Dynamics of metabolic compensation and hematological changes in chicken (*Gallus gallus*) embryos exposed to hypercapnia with varying oxygen

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ABSTRACT

In day 15 chicken embryos, we determined the time course responses of acid–base balance and hematological respiratory variables during 24 h exposure to 15, 20, 40 or 90% O_2, in the presence of 5% CO_2. Hypercapnic respiratory acidosis was initially (2 h) only slightly (∼20%) compensated by metabolic alkalosis in normoxic/hyperoxic embryos. After 6 h, respiratory acidosis was partially (∼40–50%) compensated not only in normoxic/hyperoxic embryos, but also in hypoxic embryos. However, partial metabolic compensation in 15% O_2 could not be preserved after 24 h. Preservation of metabolic compensation required oxygen concentration ($[O_2]$) above 20%, but the magnitude of partial metabolic compensation was unrelated to $[O_2]$. Hematocrit (Hct), together with mean corpuscular volume (MCV), markedly increased in hypercapnic hypoxia, and was maintained at 24 h due to a subsequent increase in red blood cell concentration ($[RBC]$). In contrast, Hct, together with MCV, decreased in hypercapnic normoxia/hyperoxia accompanied by a subsequent decrease in $[RBC]$ at 24 h. Regulation of variables takes place similarly irrespective of environmental $[O_2]$ above 20%, matching acid–base regulation.

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1. Introduction

Avian embryos developing within the eggshell are not able to employ convective ventilation, and the resultant prolonged period of diffusion-only gas exchange results in unique acid–base balance. For instance, exposure to a hyperoxic normocapnic (i.e., 40% O_2, 0% CO_2) environment for 24 h induces respiratory acidosis in late chicken embryos due to increased CO_2 production against the backdrop of a fixed eggshell gas conductance during development (Tazawa, 1986; Tazawa et al., 1992; Burggren et al., 2012).

In chicken eggs, natural variability of eggshell gas conductance is quite large (Tazawa et al., 1983; Visschedijk et al., 1985). Water vapor conductance of 395 eggs obtained from a commercial hatchery ranged from ∼7 mg d^{-1} Torr^{-1} to ∼33 mg d^{-1} Torr^{-1}, resulting in wide variations of P_{CO_2} from 18 Torr to 54 Torr, in the perichorioallantoic air space on d16–d19 of incubation (Visschedijk et al., 1985). In d16 embryos, arterialized blood P_{CO_2} varies naturally from ∼30 Torr to ∼55 Torr in eggs selected for widely varying conductance (Tazawa et al., 1983). Accordingly, exposure to extrinsic CO_2 around 5–10% is not an unrealistic imposition on chicken embryos. Some bird species lay eggs in a burrow nest where developing embryos are exposed to a hypercanic (and hypoxic) environment and therefore avian embryos may potentially tolerate hypercapnia. In fact, 20 min exposure to 21% CO_2

with 21% O_2 or even with 10% O_2 is not fatal in d14 chicken embryos (Tazawa, 1981b). Day 15 embryos survive exposure to 10% CO_2, 20% O_2 for 24 h with acid–base balance returned to the control state after 2 h recovery in air (H. Tazawa, unpublished data). Likewise, d15 embryos survive 2 h exposure to 5% CO_2, 10% O_2 producing severe metabolic acidosis with $[HCO_3^-]$ of ∼10 mmol L^{-1} (Tazawa et al., 2012). Therefore, avian embryos are a favorable model to study acid–base and hematological regulation in animals confronting extraordinary environmental respiratory gas challenges.

Respiratory acidosis of chicken embryos, induced by either CO_2 treatment or by decreasing eggshell gas conductance due to exposure of eggs to SF_6–O_2 gas mixture, is partially compensated by an increase in blood bicarbonate concentration ($[HCO_3^-]$) (Tazawa et al., 1981; Tazawa, 1982). Acid–base status for embryos exposed to 9% CO_2, or for embryos with the eggshell partially covered by gas-impermeable material, also shows metabolic compensation for respiratory acidosis, due to an increase in $[HCO_3^-]$ (Dawes and Simkiss, 1971; Tazawa et al., 1971b). When the eggshell is partially covered by impermeable material, blood hematocrit (Hct) (and calculated hemoglobin concentration ($[Hb]$)) increases (Tazawa et al., 1971b, 1988; Nakazawa and Tazawa, 1988). Even in chicken eggs selected for widely varying eggshell gas conductance, partial compensation for blood pH occurs due to non-respiratory changes in $[HCO_3^-]$, because the variations of pH with P_{CO_2} are smaller than predicted for true plasma (Tazawa et al., 1983). Unique acid–base balance responses also occur in chicken embryos after 24 h exposure to hypercapnic hypoxia (5% CO_2, 15% O_2) and

hypercapnic normoxia (5% CO_2, 20% O_2) (Burggren et al., 2012). In day 15 (d15) embryos, hypercapnic hypoxia caused uncompensated respiratory acidosis after 24 h exposure, while respiratory acidosis caused by hypercapnic normoxia was partially compensated by metabolic alkalosis after 24 h exposure. Because the CO_2 concentration ([CO_2]) was the same in both hypercapnic groups, O_2 concentration ([O_2]) appears to be a key factor in compensation of respiratory acidosis. In addition to acid–base responses determined after 24 h exposure (Burggren et al., 2012), respiratory disturbances of blood acid–base balance in chicken embryos begin soon after extrinsic gas alterations, with partial metabolic compensation reaching equilibrium within 3–6 h (Tazawa, 1981a, 1982, 1986; Tazawa et al., 1981). Such time-dependent responses of acid–base balance and associated hematological respiratory variables have not been previously investigated for chicken embryos exposed to hypercapnia with altered [O_2].

