., 2012), full understanding of the effects of acute hypoxia (or hypercapnic hypoxia) requires a developmental context.

The acid–base and Hct responses of embryos when challenged by severe hypoxia are as yet unknown. Early chicken embryos (<d6) tolerate severe hypoxia (10%) surviving >240 min. However, after ~day 6, profound bradycardia develops during severe hypoxic exposure, often ending in death. Additionally, survival time in such conditions declines with age (e.g., ~160 min on d9) (Akiyama et al., 1999 and Andrewartha et al., 2011a for review). It is likely that severe acid–base disturbances during this time-course (>3 h on d9) prove to be ultimately fatal and that the relatively greater metabolism of older embryos will result in critically low pH and/or bicarbonate levels. Thus, the present study elucidates how acute, severe hypoxia (10% O_2, with and without 5% CO_2) affects Hct and blood acid–base balance in late prenatal (d11–d19) embryos. The time-course of the resulting Hct changes and blood acid–base disturbances are examined in detail in d15 embryos, with the goal of further revealing the scope and nature of embryonic acid–base and hematocrit regulatory mechanisms. We hypothesize that the Hct of developing embryos will increase during severe hypoxia (10% O_2) and hypercapnic hypoxia (10%O_2, 5%CO_2) exposure due to an increase in both MCV and [RBC], as occurs at milder hypoxia levels in d15 embryos (Burggren et al., 2012). However, because 10% O_2 is low enough to induce anaerobic glycolysis, it is additionally hypothesized that the metabolic acidosis will be more predominant in severe hypoxia (and hypercapnic hypoxia) due to increased lactate. In some bird species, eggs are laid in a burrow nest where developing embryos are exposed to a hypercapnic environment. Thus, the effect of hypercapnia (5%CO_2) in hypoxia (10%CO_2) is not only of use in revealing emerging regulatory mechanisms, but is additionally an interesting subject to be examined from comparative point of view.

2. Materials and methods

2.1. Incubation of eggs

Fertile eggs of the domestic fowl (*Gallus gallus domesticus*) were obtained weekly from Texas A&M University (College Station, TX, USA). Eggs were weighed (±0.01 g) and then incubated at 37.5 ± 0.1 °C and relative humidity of ~55% in a forced draught incubator (1502, G.Q.F. Manuf. Co., USA). The eggs were placed vertically on an automatic turning tray which rotated the eggs every 3 h.

2.2. Blood collection and analysis

Blood was collected from the allantoic vein. A 6–8 mm diameter region of the eggshell was removed and the underlying allantoic vein gently lifted by forceps through the hole in the eggshell. Allantoic venous values represent "arterialized" values, since an allantoic vein is analogous to a vein in the pulmonary circulation (Piiper et al., 1980). Consequently, values measured are presented with the subscript "a" as for systemic arterial values. Approximately 0.15–0.35 mL of blood was collected from d11, d13 and d19 embryos and ~0.3–0.6 mL from d15 and d17 embryos, using a 1 mL plastic syringe with a fixed needle to minimize a dead-space and flushed in advance with heparinized saline (100 mg in 100 mL saline).

All embryos are used only once due to the limited blood capacity of the embryos (i.e., there was no serial sampling in this study). Sampled eggs were subsequently euthanized via exposure to a cold, anoxic environment and the embryos were removed from their eggshell. The yolk and extra-embryonic membranes were then removed, and embryo body mass measured (±0.01 g) with an electronic balance.

Collected blood was gently transferred into a 2 mL plastic vial and ~0.12 mL of blood withdrawn from the bottom of the vial to minimize contact of blood with air. This sample was immediately measured for pH, P_{CO_2} and [HCO_3^-] (calculated by the analyzer from pH and P_{CO_2}) with a blood gas analyzer (ABL5, Radiometer Medical A/S, Copenhagen, Denmark) at 37 °C. The blood was then inverted several times in the vial to ensure thorough mixing, and [RBC] and hemoglobin concentration ([Hb], g%) determined by a blood cell counter (Coulter analyzer, $A^c{\cdot}10$ T, Beckman, USA) and osmolality (Osm, mmol kg^{-1}) by a vapor pressure osmometer (Vapro 5520, Wescor, USA). Duplicate preparations of 60 μL of blood were transferred into sealed hematocrit tubes and centrifuged for 4 min at 10,000 rpm and the mean Hct determined (±0.1%, Readacrit Centrifuge, Becton Dickinson, USA). When the collected blood volume was insufficient to measure all variables (~0.26 mL), either blood gas variables and Osm were measured or the variables determined by the Coulter analyzer ([RBC] and [Hb]). Although Hct could have been determined using the Coulter analyzer, the values reported in this study were determined via centrifugation because the Coulter analyzer underestimates Hct, particularly during hypoxic exposure. Thorough comparison of these two methodologies is undertaken in the Appendix.

Mean corpuscular indices (MCV, μ^3), mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration ([MCHb], g%)) were calculated by equations from Tazawa et al. (2011), where Hct is the value obtained via centrifugation (%), [Hb] is the value determined from the Coulter analyzer (g%) and [RBC] (10^6 μL^{-1}) is calculated from an individual value determined by the Coulter analyzer, using a previously obtained regression equation relating values simultaneously determined using the Coulter analyzer and hemocytometry (Tazawa et al., 2011).

A previously constructed Davenport (pH-[HCO_3^-]) diagram (Burggren et al., 2012) was used to depict acid–base balance. Hb, the important non-carbonate buffer in blood, increases during the last half of incubation (Tazawa et al., 2011). Therefore, the blood buffer value is also predicted to increase during embryonic development (as demonstrated in Erasmus et al., 1970/71; Tazawa and Piiper, 1984; Tazawa, 1986). Previous studies have not always demonstrated this increase (e.g., Tazawa et al., 1983; Andrewartha et al., 2011b). A value of −16.0 mmol L^{-1} pH^{-1} was used in this report (as determined in Burggren et al., 2012).

2.3. Acute hypoxic (10%O_2) challenges in developing embryos (d11–d19)

Eggs were candled to locate the allantoic vein on one of d10, d12, d14, d16 and d18, and transferred to a desk-top incubator warmed at 37.5 °C and ventilated with air at a relative humidity of ~60% (Hova-Bator 1590, G.Q.F. Manuf. Co., USA). The eggs were placed on a cardboard holder in the incubator for one further day. On the following target day (one of d11, d13, d15, d17 or d19), the eggs were randomly divided into control or experimental groups. The control eggs remained in the incubator until blood collection. The experimental eggs were transferred to a 3.78 L (26.8 cm × 29.7 cm) plastic Ziploc® bag (referred to as the gas-exposure bag) placed in the incubator. The gas-exposure bag was fitted with diagonally placed inlet and outlet conduits and was ventilated in advance at a rate of ~600 mL min^{-1} with hypoxic gas mixture provided by a Wösthoff gas mixing pump (oHG, Bochum, Germany) (after

Burggren et al., 2012). After a 2 h hypoxic exposure (duration of exposure was determined on the basis of preliminary experiment with d15 embryos, see Section 3.2), the eggs were removed from the gas-exposure bag and immediately wrapped in aluminum foil to minimize any adverse effects of changes in environmental gases on blood gas properties within the 2 min required for blood collection and injection into the blood gas analyzer (see Appendix in Burggren et al., 2012).

2.4. Time-course during hypoxic challenges and recovery in air (d15)

The time-course of acid–base disturbances and resulting Hct regulation were examined using d15 embryos incubated as detailed above. On d15, blood was sampled from control embryos, embryos exposed to hypoxia (10% O_2) or hypercapnic hypoxia (5% CO_2, 10% O_2) for 2 h in the gas-exposure bag or embryos returned to normoxia after hypoxic exposure. Blood was sampled from hypoxic or hypercapnic hypoxic embryos at 10, 30, 60, 90 and 120 min. Additional embryos were sampled during 10, 30, 60, 90 and 120 min recovery in air following 120 min hypoxia. Recovery of embryos exposed to hypercapnic hypoxia was initiated at 90 min of exposure, because during preliminary testing it was established that embryos did not always survive recovery after 120 min exposure to hypercapnic hypoxia. Similar to hypoxia recovery, blood was sampled at 10, 30, 60, 90 and 120 min of recovery in air. Approximately 0.4 mL of blood was collected from individual embryos. In addition to blood gas, hematological respiratory variables and Osm, lactate concentration ([La^-], mmol L^{-1}) was determined on one drop of blood using a lactate meter (Nova lactate plus meter, Nova Biomedical, UK).

2.5. Statistical analysis

All data were tested for normality and equal variance. Differences in egg mass, body mass and blood Osm between control and hypoxic embryos during development from d11 to d19 were examined by two-way ANOVA. Differences in P_{CO_2} between hypoxic (or hypercapnic hypoxia) exposure and recovery and differences between time-points during 120 min exposure in d15 embryos were examined by two-way ANOVA. Differences in egg mass and body mass of d15 embryos and blood variables (Hct, MCV, [RBC], [Hb], MCH and [MCHb]) between the 11 groups of eggs (including control) used for the time-course responses to hypoxic (or hypercapnic hypoxia) exposure and recovery were examined by one-way ANOVA with post hoc multiple comparison procedures (Tukey test or Holm–Sidak method). One-way ANOVA was also used to examine the differences between variables across the age groups. Differences of mean values between two groups, e.g., mean P_{CO_2} between control and hypoxic groups at individual ages, were examined by un-paired *t*-test with Mann–Whitney test, if necessary. Significance was assumed at $P < 0.05$. All data were presented as mean ± 1 S.E.M

3. Results

3.1. Egg and body mass

There was no difference in initial fresh egg mass between embryos assigned to control (54.41 ± 0.40 g, N = 143) and acute hypoxic (10%O_2) exposure for 2 h (54.85 ± 0.40 g, N = 142) ($P = 0.435$) during development from d11 to d19. There was also no difference between individual days in these populations ($P = 0.098$) (Table 1). Similarly, there was no difference in mean body mass between control and hypoxic embryos ($P = 0.065$), although there was the expected significant difference between individual developmental ages ($P < 0.001$).

There were no differences in fresh egg mass or body mass between the 11 groups (including control) of eggs examined on d15 for the time course of responses to hypoxic (10%O_2) or hypercapnic hypoxia (5%CO_2, 10%O_2) exposure and recovery. The mean egg and body masses of embryos exposed to hypoxia for 2 h with 2 h-recovery were 58.20 ± 0.32 g ($N = 182$, $P = 0.410$ for 11 groups) and 13.01 ± 0.10 g ($P = 0.075$), respectively. Those examined for hypercapnic hypoxia exposure were 57.80 ± 0.28 g ($N = 197$, $P = 0.479$ for 11 groups) and 12.75 ± 0.10 g ($P = 0.582$), respectively.

3.2. Survival rates in hypoxia

A preliminary experiment examined the tolerance of d15 embryos to hypoxia (with and without CO_2) to determine exposure period, i.e., 2 h as mentioned in Section 2.3. The eggs were candled to check and ensure the embryos were alive by investigating the vivid shadow of major blood vessels prior to and after exposure to hypoxia (10%O_2) or hypercapnic hypoxia (5%CO_2, 10%O_2). After exposure, embryo viability was again assured by opening the eggs. None of the 12 embryos examined survived 300 min hypoxic exposure (10% O_2). Approximately 42% (5 of 12) and 92% (11 of 12) of embryos were alive after 240 min and 180 min, respectively, but none of these embryos ($N = 12$) survived the following 120 min-recovery in air. On the other hand, all 12 embryos examined survived 120 min hypoxia exposure, and ~83% (10 of 12) survived the following 120 min recovery period in air. Consequently, we chose an exposure time for this study of 2 h of hypoxia exposure followed by 2 h of recovery.

Survival time was decreased with the addition of CO_2 with only ~60% of embryos (7 of 12) surviving 120 min hypercapnic hypoxia (10%O_2 with 5%CO_2) exposure and all 12 embryos surviving 90 min exposure. Approximately ~10% of embryos (1 of 12) did not survive the 120 min recovery period in air after 90 min hypercapnic hypoxia.

In subsequent studies, all embryos ($N = 93$) survived 120 min of severe hypoxia (10%) and the subsequent 120 min recovery ($N = 58$) period. Similarly, all embryos ($N = 90$) survived 90 min hypercapnic hypoxia (5%CO_2, 10%O_2). However, only 2 of 63 failed to survive the 120 min recovery period in air. Embryos were additionally exposed to 120 min hypercapnic hypoxia (although they were not recovered from this timepoint) and ~35% of embryos (9 of 26) could not survive this extended exposure time.

3.3. Plasma osmolality

Osmolality (Osm) during incubation from d11 to d19 was higher in hypoxic (268 ± 1 mmol kg^{-1}) compared with control embryos (266 ± 1 mmol kg^{-1}) ($P = 0.006$). Further, Osm changed significantly with age ($P < 0.001$). Thus, Osm on d17 (270 ± 1 mmol kg^{-1}) and d19 (274 ± 1 mmol kg^{-1}) was higher than that on d11 (261 ± 1 mmol kg^{-1}), d13 (264 ± 1 mmol kg^{-1}) and d15 (267 ± 1 mmol kg^{-1}) (Table 1). Osm changed significantly during time-course in both hypoxia and hypercapnic hypoxia experiments.

3.4. Acid–base disturbances during hypoxic challenges

3.4.1. Control embryos

Across normal development, Pa_{CO_2} increased from 10.4 ± 0.5 mmHg ($N = 14$) in d11 embryos to 38.0 ± 1.2 ($N = 28$) mmHg in d17 embryos (Fig. 1). Concurrently, pH_a decreased from 7.78 ± 0.02 to 7.54 ± 0.01 and was accompanied by a doubling in [HCO_3^-]$_a$ from 16.2 ± 0.6 mmol L^{-1} to 32.0 ± 1.0 mmol L^{-1}. Thus, a relative acidosis developed across the

Table 1
Fresh egg mass, body mass and osmolality (Osm) of control chicken embryos and embryos exposed to hypoxia (10% O_2) for 2 h. Values are mean ± 1 S.E.M. (*N*).

Age (d)	Experiment	Egg mass (g)	Body mass (g)	Osm (mmol kg^{-1})
11	Control	53.93 ± 0.82 (23)	3.21 ± 0.04 (23)	260 ± 2 (21)
	10% O_2	53.69 ± 0.78 (24)	3.18 ± 0.07 (24)	262 ± 2 (18)
13	Control	53.50 ± 0.98 (24)	6.00 ± 0.17 (24)	263 ± 1 (23)
	10% O_2	53.55 ± 1.00 (21)	6.64 ± 0.16 (21)	265 ± 1 (18)
15	Control	54.30 ± 0.88 (20)	11.68 ± 0.32 (20)	265 ± 1 (20)
	10% O_2	56.05 ± 0.81 (20)	12.95 ± 0.27 (20)	268 ± 1 (20)
17	Control	54.95 ± 0.98 (36)	18.96 ± 0.35 (36)	269 ± 1 (36)
	10% O_2	55.51 ± 0.62 (36)	19.47 ± 0.30 (36)	272 ± 1 (35)
19	Control	55.35 ± 0.67 (40)	26.33 ± 0.48 (40)	272 ± 1 (37)
	10% O_2	55.43 ± 0.84 (41)	25.99 ± 0.31 (41)	276 ± 2 (36)

latter half of incubation (d11–d17). The acid–base status of d19 embryos was similar to d17 embryos, with an additional metabolic acidosis.

3.4.2. Acute hypoxic challenges in developing embryos

Severe metabolic acidosis resulted from 2 h of hypoxic (10%O_2) exposure, with further exposure resulting in embryo mortality (Fig. 1). The mean pH_a of living embryos prior to death ("critical pH_a") ranged from 7.21 ± 0.02 (*N* = 15) in d11 embryos to 7.19 ± 0.01 (*N* = 32) in d17 embryos (Fig. 1). The critical $[HCO_3^-]_a$ (corresponding to critical pH_a) ranged from ~5 to 11 mmol L^{-1} in d11 and d17 embryos, respectively. Pa_{CO_2} remained unchanged from control in d11 and d13 embryos after 2 h of exposure, e.g., control = 10.4 ± 0.4 mmHg cf. hypoxia = 10.5 ± 0.4 mmHg in d11 embryos (*P* = 0.982). However, an additional respiratory alkalosis (i.e., decrease in Pa_{CO_2}) occurred in d15-d19 embryos, e.g., control = 38.0 ± 1.2 mmHg cf. hypoxia = 30.3 ± 0.9 mmHg in d17 embryos (*P* < 0.001) (Fig. 1).