It is apparent from the aforementioned emerging studies that isolated examination of acid–base balance, hematology or blood O_2 alone, rather than in concert, allows only fragmented understanding of their very complex interactions and dynamics during embryonic development. To address the roles of [O_2], hematology and gas exchange perturbation in acid–base responses, this study examines the time course of acid–base balance changes in d15 embryos exposed to varying [O_2] (i.e., 15, 20, 40 and 90%) in the presence of increased [CO_2] (5%). In addition to the previous study showing acid–base responses and Hct regulation after 24 h exposure to moderate hypoxia (15% O_2, with or without 5% CO_2) (Burggren et al., 2012), we also investigated acid–base responses and Hct regulation in embryos exposed to severe hypoxia (10% O_2, with or without 5% CO_2) (Tazawa et al., 2012). While moderate hypoxia produced metabolic acidosis with a slight increase in lactate concentration ([La^-]), which accounted for ∼1/6th of the decrease in [HCO_3^-] (Burggren et al., 2012), metabolic acidosis produced by severe hypoxia (and hypercapnic hypoxia (5% CO_2, 10% O_2)) was accompanied by predominant increase in [La^-] which matched the decrease in [HCO_3^-] (Tazawa et al., 2012). Accordingly, a mechanism causing metabolic acidosis differs between moderate (15%) and severe (10%) hypoxia. While anaerobic glycolysis determines the metabolic alterations (i.e., [HCO_3^-]) and acid–base status during severe hypoxia, there is a possibility that [HCO_3^-] may change to compensate for acidosis within 24 h (e.g., 3–6 h) of moderate hypoxia and hypercapnic hypoxia (5% CO_2, 15% O_2) as in embryos exposed to hypercapnic normoxia (4% CO_2, 21% O_2) (Tazawa, 1982) or to 20% O_2 balanced by SF_6 (Tazawa et al., 1981). That is, a mechanism responding to moderate hypoxia and hypercapnic hypoxia may be the same as to hypercapnic normoxia, although the magnitude of responses may depend on [O_2]. Thus, we predict that metabolic compensation will occur even in hypoxic (15% O_2) embryos during time-course of 24 h responses, and that compensation will increase in a dose-related fashion as [O_2] rises to normoxia (20% O_2) and beyond to hyperoxia (40% and 90% O_2). Because Hb serves as a non-carbonate buffer in blood, with an increasing concentration during the last half of incubation (e.g., Tazawa et al., 2011), there may also be a possible contribution of [Hb] to the increase in non-respiratory [HCO_3^-] in metabolic compensation for respiratory acidosis during 24 h responses. Consequently, we also undertook time course measurements of hematological respiratory variables, including Hct, [Hb], red blood cell concentration ([RBC]), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration ([MCHb]) and osmolality (Osm), in embryos challenged by extrinsic alterations of [O_2] in the presence of 5% CO_2. By examining multiple hematological variables we may identify the mechanisms underlying acid–base compensation.

2. Materials and methods

2.1. Egg incubation and exposure to gas mixtures

Fertile eggs of the domestic chicken (*Gallus gallus*) were obtained from a hatchery at Texas A&M University (College Station, Texas, USA). Eggs were numbered, weighed (± 0.01 g) by an electronic balance upon arrival and set in an incubator within the week according to an experimental schedule. Temperature of the incubator (model 1502, G.Q.F. Manuf. Co., USA) was kept at $37.5 \pm 0.1\,°C$ with a relative humidity of approximately 55%. Eggs were turned automatically every 3 h until d14.

On d14 of incubation, eggs were candled to locate an allantoic vein and the eggshell over the vein was marked. The eggs were divided into "control" eggs and "gas–exposed" eggs, and moved to a second desk-top incubator (1588 Electr. Hova-Bator, G.Q.F. Manuf. Co., USA) warmed to 37.5 °C. Half of the incubator space was filled by a 3.78-L gas exposure bag to hold the gas exposed eggs as described previously (Burggren et al., 2012), while a cardboard egg stand held control eggs in the other half. The gas exposure bag was ventilated with one of four gas mixtures: 15, 20, 40 or 90% O_2, all with 5% CO_2 balanced by N_2. Gas mixtures were created with a Wösthoff gas mixing pump (oHG, Bochum, Germany) at a rate of 600 ml/min (Burggren et al., 2012). Gas exposed eggs were either placed in the bag at 12:00 on d14 of incubation (24 h exposure) or they were exposed to the gas mixtures for 2 h or 6 h on d15 (2 h or 6 h exposure). Accordingly, the experiments were carried out for four gas exposure groups; group 1 = hypercapnic hypoxia (5% CO_2, 15% O_2/80% N_2), group 2 = hypercapnic normoxia (5% CO_2, 20% O_2/75% N_2), group 3 = hypercapnic hyperoxia (5% CO_2, 40% O_2/55% N_2), and group 4 = hypercapnic hyperoxia (5% CO_2, 90% O_2/5% N_2). Each group included four sub-groups; control (0 h), 2 h, 6 h and 24 h exposure.

2.2. Blood collection and analysis

Approximately 0.4 mL of blood was collected from the allantoic vein of the control and gas exposed embryos on d15. Immediately after removal from the incubator, gas exposed eggs were wrapped loosely in aluminum foil to allow for a greater gas reservoir between the foil and the eggshell, preserving the blood gases during rapid blood collection (<2 min since removal from the incubator), as described by Burggren et al. (2012). In order to minimize a dead space between needle and syringe, we used a syringe on which a needle was directly fixed. Accordingly, collected blood had to be emptied once into a conic-ended plastic vial. To minimize contact of blood with air, a tip of the needle was placed at the bottom of the vial and the blood was gently transferred into the vial so that the blood would move gently from the bottom to the upper surface. For measurement, the blood was withdrawn from the bottom of the vial. Because the upper surface of blood contacted air, we made a preliminary measurement of blood gas variables to estimate the effect of air contact and assured that careful treatment of blood increased accuracy of the measurement and did not negate the measurement.

Because the blood collected from the allantoic vein was arterialized by chorioallantoic capillaries (Piiper et al., 1980), measured variables represent arterial values (annotated by subscript a) corresponding to adult pulmonary venous blood. Blood was immediately analyzed for pH_a, [HCO_3^-]$_a$ (mmol L^{-1}) and P_{CO_2} (mmHg) by a blood gas system (ABL5, Radiometer Medical A/S, Denmark). The relationship between pH_a and [HCO_3^-]$_a$ was depicted on a Davenport (pH_a–[HCO_3^-]$_a$) diagram, which was previously constructed by plotting P_{CO_2} isopleths calculated from the Henderson-Hasselbalch equation using a CO_2 solubility factor of 0.0308 mmol L^{-1} mmHg^{-1} and a serum carbonic acid pK′ varying with pH (Severinghaus

et al., 1956a,b; Burggren et al., 2012). A buffer line was arbitrarily drawn on the Davenport diagram to indicate the buffer value of $-16\,\text{mmol}\,L^{-1}\,\text{pH}^{-1}$, which was determined in a previous study (Burggren et al., 2012).

Remaining blood was well homogenized and measured for [RBC] ($10^6\,\mu L^{-1}$) and [Hb] (g%) by a hematology analyzer (Beckman Coulter Analyzer A^c.T), osmolality (Osm, mmol kg^{-1}) by a vapor pressure osmometer (5520 Vapro, Wescor Inc., USA) and Hct ($\pm 0.1\%$) in duplication by a centrifuge (Readacrit Centrifuge, Becton Dickinson, USA). Two centrifuge determinations were averaged for a value of Hct in individual embryos, and the value was used to calculate mean corpuscular volume (MCV, μm^3), mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration ([MCHb], g%). Hct was simultaneously determined by the Coulter analyzer to use as a comparison with Hct determined by centrifugation (Appendix A), because previous studies found a difference in Hct when measured by the two methods (Tazawa et al., 2011; Burggren et al., 2012), especially in hypoxic embryos (Tazawa et al., 2012). After blood collection, all eggs were moved to a cold environment for euthanasia. The embryo was removed from the egg and measured for mass (± 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 used where appropriate. Differences in egg mass, embryo mass, Osm and hematological variables across gas exposure groups and at each exposure time was tested using a two-way ANOVA with an interaction term at a level of $P < 0.05$. Comparisons of means within each treatment and within each exposure time were performed using the Tukey test or Dunn's method. All other data presented as mean ± 1 S.E.M.