3.4.3. Time-course during hypoxic challenges and recovery

In d15 embryos exposed to severe hypoxia (10%), pH_a (control = 7.59 ± 0.01, *N* = 30) and $[HCO_3^-]_a$ (26.4 ± 0.4 mmol L^{-1}) began to decrease within 10 min of hypoxic exposure (7.49 ± 0.01 and 23.6 ± 0.5 mmol L^{-1}, *N* = 16) (Fig. 2A). By 90 min, pH_a deceased by 0.36 unit (7.23 ± 0.02, *N* = 21) and $[HCO_3^-]_a$ more than halved (11.7 ± 0.6 mmol L^{-1}). pH_a remained constant during the next 30 min of exposure (between 90 min and 120 min (7.22 ± 0.01, *N* = 20, *P* = 0.660), whereas $[HCO_3^-]_a$ decreased further to 9.6 ± 0.5 mmol L^{-1} (*P* < 0.014). Meanwhile, Pa_{CO_2} (control = 27.6 ± 0.6 mmHg) remained unchanged during the first 90 min of hypoxic exposure (mean = 29.0 ± 0.6 mmHg, *N* = 74, *P* = 0.250) and subsequently decreased during the following 30 min to 24.2 ± 0.7 mmHg (*N* = 20, *P* = 0.049) at the end of exposure (120 min). When returned to air, pH_a and $[HCO_3^-]_a$ remained unchanged during the first 10 min (7.26 ± 0.01 and 9.6 ± 0.7 mmol L^{-1}, *N* = 10) and began to increase within 30 min (7.37 ± 0.02 and 12.7 ± 1.0 mmol L^{-1}, *N* = 10), returning to control values within the 120 min recovery in air (7.55 ± 0.01 and 22.4 ± 0.9 mmol L^{-1}, *N* = 18) (Fig. 2A).

In embryos exposed to hypercapnic hypoxia (5%CO_2, 10%O_2) (Fig. 2B), in contrast to that of pure hypoxia, Pa_{CO_2} increased

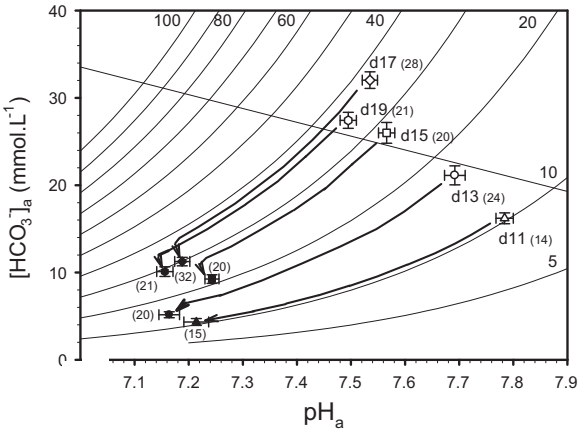

Fig. 1. Mean acid–base status of d11–d19 embryos in air (open symbols) and disturbances produced by exposure to severe hypoxia (10% O_2) for 2 h (closed symbols). Solid arrows indicate the magnitude of the disturbance in hypoxia for individual development days. Numbered curves are the Pa_{CO_2} isopleths, in mmHg. The solid regression is an arbitrary buffer line with the determined mean slope of −16 mmol L^{-1} pH^{-1} (Burggren et al., 2012). Values are mean ± 1 S.E.M. and *N* values are indicated in parentheses.

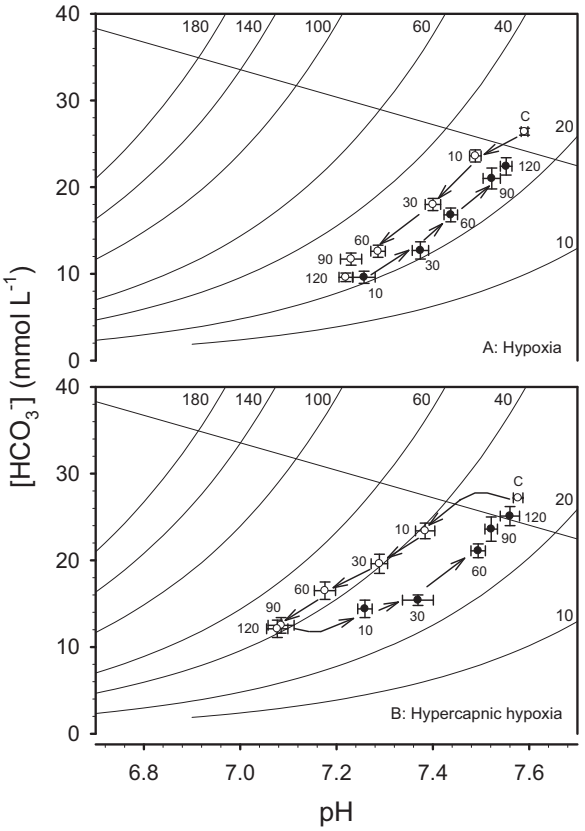

Fig. 2. The time-course of the acid–base disturbance in d15 embryos in response to (A) hypoxia (10% O_2) or (B) hypercapnic hypoxia (5%CO_2, 10%O_2) (open symbols) with recovery in air (closed symbols). Numbered curves are the Pa_{CO_2} isopleths in mmHg. The solid regression is the same as in Fig. 1. Values are mean ± 1 S.E.M. and *N* values are as for Hct in Fig. 5A.

significantly within 10 min from pre-exposure values of 29.2 ± 0.9 mmHg (N = 29) to 40.0 ± 1.0 mmHg (N = 24, P < 0.001) and continued to increase until 60 min exposure (45.8 ± 0.9 mmHg, N = 22). A subsequent slight decrease occurred towards the end of exposure at 120 min (42.2 ± 1.6 mmHg, N = 17) (P = 0.004). Mean Pa_{CO_2} during 120 min of hypercapnic hypoxia exposure resulted in 42.8 ± 0.5 mmHg across all embryos (N = 107), which was significantly higher than mean Pa_{CO_2} isopleth (28.7 mmHg) during recovery in air (see later) (P < 0.001). pH_a (control = 7.58 ± 0.01) significantly decreased within 10 min (7.38 ± 0.01, P < 0.001) and continued to decrease during 90 min (7.08 ± 0.02, N = 22) to 120 min (7.08 ± 0.01) exposure. Decreasing pH_a was accompanied by a large decrease in $[HCO_3^-]_a$ from control value of 27.2 ± 0.5 mmol L^{-1} to 23.4 ± 0.6 mmol L^{-1} at 10 min (P < 0.001) and finally to 12.5 ± 0.5 and12.0 ± 0.7 mmol L^{-1} at 90 and 120 min, respectively. During recovery in air, pH_a significantly increased by 10 min (7.26 ± 0.01, N = 10, P < 0.001) and continued to increase to 7.56 ± 0.01 (N = 21) after 120 min in air. Although unchanged during the first 10 min (13.9 ± 0.8 mmol L^{-1}) of recovery from 90 min of hypercapnic hypoxia (P = 0.083), $[HCO_3^-]_a$ began to increase within 30 min recovery in air (15.5 ± 1.5 mmol L^{-1}, N = 10) with further increases at 60 min (21.1 ± 0.7 mmol L^{-1}, N = 10) and 120 min (25.0 ± 0.7 mmol L^{-1}) of recovery.

Pa_{CO_2} rapidly decreased within the first 10 min (32.0 ± 1.4 mmHg, P < 0.001) of recovery in air and remained unchanged across the remaining recovery period (P = 0.174). The mean Pa_{CO_2} during recovery (28.7 ± 0.7 mmHg, N = 51) was lower than Pa_{CO_2} (42.8 mmHg) during hypercapnic hypoxic exposure. Consequently, all embryos survived 120 min recovery in air after 90 min hypercapnic hypoxic exposure with pH_a, $[HCO_3^-]_a$ and Pa_{CO_2} returning to control values (Fig. 2B).

3.5. Hematological variables during hypoxic challenges

3.5.1. Acute hypoxia

Changes in Hct, MCV and [RBC] by hypoxic exposure (shown with the subscript "hypoxia") from the control (with subscript, c) were expressed as a Δ, e.g., $\Delta Hct = 100 \times (Hct_{hypoxia} - Hct_c)/Hct_c$, to allow comparisons of a % between variables (i.e., ΔMCV and Δ[RBC]) (Burggren et al., 2012).

Control Hct significantly increased with embryonic development from 17.9 ± 0.6% on d11 to 31.7 ± 0.4% on d17 and 30.2 ± 0.4% on d19 (P < 0.001) (Fig. 3A). In response to 2 h hypoxic exposure, Hct markedly increased by a ΔHct of ~30–28%, 18% and 24% in d11–d15 (e.g., from control Hct of 27.7 ± 0.7% to hypoxic Hct of 35.5 ± 0.6% in d15 embryos), d17 (from 31.7 ± 0.4% to 37.3 ± 0.8%) and d19 (from 30.2 ± 0.4% to 37.6 ± 0.7%) embryos, respectively. Control MCV was only significantly larger in d11 embryos (147 ± 2 μ^3) compared with the other age groups (mean MCV from d13 to d19 = 137 ± 1 μ^3) (Fig. 3B). During 2 h hypoxic exposure, MCV also markedly increased by ~30–28%, 17% and 13% in d11–d15 (e.g., from control MCV of 139 ± 1 μ^3 to 179 ± 1 μ^3 during hypoxia in d15 embryos), d17 (from 138 ± 1 μ^3 to 161 ± 1 μ^3) and d19 (from 136 ± 1 μ^3 to 153 ± 1 μ^3) embryos, respectively. Control [RBC] significantly increased with development of embryos from 1.27 ± 0.05 × 10^6 μL^{-1} on d11 to 2.30 ± 0.03 × 10^6 μL^{-1} on d17 and 2.22 ± 0.03 × 10^6 μL^{-1} on d19 (P < 0.001) (Fig. 3C). However, changes in [RBC] during hypoxia were insignificant except for a ~11% increase (from 2.22 ± 0.03 × 10^6 μL^{-1} to 2.46 ± 0.04 × 10^6 μL^{-1}) in d19 embryos.

Control [Hb] significantly increased with development from 5.9 ± 0.2 g% on d11 to 10.0 ± 0.1 g% on d17 and 9.8 ± 0.1 g% on d19 (P < 0.001). However, increased [Hb] in response to hypoxic exposure occurred in d19 embryos only (from 9.8 ± 0.1 g% to 10.8 ± 0.2 g%, P < 0.001) (Fig. 4A). Control MCH was significantly larger in d11 embryos (49.5 ± 0.7 pg) compared with the other age groups. Further, the control MCH of d13 embryos (46.0 ± 0.4 pg) was also significantly larger compared with d17 (44.0 ± 0.3 pg) and d19 (44.0 ± 0.2 pg) embryos only. Hypoxic exposure did not significantly influence MCH on any incubation day (Fig. 4B). There was no difference in control [MCHb] across embryonic development (P = 0.057), resulting in a mean [MCHb] across all embryos of 32.7 ± 0.1 (N = 129). [MCHb] markedly decreased in response to hypoxic exposure and the decrease was larger in younger embryos; i.e., ~25% in d11–d15 embryos, 16% in d17 and 12% in d19 embryos (Fig. 4C).

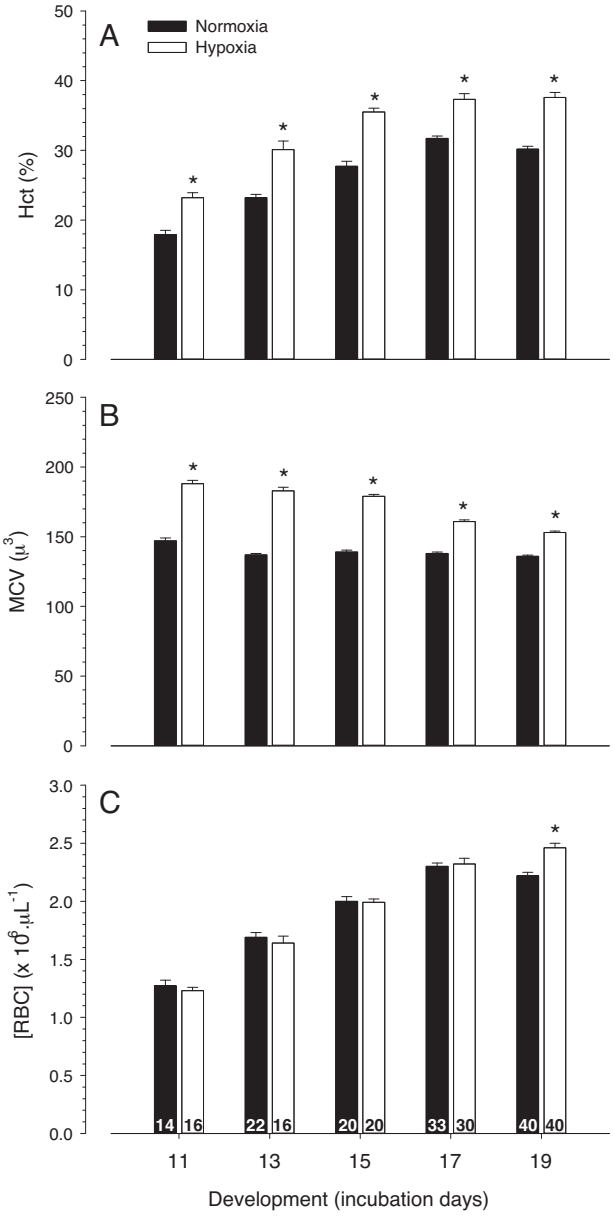

Fig. 3. Hematocrit (Hct), mean corpuscular volume (MCV) and red blood cell concentration ([RBC]) of developing embryos (d11–d19) in air and after exposure to severe hypoxia (10%O_2) for 2 h. An * indicates a difference of hypoxia-exposed from control (normoxic) embryos on a given developmental day. Values are mean ± 1 S.E.M. and N is indicated at the bottom of the bars on panel C.

3.5.2. Time-course during hypoxic challenges

In response to hypoxia (10%O_2), Hct (control = 26.9 ± 0.4%, N = 30) increased significantly after 30 min (33.7 ± 0.5%, N = 17) and remained high throughout further exposure, peaking at 120 min (35.3 ± 0.7%, N = 20) (Fig. 5A). During recovery in air, Hct remained

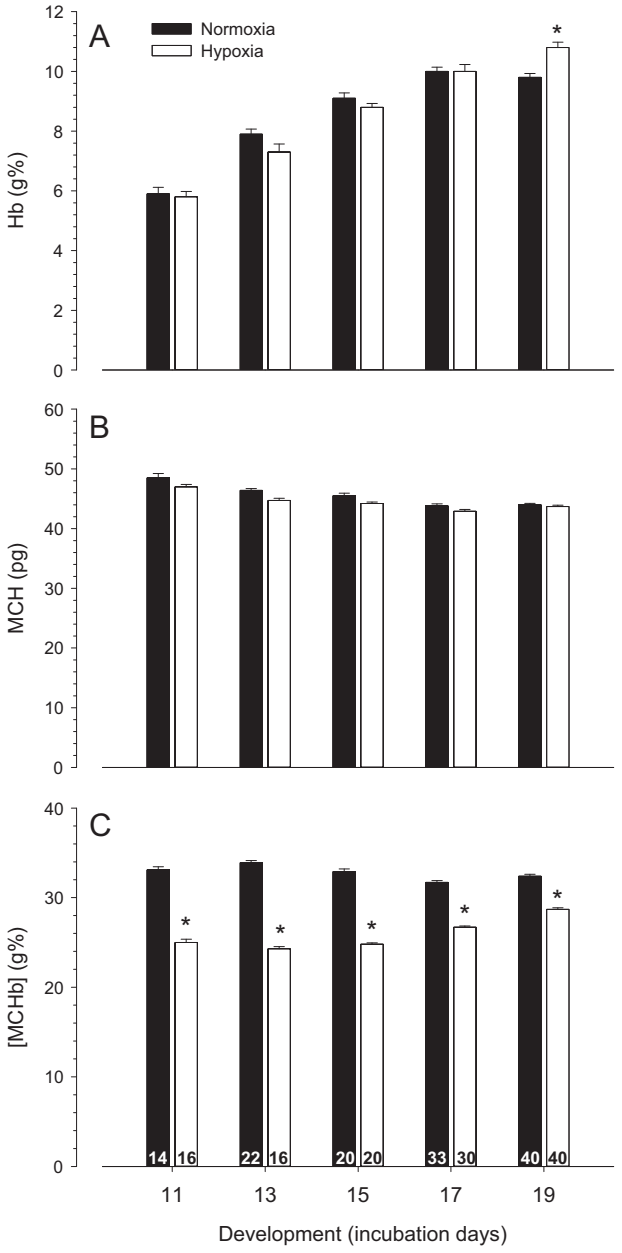

Fig. 4. Hemoglobin concentration ([Hb]), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration ([MCHb]) of developing embryos (d11–d19) in air and after exposure to severe hypoxia (10%O_2) for 2 h. An * indicates a difference of hypoxia-exposed from control (normoxic) embryos on a given developmental day. Values are mean ± 1 S.E.M. and *N* is indicated at the bottom of the bars on panel C.

unchanged during the first 10 min (34.8 ± 1.2%, *N* = 10) and subsequently began to decrease (31.9 ± 0.8%, *N* = 10, at 30 min) and by 60 min (28.4 ± 0.7%, *N* = 10) was sufficient for Hct to return to control levels. [RBC] was not affected by hypoxic exposure and averaged 2.11 ± 0.02 × 10^6 μL^{-1} (*N* = 110) across the exposure and recovery period (*P* = 0.06) (Fig. 5B). MCV (control = 134 ± 2 μ^3, *N* = 10) began to increase within 10 min (141 ± 3 μ^3, *N* = 10), with a subsequent increase at 30 min (149 ± 1 μ^3, *N* = 10), and remained high throughout further exposure, peaking at 120 min (166 ± 3 μ^3, *N* = 10). During recovery, MCV began to decrease within 30 min (156 ± 3 μ^3, *N* = 10) and by 60 min (140 ± 3 μ^3, *N* = 10) was sufficient to return to the control level (Fig. 5C). MCH was not affected by hypoxic exposure (*P* = 0.06) and averaged 44.9 ± 0.2 pg (*N* = 110) across both the exposure and recovery period. Accordingly, [Hb] remained unchanged during hypoxic exposure and recovery in air (9.5 ± 0.2 g%, *N* = 110, *P* = 0.13) and [MCHb] (control = 33.8 ± 0.8 g%, *N* = 10) decreased significantly during hypoxic exposure (e.g., 26.7 ± 0.6 g%, *N* = 10, at 120 min) and subsequently increased during recovery (e.g., 32.2 ± 0.7 g%, *N* = 10, at 120 min in air).