3. Results

3.1. Fresh egg mass, body mass of embryos and Osm

A total of 375 embryos were used in the study (mean fresh egg mass = 58.60 ± 0.23 g). The mean egg masses between the four gas exposure groups and within the four groups were not significantly different (Table 1). Embryo wet mass differed between exposure times within group 1 ($P < 0.001$), but the body masses in the remaining three gas-exposure groups (2, 3 and 4) were not significantly different between exposure times (Table 1). Osm did

not differ between the four exposure subgroups in group 1 or 2 (Table 1). However, Osm was significantly different between the subgroups in group 3 and 4, due to Osm at 24 h exposure being higher than the control.

3.2. Acid–base response dynamics to exposures to hypercapnia with altered [O_2]

The response of acid–base balance to hypercapnic hypoxia (5% CO_2, 15% O_2, group 1) exposure resulted in respiratory acidosis at 2 h of exposure. This was coincident with a status of no metabolic compensation predicted by buffer capacity (i.e., the change from the control status along the buffer line) (Fig. 1A). The uncompensated pH_a (referred to as pH_0) at 2 h was 7.37 and thus the uncompensated change in pH_a (referred to as ΔpH_0) from the control pH_a (referred to as pH_C, 7.57) was 0.20 ($\Delta pH_0 = pH_C - pH_0$). After 6 h of exposure, acid–base response was partially compensated and pH_a increased to 7.46. Accordingly, compensated change in pH_a ($\Delta pH_a = pH_a - pH_0$) was 0.09. When compensation is expressed by a percent ratio of ΔpH_a to ΔpH_0 ($100 \times \Delta pH_a/\Delta pH_0$) (Tazawa et al., 1981), compensation is ~45%. However, even this partial metabolic compensation could not be maintained at 24 h of hypercapnic hypoxia, resulting in a decrease in blood pH_a to 7.42 (and thus $\Delta pH_a = 0.05$), decreasing calculated compensation to ~25%.

In response to hypercapnic normoxia (5% CO_2, 20% O_2, group 2), pH_0 of uncompensated respiratory acidosis was 7.34, estimated from intersection of the buffer line passing through the control status and P_{CO_2} isopleth ($pH_C = 7.57$). This translated into a ΔpH_0 of 0.23 (Fig. 1B). The respiratory acidosis was already partially compensated by metabolic alkalosis 2 h later, with pH_a increasing to 7.39. Thus, ΔpH_a was 0.05 and compensation was ~22%. At 6 h and 24 h later, pH_a was additionally compensated to 7.45 ($\Delta pH_a = 0.11$, compensation = ~48%) and 7.46 ($\Delta pH_a = 0.12$, compensation = ~52%), respectively.

For [O_2] exceeding 20%, the magnitude of metabolic compensation for respiratory acidosis remained unchanged from that at 20% O_2 (Fig. 1C and D). In response to exposure to hypercapnic hyperoxia (5% CO_2, 40% O_2, group 3), pH_0 of uncompensated respiratory acidosis was estimated to be 7.38 (while $pH_C = 7.56$) and ΔpH_0 was 0.18. The respiratory acidosis was partially compensated at 2 h, 6 h and 24 h later, with pH_a values of 7.42, 7.47 and 7.48, respectively (Fig. 1C), resulting in ΔpH_a values of 0.04, 0.09 and 0.10. Thus metabolic compensation was estimated to be ~22%, 50% and 55%

Table 1
Fresh egg mass (g), body mass (g) and osmolality (Osm, mmol kg^{-1}) of control embryos and embryos exposed for 2 h, 6 h and 24 h to 5% CO_2 with four altered O_2 concentrations (15% (group 1), 20% (group 2), 40% (group 3) and 90% (group 4)). Data presented as mean ± 1 S.E.M. Different superscript letters indicate significant differences between variables within each group.

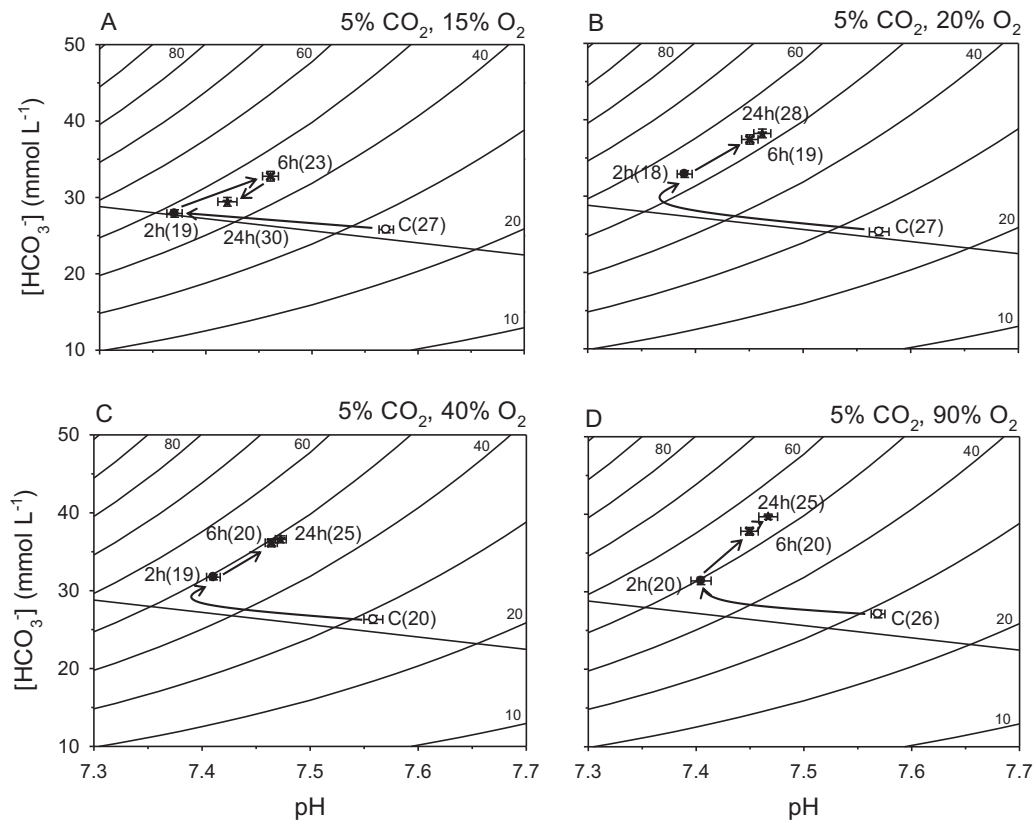|		Group 1	Group 2	Group 3	Group 4
Control	Egg mass	57.74 ± 0.86	57.87 ± 0.85	58.67 ± 0.91	58.07 ± 0.96
	Body mass	12.58 ± 0.27[a]	11.67 ± 0.30	11.95 ± 0.37	12.33 ± 0.27
	Osm	273 ± 2	275 ± 1	274 ± 1[a]	271 ± 1[a]
	N	27	27	22	26
2 h	Egg mass	59.03 ± 0.92	57.41 ± 1.00	59.54 ± 0.95	56.95 ± 0.85
	Body mass	12.90 ± 0.33[a]	12.33 ± 0.31	11.59 ± 0.27	13.01 ± 0.15
	Osm	278 ± 1	276 ± 1	280 ± 2[a,b]	271 ± 2[a,b]
	N	21	18	20	20
6 h	Egg mass	59.41 ± 0.82	59.64 ± 0.90	59.76 ± 1.02	58.88 ± 1.08
	Body mass	12.23 ± 0.28[a]	11.72 ± 0.30	12.12 ± 0.25	13.27 ± 0.29
	Osm	274 ± 2	278 ± 1	278 ± 1[a,b]	274 ± 1[a,b]
	N	23	20	20	20
24 h	Egg mass	59.39 ± 0.91	59.25 ± 0.90	59.31 ± 0.63	56.67 ± 0.75
	Body mass	11.07 ± 0.23[b]	11.85 ± 0.26	11.57 ± 0.24	12.94 ± 0.23
	Osm	277 ± 1	277 ± 1	279 ± 1[b]	276 ± 1[b]
	N	32	29	25	25