Lactate concentration ([La$^-$]) was 1.2 ± 0.1 mmol L^{-1} in d15 control embryos. [La$^-$] increased rapidly during the first 30 min period of exposure, with subsequent significant increase at 60 min (14.2 ± 0.3 mmol L^{-1}) to a plateau during the remaining period (i.e., 16.2 ± 0.3 mmol L^{-1} at 90 min and 16.7 ± 0.3 mmol L^{-1} at 120 min) (Fig. 6A). During recovery in air, [La$^-$] remained high during the first 30 min (15.8 ± 0.3 mmol L^{-1}) with a subsequent slight decrease at 60 min (14.0 ± 0.7 mmol L^{-1}) to control values at 90 min (9.0 ± 0.8 mmol L^{-1}) and 120 min (9.3 ± 1.1 mmol L^{-1}).

Osm (control = 263 ± 1 mmol kg^{-1}) significantly increased during 90 min (268 ± 1 mmol kg^{-1}) to 120 min (269 ± 1 mmol kg^{-1}) (Fig. 6B). Immediately upon recovery in air, Osm returned to control values (e.g., 266 ± 1 mmol kg^{-1} at 10 min).

A similar pattern occurred during hypercapnic hypoxia (5%CO_2, 10%O_2). An increase in Hct (control = 27.4 ± 0.4%) had begun by 10 min (30.7 ± 0.6%) with a significant increase after 60 min exposure (32.9 ± 0.5%). Hct remained high throughout further exposure, peaking at 120 min (35.6 ± 0.5%) (Fig. 5A). High mortality resulted from 120 min hypercapnic hypoxia, so recovery was initiated at 90 min. Hct dropped to control levels within 10 min of recovery in air (32.5 ± 0.6%). Similar to hypoxic (10% O_2) exposure, [RBC] was not affected by hypercapnic hypoxia and averaged 1.99 ± 0.02 × 10^6 μL^{-1} (*N* = 110) across the exposure and recovery period (*P* = 0.22) (Fig. 5B). MCV (control = 132 ± 2 μ^3) increased significantly within 10 min (151 ± 3 μ^3) and remained high throughout further exposure peaking at 90 min (173 ± 3 μ^3) and 120 min (172 ± 2 μ^3) (Fig. 5C). During recovery, MCV began to decrease significantly within 30 min (157 ± 4 μ^3) and 60 min (144 ± 2 μ^3) was sufficient to return to the control level. Similar to embryos in hypoxia, MCH was not affected by hypercapnic hypoxia (5%CO_2, 10%O_2) and averaged 45.3 ± 0.2 pg (*N* = 110) across the exposure and recovery period (*P* = 0.42). Accordingly, [Hb] remained unchanged during exposure and recovery (9.0 ± 0.1 g%, *N* = 110, *P* = 0.56) and [MCHb] (control = 34.3 ± 0.4 g%) decreased significantly during hypoxic exposure (e.g., 26.2 ± 0.4 g% at 120 min) and subsequently increased during recovery (e.g., 33.3 ± 0.4 g% at 120 min in air).

In response to hypercapnic hypoxia (5%CO_2, 10%O_2), lactate concentration (control = 1.2 ± 0.1 mmol L^{-1}) increased rapidly during the first 30 min period with subsequent significant increase by 60 min (12.9 ± 0.3 mmol L^{-1}) and reached a plateau during the remaining period (i.e., 16.1 ± 0.3 mmol L^{-1} at 90 min and 16.2 ± 0.3 mmol L^{-1} at 120 min) (Fig. 6A). [La$^-$] remained elevated during the first 10 min recovery in air (15.3 ± 0.3 mmol L^{-1}), and subsequently decreased significantly at 60 min (9.6 ± 0.6 mmol L^{-1}), 90 min (7.3 ± 0.8 mmol L^{-1}) and 120 min (5.6 ± 0.5 mmol L^{-1}), resulting in lower [La$^-$] compared with hypoxia recovery. Osm (control = 261 ± 1 mmol kg^{-1}) increased significantly during 90 min (271 ± 1 mmol kg^{-1}) to 120 min (271 ± 1 mmol kg^{-1}) of exposure (Fig. 6B). Immediately upon recovery in air, Osm returned to control values (e.g., 265 ± 1 mmol kg^{-1} at 10 min) similar to recovery from hypoxia.

4. Discussion

4.1. Hypoxia tolerance

Chicken embryos can survive chronic, moderate hypoxia (~14–15% O_2) throughout incubation (e.g., Stock and Metcalfe, 1987; Burton and Palmer, 1992; Rouwet et al., 2002; Dzialowski et al., 2002; Miller et al., 2002; Ruijtenbeek et al., 2003; Villamor

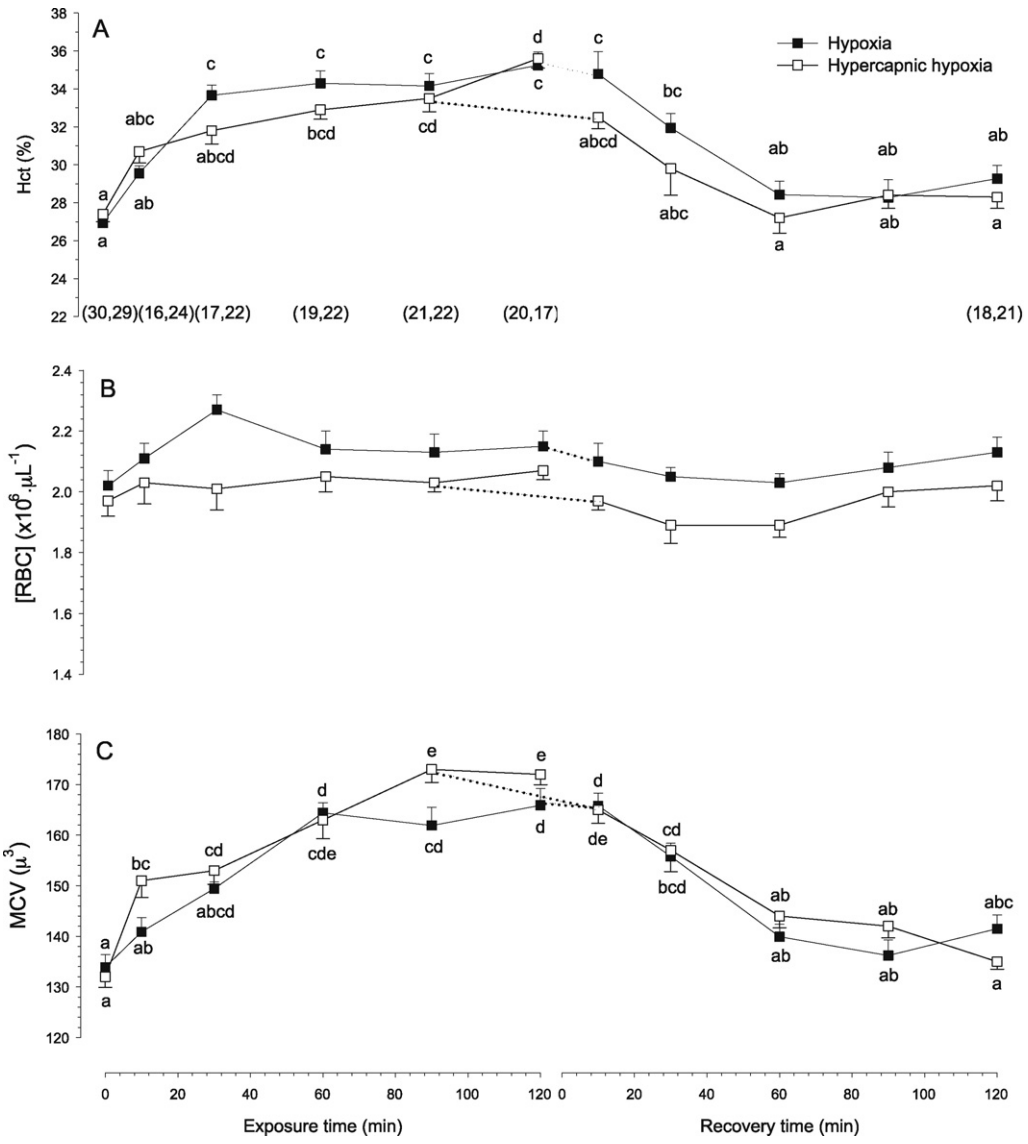

Fig. 5. The time-course of (A) hematocrit (Hct), (B) red blood cell concentration ([RBC]) and (C) mean corpuscular volume (MCV) response to 120 min hypoxia (10% O_2) or 120 min hypercapnic hypoxia (5%CO_2, 10%O_2) with 120 min recovery in air in d15 embryos. The recovery data points are from embryos returned to air for 120 min following either the 120 min hypoxia or 90 min hypercapnic hypoxia exposure as connected by a dotted line. Values are mean ± 1 S.E.M. and *N* values are 10 for each data point, unless otherwise indicated by values given in parentheses (hypoxia, hypercapnic hypoxia). Different lowercase letters indicate statistically significant means for a given variable and gas exposure.

et al., 2004; Chan and Burggren, 2005; Azzam and Mortola, 2007; Ferner and Mortola, 2009). However, prolonged exposure (4 h) to more severe hypoxia (10% O_2) is fatal (Tazawa et al., 1992). In the present study, all embryos (d11–d19, $N = 122$) survived acute (120 min) severe hypoxia (10% O_2). Notably, the addition of 5% CO_2 weakened embryonic tolerance to hypoxia. Consequently, d15 embryos could survive exposure to hypoxia (10% O_2) for 120 min but hypercapnic hypoxia (5% CO_2, 10% O_2) for only 90 min. Further, the blood gas and Hct values of the survivors returned to control, suggesting that the hypoxia-induced disturbances were only transient at 120 min (or 90 min) and may not affect long term survival (Figs. 1 and 2).

4.2. Acid–base disturbances in severe hypoxia and hypercapnic hypoxia

During 2 h severe hypoxia (10%O_2), developing (d11–d19) embryos underwent a severe uncompensated metabolic acidosis (Fig. 1). However, they recovered if returned to normoxia, as exemplified in d15 embryos (Fig. 2A). The onset of the metabolic acidosis was rapid, apparent after just 10 min. For example in d15 embryos there was a significant decrease in both pH_a (from 7.59 to 7.49, $P < 0.001$) and $[HCO_3^-]_a$ (from 26.4 mmol L^{-1} to 23.6 mmol L^{-1}, $P < 0.001$) in early exposure, decreasing to minimal levels by 90 min exposure (i.e., pH_a further decreased to 7.23 and $[HCO_3^-]_a$, to 11.7 mmol L^{-1}) (Fig. 2A). A relative respiratory alkalosis (decreased Pa_{CO_2}) occurred during continued exposure, with Pa_{CO_2} decreasing from 28.2 mmHg at 90 min to 24.2 mmHg at 120 min (Figs. 1 and 2A). The relative respiratory alkalosis was likely the result of hypoxia-induced metabolic depression (i.e., hypometabolism triggered by hypoxia) and the consequent relative loss of CO_2 (i.e., decreased Pa_{CO_2}) due to a reduction in CO_2 production against a backdrop of fixed eggshell gas conductance. It is likely that hypometabolism occurring in the presence of hypoxia and alkalosis is not triggered by the acidemia induced by hypoxia, but rather due to hypoxemia. The present evidence thus implies a role of hypoxemia per se in the induction of hypometabolism in chicken embryos.

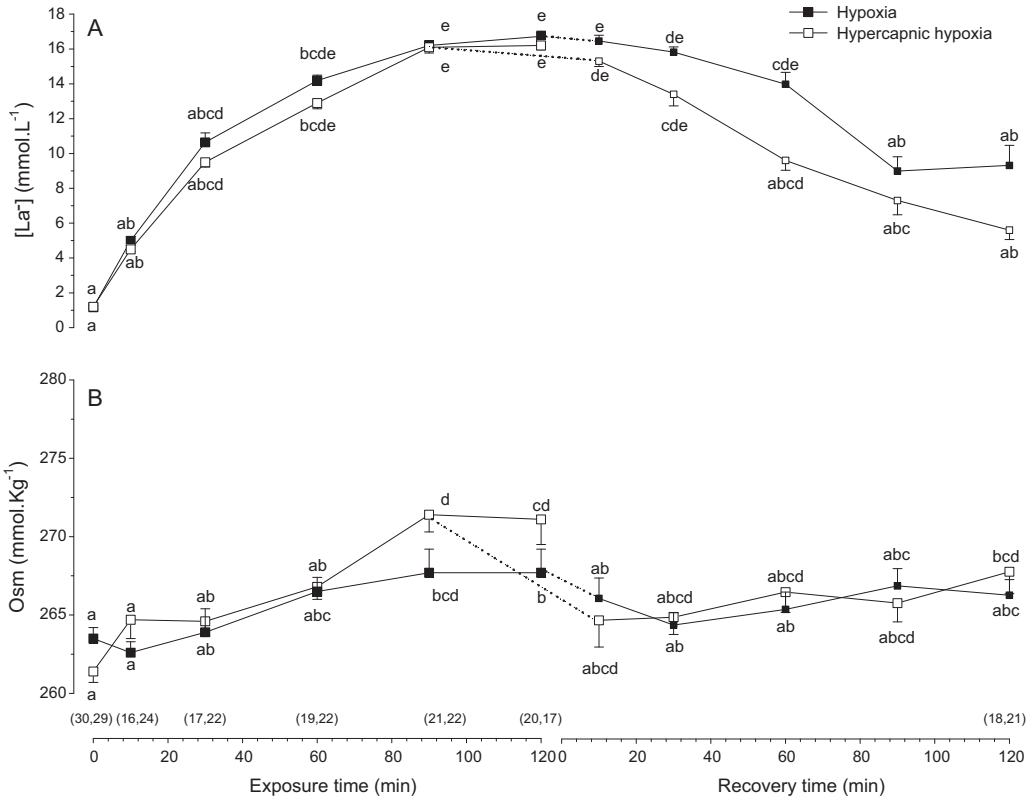

Fig. 6. The time-course of (A) lactate concentration ([La$^-$]) and (B) osmolality (Osm) in response to 120 min hypoxia (10% O_2) or 120 min hypercapnic hypoxia (5%CO_2, 10%O_2) with 120 min recovery in air in d15 embryos. The recovery data points are from embryos returned to air for 120 min following either the 120 min hypoxia or 90 min hypercapnic hypoxia exposure as connected by a dotted line. Values are mean ± 1 S.E.M. and *N* values are 10 for each data point, unless otherwise indicated by values given in parentheses (hypoxia, hypercapnic hypoxia). Different lowercase letters indicate statistically significant means for a given variable and gas exposure.

Acid–base disturbance at 2 h hypoxic exposure was assumed to be maximal for embryos across the developmental range (Fig. 1). In fact, mean pH_a decreased to a similar level of ~7.2 (ranging between ~7 and 7.3 for a given age within a narrow Pa_{CO_2} range) across development. Concurrently, $[HCO_3^-]_a$ decreased to ~5 mmol L^{-1} and 10 mmol L^{-1} in d11–d13 and d15–d19 embryos, respectively (Figs. 1 and 2A). Embryos do not survive for longer than 2 h in severe hypoxia, so we can assume that a pH_a of ~7.2 and $[HCO_3^-]_a$ of ~5–10 mmol L^{-1} represents critical pH_a and $[HCO_3^-]_a$ with mortality occurring at the critical level. Due to the highly conserved $[HCO_3^-]_a$ (e.g., varied by ~4 mmol L^{-1} (from 3 to 7) for d11–d13 and ~10 mmol L^{-1} (6–16) for d15–d19 embryos) compared to pH_a (varied by up to 0.3 unit within a given age group), it appears that $[HCO_3^-]_a$ was a more tightly regulated variable. Failure to regulate $[HCO_3^-]_a$ may thus have more severe consequences than wide changes in pH_a.