Fig. 1. The time course of acid–base disturbance during 24 h exposure of d15 embryos to (A) hypercapnic hypoxia (5% CO_2, 15% O_2), (B) hypercapnic normoxia (5% CO_2, 20% O_2), (C) hypercapnic hyperoxia (5% CO_2, 40% O_2) and (D) hypercapnic hyperoxia (5% CO_2, 90% O_2). Acid–base status was determined prior to exposure (control, C) and at 2 h, 6 h and 24 h. The buffer line with a slope of -16 mmol L^{-1} pH^{-1} (buffer capacity referred to in Burggren et al., 2012) was arbitrarily drawn. The number beside the P_{CO_2} isopleths indicates P_{CO_2} in mmHg. The number in the parentheses shows sample size with bars representing ± 1 S.E.M.

at 2 h, 6 h and 24 h, respectively. Similarly, metabolic compensation for respiratory acidosis induced by exposure in group 4 (5% CO_2, 90% O_2) was estimated to be ~19%, 38% and 52% at 2 h, 6 h and 24 h, respectively (Fig. 1D).

3.3. Dynamics of respiratory hematology related to hypercapnia with altered [O_2]

Gas treatment, exposure time, and the interaction between the two, significantly affected all hematological respiratory variables. The significant interaction ($P < 0.001$ for all variables) was due mainly to a very different time course pattern displayed by embryos in the hypercapnic hypoxia treatment.

Control Hct differed significantly between gas exposure treatments ($P = 0.009$) (Fig. 2A). After 2 h exposure to the different gas treatments, Hct greatly diverged from control values ($P < 0.001$) to be much higher in hypercapnic hypoxia (5% CO_2, 15% O_2, group 1), where it was maintained at 6 h and 24 h. The Hct increase expressed by ΔHct in % (ΔHct = 100 × (altered Hct-control Hct)/control Hct) was ~20–18% at 2 h to 24 h. The lower Hct at 2 h in the remaining groups continued to decrease through to 24 h exposure. Although subtle differences existed between the three normoxia/hyperoxia groups, the trend over time was similar for all. Hct significantly decreased at 6 h and further at 24 h with a mean ΔHct of ~15%.

[RBC] in control embryos was also significantly different between the four groups ($P = 0.013$) (Fig. 2B). The time course of [RBC] was substantially different in hypercapnic hypoxia (group 1) compared to the other groups. [RBC] changed little at 2 h and 6 h, but significantly increased after 24 h in group 1 ($P = 0.001$, Fig. 2B).

The Δ[RBC] ($100 \times$ (altered [RBC] − control [RBC])/control [RBC] in %) was ~9% at 24 h, as predicted from Δ[RBC] = ΔHct − ΔMCV. In comparison, [RBC] in other normoxia/hyperoxia groups was also unchanged during early exposure, but significantly decreased after 24 h ($P < 0.001$, Fig. 2B), corresponding to the mean percent decrease (Δ[RBC]) of ~10%.

Control MCV values were very similar between the gas exposure groups ($P > 0.05$), before diverging at 2 h. MCV in group 1 was significantly higher than the other 3 groups ($P < 0.001$), which did not differ from each other (Fig. 2C). In group 1 MCV was maintained at 6 h with a subsequent slight decrease 24 h later, though the value was still significantly higher than the control ($P < 0.001$). These changes in MCV (ΔMCV = 100 × (altered MCV − control MCV)/control MCV) at 2 h to 24 h corresponded to increases of ~18% to 9%. As such, these values were almost identical with the value predicted from ΔMCV = ΔHct − Δ[RBC]. MCV in hypercapnic normoxia/hyperoxia significantly decreased at 2 h and was maintained at a lower value than the control treatments (Fig. 2C). ΔMCV was ~−6% in these groups.

[Hb] control values, as well as at 2 h and 6 h exposure times, did not differ significantly between groups ($P > 0.05$, Fig. 3A). In group 1, [Hb] at 24 h was significantly higher than at 6 h ($P = 0.006$), but not higher than the control value ($P > 0.05$, Fig. 3A). In groups 2, 3 and 4, [Hb] significantly decreased at 24 h ($P < 0.001$) and did not differ between the three groups.