Exposing d15 embryos to hypercapnia (i.e., hypercapnic hypoxia; 5%CO_2, 10%O_2) further decreased the critical pH to ~7.1 (with a wide range from ~6.9 to 7.2) and respiratory acidosis occurred in addition to metabolic acidosis concurrent with the increased Pa_{CO_2} (Fig. 2B). A mean critical $[HCO_3^-]_a$ of ~10 mmol L^{-1} was observed in d15 embryos in hypercapnic hypoxia, similar to hypoxia alone (where mean critical pH for hypoxic (10%O_2) and hypercapnic hypoxia (5%CO_2, 10%O_2) exposure was ~7.2 and 7.1, respectively), further supporting the indication that the $[HCO_3^-]_a$ level potentially determines embryo survival. Because blood $[HCO_3^-]_a$ increases during normal (i.e. normoxic) development (e.g., Tazawa et al., 1971a,b; Tazawa, 1973, 1980; Burggren et al., 2012), it is likely that the critical $[HCO_3^-]_a$ also increases from ~5 mmol L^{-1} in d11–d13 embryos to ~10 mmol L^{-1} in advanced (d15–d19) embryos, although this remains to be studied. The reduction in $[HCO_3^-]_a$ during hypoxia occurred concurrently with O_2 depletion, as indicated by increased lactic acid production via anaerobic pathways. At any time throughout the hypoxic exposure and recovery time-course, the change in $[HCO_3^-]_a$ was matched by changes in [La$^-$] (Fig. 7). For example, during 120 min of hypercapnic hypoxic exposure, the decrease in $[HCO_3^-]_a$ of ~15 mmol L^{-1} was matched by an increase in [La$^-$] of ~15 mmol L^{-1} and vice versa during recovery in air (i.e., increase in $[HCO_3^-]$ of ~11 mmol L^{-1} cf. decrease in [La$^-$] of ~10 mmol L^{-1}). This indicates that anaerobic glycolysis is a key factor determining the metabolic alterations and overall acid–base status of embryos during severe hypoxia. The decrease in $[HCO_3^-]_a$ likely occurs too rapidly to be attributed to renal function, instead being accredited to the well developed chorioallantoic membrane (CAM) which actively transports ions between the capillary blood and allantoic fluid (Stewart and Terepka, 1969; Hoyt, 1979; Davis et al., 1988; see Gabrielli and Accili (2010) for review). Similar rapid (<4 h) alterations in acid–base balance (mild respiratory and/or metabolic disturbances) have previously been reported during environmental gas alterations (e.g., CO_2, He or SF_6), as a result of altered eggshell gas conductance, or after infusion of hypertonic electrolyte solutions (Tazawa, 1981, 1982; Tazawa et al., 1981).

A small, partial respiratory alkalosis occurred in d15–d19 embryos during hypoxia (Fig. 1), with the decrease in Pa_{CO_2} likely resulting from a metabolic down-regulation, known to occur in response to hypoxia across a broad range of embryonic, neonatal and adult embryos (see Mortola (2005) for review). The hypometabolism due to hypoxia must entail a decrease in oxygen consumption and thus should lessen the needs for anaerobic energy supply and lactic acid production. However, as shown in recovery (Fig. 6), lactic acid production does not decrease rapidly

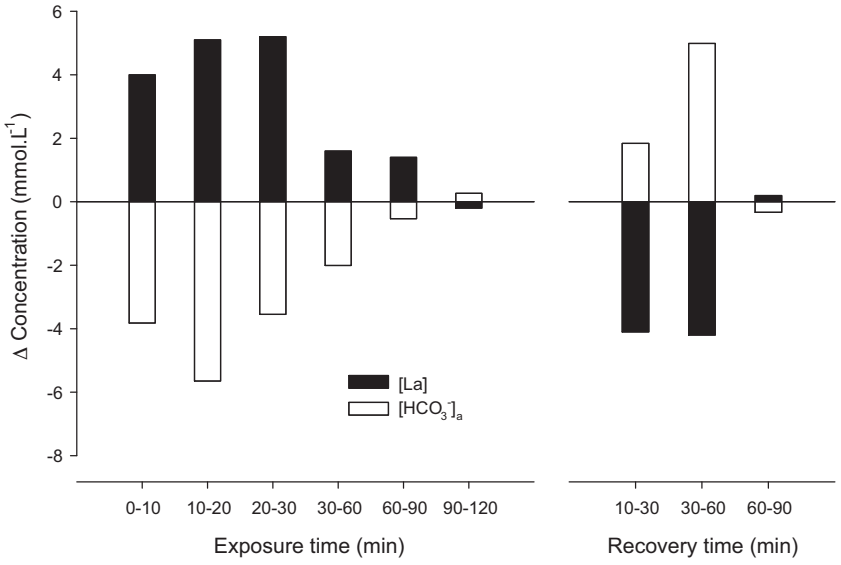

Fig. 7. Change in the lactate ($[La^-]$) and bicarbonate concentration ($[HCO_3^-]_a$) during hypoxic exposure and recovery in air in d15 embryos. Values are presented as the difference in concentration between two consecutive time-points. *N* values for lactate are 10 for each data point and for bicarbonate they are the same as for Hct in Fig. 5A.

with a return to normoxia. Accordingly, it is likely that decrease in oxygen consumption due to hypometabolism is not accompanied by a decrease in anaerobic energy supply and lactate production. Interestingly, although younger embryos (e.g., d12) also down-regulate metabolism during hypoxia (Tazawa et al., 1992), in the present study Pa_{CO_2} was constant (d11 and d13) and no respiratory alkalosis occurred. Consequently, different acid–base responses to hypoxia occurred in younger versus more advanced embryos, as has been demonstrated previously using moderate hypercapnic hypoxia (5%CO_2, 15%O_2) (Burggren et al., 2012). The differences in response likely arise due to changes in normoxic arterialized blood gas values which change across development.

Many previous studies have reported that blood pH of developing embryos either showed little decrease or displayed no particular pattern of change during the late incubation period (Dawes and Simkiss, 1969, 1971; Freeman and Misson, 1970; Erasmus et al., 1970/71; Girard, 1971; Boutilier et al., 1977; Bruggeman et al., 2007; Everaert et al., 2008). However, the present study confirms findings of some previous studies (e.g., Tazawa et al., 1971a,b; Tazawa, 1973, 1980; Burggren et al., 2012) indicating that dissolved CO_2 accumulates, resulting in decreased blood pH across development (Fig. 1). This occurs due to the increase in metabolism across development in the face of fixed eggshell gas conductance, creating an increased Pa_{CO_2}. The decrease in pH during embryonic development is much smaller than estimated from the buffer value because of large daily increases in $[HCO_3^-]$ (Fig. 1) (Tazawa et al., 1971a,b; Tazawa, 1973, 1980; Burggren et al., 2012). The source of HCO_3^- during normal embryonic development has been variously attributed to renal activity, absorption of the eggshell (Dawes and Simkiss, 1969, 1971; Erasmus et al., 1970/71; Dawes, 1975), absorption of chorioallantoic fluid HCO_3^- through the choriallantoic vascular system (Boutilier et al., 1977) and intracellular exchange of H^+ and K^+ (Everaert et al., 2008). A combination of these mechanisms, involving functions of the CAM, are likely responsible for the daily increases in non-respiratory $[HCO_3^-]_a$ which mitigate the drop in pH_a.

4.3. Hematocrit and severe hypoxia or hypercapnic hypoxia

Hct, [RBC] and [Hb] significantly increased during d11-d17, with a slight decrease on d19 in all three variables (Figs. 3A and C and 4A). The sigmoidal pattern of increase has been demonstrated previously across the second half of development (d10–d19) (Tazawa et al., 2011). MCV, MCH and [MCHb] (which collectively indicate how Hb is packaged within the RBCs) were actually constant or initially decreased slightly during d11–d19, as has been shown previously during d10–d19 (Tazawa et al., 2011). For instance, MCV initially decreased until d13 then remained constant at a mean value of ~137 μ^3 (Tazawa et al., 2011) during the remaining d13–d19 of incubation (Fig. 3B). Similarly, MCH initially decreased until d13, then remained unchanged at mean value of ~44 pg from d15 to d19 (Tazawa et al., 2011) (Fig. 4B). Accordingly, [MCHb] remained constant at ~33 g% during d10–d19 (Tazawa et al., 2011) or during d11–d19 in the present experiment (Fig. 4C).