Control MCH values did not differ between gas exposure treatments, as was also the case for groups 2, 3 and 4 at all exposure times (Fig. 3B). In all 3 groups, MCH decreased slightly during early exposure with subsequent slight increase. In contrast, MCH in group 1
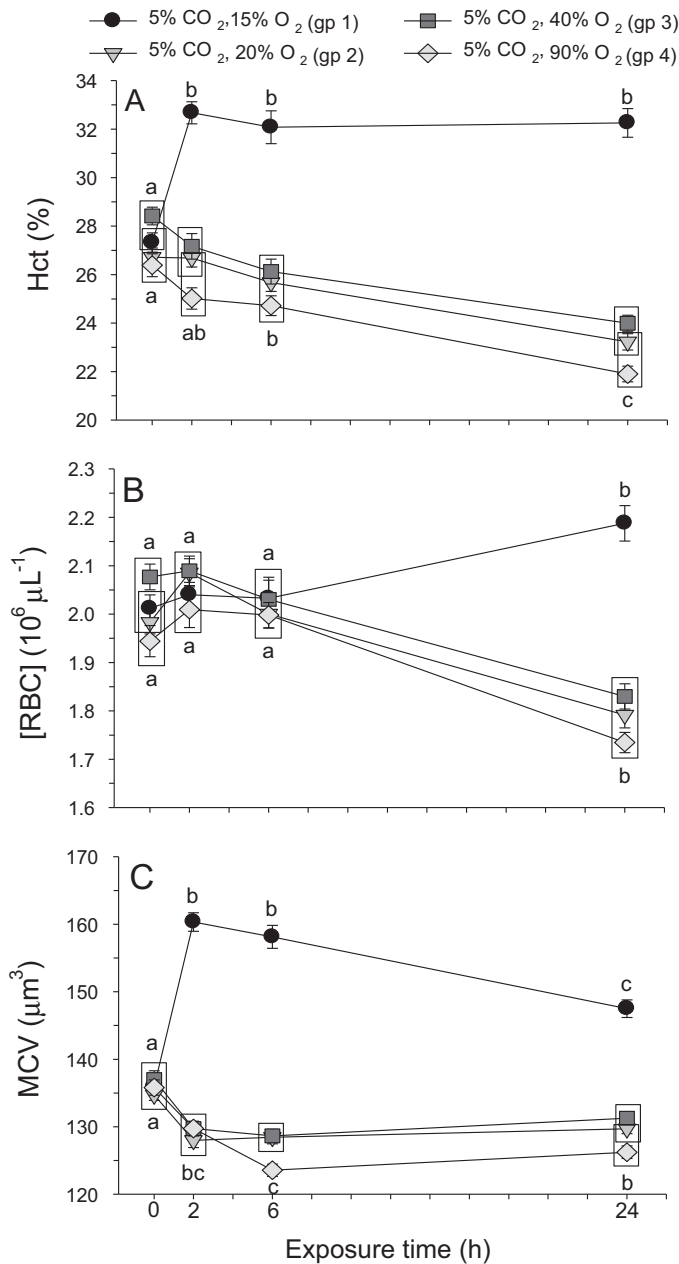
Fig. 2. The time course of changes in (A) hematocrit (Hct), (B) red blood cell concentration ([RBC]) and (C) mean corpuscular volume (MCV) of d15 embryos exposed to 5% CO_2 with altered extrinsic O_2 concentrations (15, 20, 40 and 90%) for 24 h. Symbols represent mean values. Bars represent 1 S.E.M. Means at each exposure time that are not significantly different are grouped within boxes. The set of letters along the hypercapnic hypoxia group indicates differences between exposure times within that group. The set of letters following the trend of the remaining three groups (hypercapnic normoxia/hyperoxia) indicates differences between exposure times for the three groups combined. The mean Hct values of three groups were 27.1 ± 0.3%, 26.3 ± 0.3%, 25.5 ± 0.3% and 23.1 ± 0.2% for control, 2 h, 6 h and 24 h exposure, respectively. Similarly, the mean [RBC] values were $2.00 ± 0.02 × 10^6 \, \mu L^{-1}$, $2.06 ± 0.02 × 10^6 \, \mu L^{-1}$, $2.01 ± 0.02 × 10^6 \, \mu L^{-1}$ and $1.79 ± 0.01 × 10^6 \, \mu L^{-1}$ for control, 2 h, 6 h and 24 h exposure, respectively. The mean MCV values were $136 ± 1 \, \mu m^3$, $127 ± 1 \, \mu m^3$, $127 ± 1 \, \mu m^3$ and $129 ± 1 \, \mu m^3$ for control, 2 h, 6 h and 24 h, respectively.
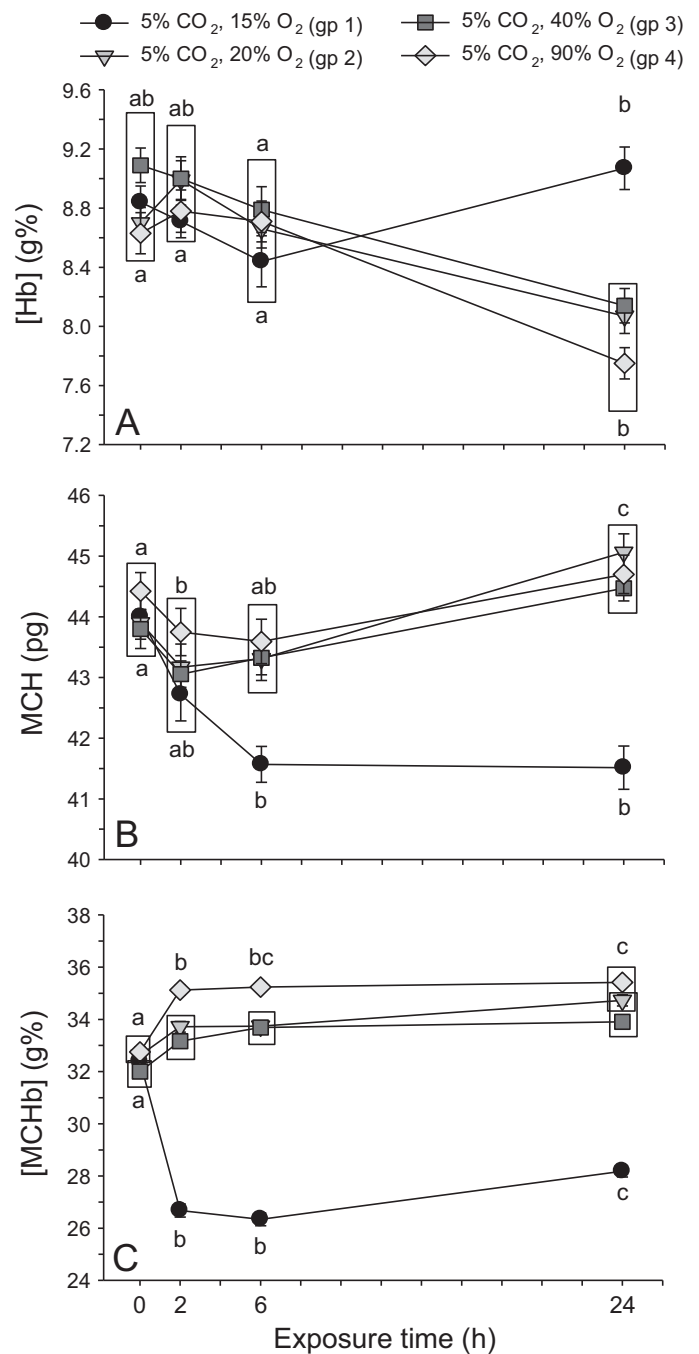
Fig. 3. The time course of changes in (A) hemoglobin concentration ([Hb]), (B) mean corpuscular hemoglobin (MCH) and (C) mean corpuscular hemoglobin concentration ([MCHb]) of d15 embryos exposed to 5% CO_2 with altered environmental O_2 concentrations (15, 20, 40 and 90%) for 24 h. Symbols represent mean values, bars represent 1 S.E.M. Means at each exposure time that are not significantly different are grouped within boxes. The set of letters along the hypercapnic hypoxia group indicates differences between exposure times within that group. The set of letters following the trend of the remaining three groups (hypercapnic normoxia/hyperoxia) indicates differences between exposure times for the three groups combined. The mean [Hb] values of the three groups were 8.8 ± 0.1 g%, 8.9 ± 0.1 g%, 8.7 ± 0.1 g% and 8.0 ± 0.1 g% for control, 2 h, 6 h and 24 h exposure, respectively. The mean MCH values were 44.1 ± 0.2 pg, 43.3 ± 0.2 pg, 43.4 ± 0.2 pg and 44.7 ± 0.2 pg for control, 2 h, 6 h and 24 h exposure, respectively. The mean [MCHb] values were 32.5 ± 0.1 g%, 34.0 ± 0.2 g%, 34.2 ± 0.1 g% and 34.7 ± 0.1 g% for control, 2 h, 6 h and 24 h exposure, respectively.

decreased significantly from control values at 6 h ($P < 0.001$) and was maintained at that level after 24 h exposure.

[MCHb] control values did not differ between groups. However, the decrease in MCH together with large increase in MCV under hypercapnic hypoxia (group 1) resulted in a marked decrease in [MCHb] from control at 2 h ($P < 0.001$) and 6 h ($P < 0.001$), with a

slightly mitigated decrease 24 h later, though still different from the control value ($P < 0.001$, Fig. 3C). In comparison, [MCHb] was elevated in the other three gas treatment groups, with a greater increase in group 4. However, the increasing trend in [MCHb] was similar in the three groups.

4. Discussion

4.1. Embryonic body mass

Egg mass was constant across gas exposure treatments, but some differences in embryo mass did exist. One would anticipate that egg and embryo masses would be identical between treatment groups to avoid an unfavorable influence on the interpretation of embryonic physiological variables and functions. However, it was recently shown that hematological respiratory variables on a given incubation day are independent of embryo mass, using either natural mass variation or experimental growth acceleration (Tazawa et al., 2011). Accordingly, it is reasonable to assume that the small differences found in body mass between sub-groups in group 1 do not influence any conclusions derived from the present experiment.

The lower body mass ($\sim$1.5 g lower compared with control) in 24 h hypercapnic hypoxia exposed embryos may represent a negative influence of gas treatment on growth or embryo's water balance (Table 1). A 24 h exposure to hypercapnia (5% CO_2, 20% O_2) alone previously had no effect on body mass, but a decrease in body mass ($\sim$0.8–1 g) was measured in d15 embryos exposed to hypoxia (15%) for 24 h, irrespective of 5% CO_2 (Burggren et al., 2012). Longer term (>24 h) hypoxia exposure has been shown to either have no effect (Chan and Burggren, 2005) or decrease growth in chicken embryos (Stock and Metcalfe, 1987; Burton and Palmer, 1992; Dzialowski et al., 2002; Miller et al., 2002; Azzam and Mortola, 2007). Incubation in elevated CO_2 can increase or have no effect on growth (Bruggeman et al., 2007; Szyzuy and Mortola, 2008). Compared with these longer exposure experiments, body mass decreased by $\sim$0.8–1.5 g for only 24 h while natural developmental increase results in $\sim$3.5 g from d14 (Tazawa et al., 1971a). This change in body mass corresponds to $\sim$20–40% of 24 h natural growth on d15. Because the decrease is quite large, we predict that hypoxia not only delays growth, but also disturb embryo's water balance which results in dehydration of embryo, although the mechanism requires further study.

4.2. Dynamics of acid–base disturbances during 5% CO_2 exposure with altered [O_2]

The time course response of respiratory acidosis differs with altered [O_2] under hypercapnic conditions. Embryos exposed to hypercapnic hypoxia demonstrate a different pattern of metabolic compensation than those exposed to either hypercapnic normoxia or hypercapnic hyperoxia. Previously, it was found that respiratory acidosis induced by exposure to hypercapnic hypoxia (5% CO_2, 15% O_2) for 24 h was partially ($\sim$38%) compensated by metabolic alkalosis in d13 embryos. In d15 embryos, metabolic compensation was not obvious, but there was a slightly larger [HCO_3^-] than that predicted from buffer value of -16 mmol L^{-1} pH^{-1} (Burggren et al., 2012). Together with this fact, we predicted that even d15 embryos would have a chance to compensate for respiratory acidosis under hypercapnic hypoxia. In fact, metabolic compensation of $\sim$45% occurred during a 6 h exposure. However, unlike the embryos exposed to hypercapnic normoxia/hyperoxia, compensation under hypercapnic hypoxia is short-lived and deteriorates after 24 h. Importantly, this indicates that the preservation of metabolic compensation requires environmental [O_2] at normoxic levels or above.

Exposure of embryos to hypercapnic normoxia (5% CO_2, 20% O_2) produces a more rapid metabolic compensation for respiratory acidosis, reaching $\sim$20% by 2 h and $\sim$50% at 6 h, with slow progress thereafter (Fig. 1B). Dawes and Simkiss (1971) exposed d9 chicken embryos to a mixture of 9% CO_2 in air (assumed to be equivalent to 20% O_2), and measured acid–base variables during the period of d12–d17 of incubation. If we plot these variables on the Davenport diagram, and assume a buffer value of -16 mmol L^{-1} pH^{-1}, then a metabolic compensation of $\sim$40% occurs for respiratory acidosis when the embryos were exposed for 3–7 day period. This compensation level is similar to the present 24 h hypercapnic normoxia exposure. Therefore, we postulate that the metabolic compensation measured in this study would have remained incomplete, and respiratory acidosis would not have been fully compensated, even if exposure was continued for a few more days beyond d15.

The present result does not support our initial hypothesis that compensation would increase with increasing [O_2]. Moreover, the magnitude of metabolic compensation was not influenced by [O_2] above 20%. In hyperoxic (both 40% and 90%) environments, as in the normoxic environment, respiratory acidosis produced by hypercapnic exposure is compensated by $\sim$20% at 2 h and $\sim$40–50% at 6 h with a further slight increase in compensation 24 h later (Fig. 1C and D). This suggests that the embryos cannot increase metabolic compensation under a hyperoxic hypercapnic environment compared to normoxic hypercapnia, at least during short-term (<24 h) exposures.