The increase in Hct during hypoxia changed with embryo age, with the greatest increase in younger embryos (e.g., ~30% by ΔHct in d11–d13 and ~28% in d15) compared with older embryos (e.g., ~18% in d17 and ~24% in d19) (Fig. 3A). Similarly, MCV increased in all embryos with the greatest increase in d11–d13 embryos (~30%) compared with d17 (~17%) and d19 (~13%) (Fig. 3B). However, the response of [RBC] to severe hypoxia was different from Hct and MCV, with a small decrease in [RBC] in younger embryos (d11–d15) and a large increase (~11%) in d19 embryos (Fig. 3C). Consequently, the increase in Hct in younger (d11–d15) embryos was attributed to the increase in MCV alone. For example, the ~28% increase in Hct in d15 embryos was due to a ~29% increase in MCV. In contrast, the 24% increase in Hct in more advanced d19 embryos was attributed to a combination of a 13% increase in MCV and 11% increase in [RBC] (Fig. 3). Furthermore, comparison between these percent changes in Hct, MCV and [RBC] indicated that the increase in Hct was attributed to RBC volume (MCV) and number ([RBC]) with little contribution of plasma volume changes, as also has been suggested previously in moderate (15%O_2) hypoxic exposure experiment (Burggren et al., 2012).

The time-course of the Hct response was very rapid, beginning within 30 min of the onset of hypoxia or hypercapnic hypoxia (e.g., Fig. 5A). Increased Hct due to an increase in [RBC] may potentially improve O_2 transport. However, contrary to expectation, increased Hct during acute severe hypoxia resulted from an increase in MCV only in d11–d17 embryos (Fig. 3) and therefore the increased Hct is unlikely to play a role in facilitating O_2 delivery during acute severe hypoxia in younger embryos. Similarly, increased Hct through increased MCV was previously demonstrated in d15 and d17 embryos during 1d exposure to moderate (13% O_2) hypoxia

(Ackerman, 1970). In contrast however, d19 embryos increased [RBC] by 0.24×10^6 μL^{-1} (in addition to an increase in MCV) requiring the release of an estimated 720 million RBC (with a volume of 0.11 mL) across the 2 h period (assuming a total circulating blood volume of 3 mL). It remains to be determined which tissues or organs are responsible for the release of this relatively large volume of RBCs in response to hypoxia. The spleen is an important site of erythrocyte storage in adult vertebrates (see e.g., Stewart and McKenzie (2002) for review) and may potentially play a role in embryonic RBC sequestration and release. It appears that embryos younger than d19 either have not yet produced enough RBCs for release during hypoxia or that the sequestration site (potentially the still-developing spleen) is not sufficiently developed to either sequester or release RBCs. Similar age-specific Hct responses have been demonstrated in response to acute hypoxic hypercapnia (produced via a decrease in eggshell gas conductance) where d17 embryos increased Hct via changes in MCV only and d19 embryos increased both MCV and [RBC] (Tazawa et al., 1988).

Increased Hct through increased [RBC] results in an increase in [Hb] in the circulation (see d19 embryos in Figs. 3C and 4A) and likely improves O_2 delivery. However, an increased Hct through increased MCV may result simply from changes in pH increasing cell volume, for example, and may provide no resulting boost to O_2 delivery. Possibly, increased MCV enhances O_2 loading when the RBCs pass through the CAM vasculature. Although the affinity of Hb for O_2 is unchanged by MCV, the rate of O_2 uptake by the RBC is a function of the surface area to volume ratio of the RBC (Vandegriff and Olson, 1984). Greater O_2 uptake (and improved O_2 transport efficiency) may occur in larger RBCs due to faster uptake while the RBC is within the vasculature of the CAM, although the loading capacity for O_2 may be contrarily decreased when MCV increases. Physiological contribution of MCV increasing with hypoxic exposure to O_2 transport may be a future subject to be studied.

Exactly how MCV increases during hypoxia is yet to be elucidated. MCV is influenced by multiple mechanisms, including the potassium-chloride co-transport, taurine transport and sodium-dependent beta-amino acid transport systems, Na^+, K^+-ATPase activity and changes in blood pH and [HCO_3^-] which influence Donnan equilibrium mechanism (see Hoffmann et al., 2009 and Nikinmaa, 1992 for review). These systems are modulated by neuronal, hormonal and autocrine stimulation and via changes in blood osmolality, P_{O_2}, P_{CO_2} and pH (see Cossins and Gibson, 1997; Lambert et al., 2008; Nikinmaa, 1992 for review). Changes in MCV involve ion and water movement, but total blood Osm does not always change in parallel (Fig. 6) (e.g., Van Beaumont and Rochelle, 1974; Staübli and Roessler, 1986; Andrewartha et al., 2011b). The increase of MCV and Hct followed a similar time-course in d15 embryos regardless of the presence or absence of CO_2. Similar to previous moderate hypoxia studies (e.g., Burggren et al., 2012), it appears that [O_2] is a more important driving force for MCV and consequently Hct, which remains to be studied. The relative contribution of these many mediators to MCV and Hct regulation during hypoxia certainly warrants additional study.

Acknowledgement

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Appendix. Underestimation of Hct by Coulter counter analysis

The Coulter analyzer overestimates Hct in normoxic embryos across the second half of embryonic development, with the differences slightly augmented at the high Hct values (Tazawa et al., 2011). Therefore, to determine whether the changes in Hct during hypoxia were similarly overestimated, comparisons were made between Hct determined by the Coulter analyzer and via traditional centrifugation (see methods). Hct was simultaneously determined using the two methodologies (Coulter counter analysis and centrifugation) for 10 blood samples from each time-point in the hypoxia and hypercapnic hypoxia time-course experiment. Thus, for each gas exposure there was a control group, 5 hypoxic (or hypercapnic hypoxic) time-points and 5 recovery time-points (Fig. A.1).

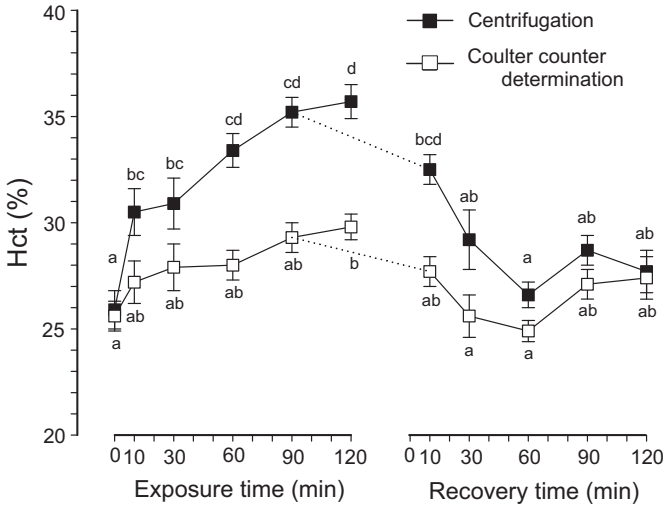

Fig. A.1. The simultaneous determinations of hematocrit (Hct) by a Coulter analyzer and by the centrifugation for the blood of embryos exposed to hypercapnic hypoxia (5%CO_2, 10%O_2) for 120 min at the longest and during recovery in air. The dotted line connects 90 min hypercapnic hypoxia exposure from which embryos are placed back in air for 120 min recovery and the first measurement in air recovery (i.e., 10 min). Sample size is 10 for all the groups. The significant difference between group means determined by the Coulter analyzer and the centrifugation is indicated by different symbols.

Hct determined by centrifugation markedly increased during hypercapnic hypoxia (and hypoxia) exposure; e.g., from $25.9 \pm 0.9\%$ (control) to $35.7 \pm 0.8\%$ after 120 min of exposure (Fig. A.1). This absolute mean increase of 9.8% corresponded to a relative increase in Hct by ~38% (ΔHct). The Hct values determined by the Coulter analyzer on the same blood samples grossly underestimates the increase; e.g., from $25.6 \pm 0.8\%$ of control to $29.8 \pm 0.6\%$ at 120 min. The absolute mean increase observed at 120 min using the Coulter values was only 4.2% corresponding to a relative increase of ~16% of control (ΔHct). Thus overall, at 120 min Hct was underestimated by ~22% in terms of relative value. Consequently, Hct only increased significantly at 120 min of exposure using the values obtained by the Coulter analyzer (Fig. A.1). This is in contrast to the significant increase seen at 10 min onwards using the values determined via centrifugation.

Hct was grossly underestimated by the Coulter counter during hypercapnic hypoxia (and hypoxia) and the discrepancy increased with longer exposure times. The underestimation was such that the Coulter counter measurements were not sensitive enough to detect the significant increases in Hct that occurred in this study. Thus, although the Coulter counter affords processing of large sample sizes, it is important to be aware of the differences in results obtained by different methodologies and sometimes the more laborious, well-established techniques such as centrifugation are required to avoid the wrong conclusions being reached.

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