Blood [HCO_3^-] increases with development during the late incubation period in chicken embryos, although the reported accompanying change in blood pH is variable. Some studies indicate little change or no particular pattern of change (e.g., 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), while others report consistent decrease (e.g., Tazawa et al., 1971a,b; Tazawa, 1973, 1980; Andrewartha et al., 2011; Burggren et al., 2012). In either case, increased blood [HCO_3^-] is accompanied by increased P_{CO_2}, but [HCO_3^-] exceeds the value estimated to occur from hydration of accumulated respiratory CO_2 and blood buffer capacity. Therefore, several sources of increasing non-respiratory [HCO_3^-] during embryonic development have been proposed. These include renal activity, absorption of eggshell $CaCO_3$ (Dawes and Simkiss, 1969, 1971; Erasmus et al., 1970/71; Dawes, 1975), absorption of allantoic fluid HCO_3^- through the chorioallantoic vascular system (Boutilier et al., 1977) and intracellular exchange of H$^+$ and K$^+$ (Everaert et al., 2008). It is likely that a combination of these mechanisms is responsible for the daily increase in [HCO_3^-]. Meanwhile, the time course of changes in [HCO_3^-] in the present experiment is fast (<2 h). Similarly rapid (<4 h) changes in [HCO_3^-] (respiratory or metabolic disturbances) have previously been reported during environmental gas perturbation using, for example, CO_2, He or SF_6, in embryos with altered eggshell gas conductance or by infusing hypertonic electrolyte solutions (Tazawa, 1981a, 1982; Tazawa et al., 1981). Furthermore, blood [HCO_3^-] changes more rapidly (<10–30 min) in embryos exposed to severe hypoxia (10% O_2) (Tazawa et al., 2012) or submergence (H. Tazawa, unpublished data). The decrease in [HCO_3^-] appears to occur too rapidly to be attributed to renal function, and instead may be accredited to the chorioallantoic membrane (CAM), which actively transports ions between the capillary blood and allantoic fluid (Stewart and Terepka, 1969; Boutilier et al., 1977; Hoyt, 1979; Davis et al., 1988; Gabrielli and Accili, 2010). The HCO_3^- (or H$^+$) infused into the allantoic fluid likely diffuses into blood across the CAM, causing metabolic alkalosis (or metabolic acidosis) (H. Tazawa, unpublished data). A study on the possible contribution of the CAM to acid–base regulation after disturbance is warranted.

4.3. Dynamics of hematology regulation

In concert with the different acid–base pattern in hypercapnic hypoxia, the time course of changes in hematological variables show a strikingly different pattern when compared with exposure to hypercapnic normoxia/hyperoxia. After only 2 h exposure, the increase in Hct is in bold contrast compared to the slight decrease in Hct under hypercapnic normoxia/hyperoxia (Fig. 2A). Likewise, [RBC] and MCV under hypercapnic hypoxia show an opposite pattern to that seen in the other gas treatments (Fig. 2B and C). The strong, early (2 h) increase in MCV indicates a short-term mechanism by which Hct can be increased to counteract the low oxygen conditions. Although plasma volume (PV) is another factor in Hct regulation, contribution of PV may be little because ΔHct is matched by ΔMCV and Δ[RBC]. The contribution of these two variables to the increase in Hct under hypercapnic hypoxia changes with exposure time. These short-term changes in Hct are clearly not due to erythropoiesis, because [RBC] remains unchanged during 2 h and 6 h exposure to hypercapnic hypoxia. After 24 h exposure to hypercapnic hypoxia, MCV decreases from a peak value obtained during early exposure and an increase in [RBC] maintains the high Hct value. Likewise, an increase in Hct in d11 to d19 embryos exposed to 10% O_2 for 2 h is attributed solely to an increase in MCV, except at d19 when an increase in [RBC] also contributes (Tazawa et al., 2012). This indicates that a developmental effect may influence the driving forces behind Hct time course changes.

The pattern of changes in Hct during hypercapnic hypoxia is in accordance with a previous study showing increased Hct on d15, accompanied by increases in both [RBC] and MCV, in response to 24 h exposure to hypoxia (15% O_2). This occurs irrespective of the presence or absence of hypercapnia (5% CO_2) (Burggren et al., 2012). The contribution of [RBC] and MCV to the 18% increase in Hct at 24 h is about 50–50 in this study, compared to an 11% increase in [RBC] and a 6% increase in MCV contribution to the 17% Hct increase in the study of Burggren et al. (2012). Likewise, the contributions of [RBC] and MCV to the decrease in Hct after 24 h exposure to hypercapnic normoxia varies slightly between the present study and that of Burggren et al. (2012). The 13% Hct decrease in the present study is attributed to a 9% and 4% decrease in [RBC] and MCV, respectively. Burggren et al. (2012) reported that the same percent decrease in Hct was attributed to a greater decrease in [RBC] (11%). Although there are a few inconsistent results between the previous and present studies, in terms of changes and relative contributions of variables at 24 h, responses across studies are overall quite similar. The time course of the response to hypercapnic normoxia exposure showed unchanged Hct and [RBC] during early exposure, while MCV decreased. This is similar to a previous study that indicated no significant changes in Hct of d13–d17 embryos after short term (<6 h) exposure to hypercapnic normoxia (Andrewartha et al., 2011). The significant increase in MCV under hypercapnic hypoxia potentially increases blood viscosity, which would be disadvantageous for oxygen transport. However, the increase in [RBC] and slight decrease in MCV at 24 h exposure maintains elevated Hct while perhaps mitigating any increase in blood viscosity (Zhang et al., 2007).

Independent of the actual [O_2], in hypercapnic hyperoxic environments we found that changes in Hct, [RBC] and MCV followed a similar time course to that displayed by embryos in hypercapnic normoxia (Fig. 2). The decrease in Hct, due to decreases in both [RBC] and MCV, in response to 24 h exposure to hypercapnic hyperoxia can be attributed to [O_2] greater than 20%, but not to the 5% CO_2. A previous study in d15 embryos exposed to hyperoxic (40% O_2) gas mixture without CO_2 for 24 h also decreased Hct via decreases in both [RBC] and MCV (Burggren et al., 2012). The decrease in Hct and MCV is matched by an increase in Osm after 24 h exposure to hypercapnic hyperoxia. However, no change

in Osm was detected under hypercapnic hypoxia or normoxia. A lack of correlation between changes in Osm and Hct has been found previously in embryos exposed to hypercapnia and different temperatures. This suggests that Osm is not a strong force in Hct regulation (Andrewartha et al., 2011; Burggren et al., 2012).

In a similar fashion to Hct, the time courses of changes in [Hb], MCH and [MCHb] are notably different under hypercapnic hypoxia than under hypercapnic normoxia/hyperoxia (Fig. 3). The significant decreases in [Hb] 24 h after exposure to hypercapnic normoxia/hyperoxia are in parallel with those in [RBC]. However, [Hb] in hypercapnic hypoxic embryos does not increase from the control despite significant increase in [RBC] (Fig. 2B). From the view point of oxygen transport, it is advantageous for [RBC], and thus [Hb], to increase or decrease in response to hypoxia or hyperoxia (in terms of changes in blood viscosity). These changes in [RBC] take place after 24 h exposure, reflecting the changes in Hct. However, Hct itself begins to respond to altered O_2 after only 2 h, due to changes in MCV without changes in [RBC] in all gas mixtures. Surprisingly, in hypercapnic hypoxia, MCH decreases during 24 h exposure, resulting in a marked decrease in [MCHb] together with marked increase in MCV. The decrease in MCH is disadvantageous to O_2 transport in a hypoxic environment, but actually occurs despite significant increase in [RBC] and Hct. Consequently, in hypercapnic hypoxia, the increase in [RBC] that occurs 24 h later reflects a significant increase in Hct, but fails to increase [Hb] because of an unfavorable decrease in MCH. The decrease in MCH occurs irrespective of the presence or absence of CO_2 (Burggren et al., 2012). Further investigation into this disadvantageous decrease in MCH during hypoxic challenge is warranted.

5. Conclusions

We are presenting a series of investigations into regulation of acid–base balance and hematological respiratory variables in developing chicken embryos during altered environmental gas challenges (e.g., Andrewartha et al., 2011; Burggren et al., 2012; Tazawa et al., 2012). They are based on the idea that isolated examination of acid–base balance, hematology or blood O_2 alone allows only fragmented understanding of their complex interactions and dynamics, and that examinations in concert are necessitated. The present study investigated the effect of [O_2] (15, 20, 40 and 90% O_2) on the time-specific regulation of acid–base balance and Hct in response to exposure to hypercapnia (5% CO_2). We report that time-specific regulation of both the acid–base disturbance and Hct depends upon [O_2] below or above 20%, with the magnitude of both changes being unrelated to [O_2]. It is likely that O_2 similarly regulates acid–base disturbances and Hct responses.

Examination of hematological respiratory variables highlights that the main contributors to Hct regulation varies with time course of exposure, a finding that would not have been uncovered without measurement of multiple variables in this study. In particular, the contributions of [RBC] and MCV to Hct regulation change substantially over the time course of 24 h. In all gas exposure treatments, irrespective of how Hct is regulated, the early change in Hct occurs via changes in MCV, but by 24 h changes in [RBC] contribute more to Hct regulation. Such results suggest that examining all variables concurrently is not only advantageous, but actually necessary to fully understand the mechanism behind hematological regulation in response to gas exchange perturbation during embryonic development. Examination of blood flow and viscosity, which are balanced by [RBC] and [Hb] changes, during hypoxic challenges will contribute to further understanding of hematological regulation in terms of O_2 transport. In addition, investigation into the effect of varying [CO_2] at constant [O_2] (e.g., 20% O_2) or the effect of combined changes in [CO_2] and [O_2] below 20% will deepen our
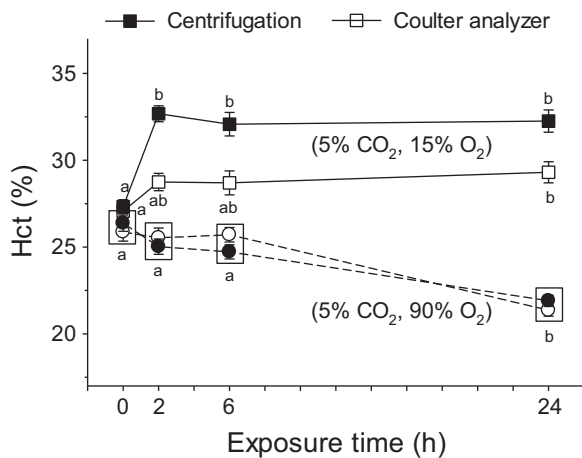
Fig. A1. The time course of Hct responses in d15 embryos exposed to hypercapnic hypoxia (5% CO_2, 15% O_2, solid lines) or hypercapnic hyperoxia (5% CO_2, 90% O_2, broken lines), determined simultaneously by the Coulter analyzer (open symbols) and the centrifugation (closed symbols). Means at each exposure time that are not significantly different are grouped within boxes. The different letters indicate significant difference between the exposure time means within each method. The time course of Hct response during hypercapnic hypoxia (solid lines) was significantly different between the Coulter analyzer and centrifuge ($P < 0.001$), but did not differ during hyperoxia exposure (broken lines) ($P = 0.731$).
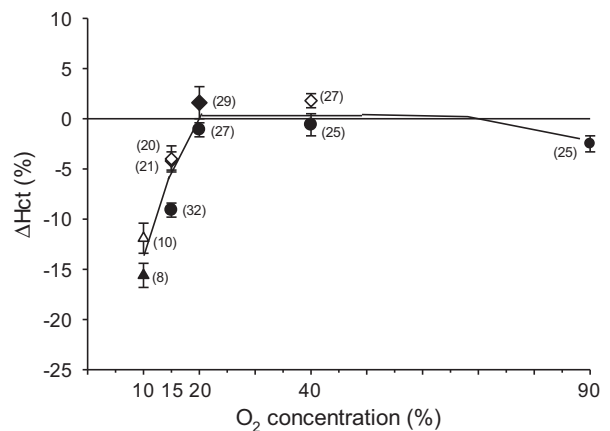
Fig. A2. The relative (proportional) difference of Hct determined by the Coulter analyzer from the value determined by the centrifuge, $\Delta Hct = 100 \times$ (coulter Hct − centrifuge Hct)/centrifuge Hct, in d15 embryos exposed to altered environmental O_2 concentrations with or without CO_2. The present results are shown by closed circles. Previous results include Hct in embryos exposed to 15% O_2 with (closed diamond) or without (open diamond) 5% CO_2, 20% O_2 with 5% CO_2 (closed diamond), 40% O_2 without CO_2 (open diamond) (Burggren et al., 2012) and embryos exposed to 10% O_2 with (closed triangle) or without (open triangle) 5% CO_2 (Tazawa et al., 2012). The number in the parentheses indicates sample size.

knowledge on the ontogeny of regulation of acid–base balance in this diffusion-only gas exchange system.

Appendix A. Simultaneous determination of Hct by Coulter analyzer and centrifugation

Hct was determined by centrifugation and the results of duplicate measurements were averaged to yield actual Hct of individual embryos. In addition, Hct of the same blood sample was measured by the Coulter analyzer, which determined [RBC] and [Hb]. The advantage of the Coulter analyzer is its higher throughput as a technique. Both Hct values were compared to evaluate our previous finding that the Coulter analyzer determined Hct differently from centrifugation. Fig. A1 presents simultaneously determined time course Hct changes in embryos exposed to hypoxia (5% CO_2, 15% O_2) or hyperoxia (5% CO_2, 90% O_2) for a period of 2 h, 6 h or 24 h. The time course of changes in Hct determined by centrifugation for embryos exposed to hypoxia and hyperoxia are the same as those shown in Fig. 2A. Briefly, exposure to hypoxia resulted in a significant increase in Hct after 2 h and maintained at a similar high level through to 24 h. However, Hct was underestimated by the Coulter analyzer. Changes in Hct from the control were not significant during early period of exposure, with a significant increase only 24 h later ($P = 0.017$). Even Hct determined 24 h later was underestimated by the Coulter analyzer (29.3%) compared with Hct by the centrifuge (32.3%), with the difference being $-9.1 \pm 0.7\%$ (ΔHct) (see Fig. A2). Meanwhile, Hct of embryos exposed to hyperoxia was not different between the Coulter analyzer and the centrifugation ($P = 0.731$) (Fig. A1). Hct determined by the Coulter analyzer was $21.4 \pm 0.3\%$ after 24 h compared to $21.9 \pm 0.3\%$ by the centrifuge, and the relative (proportional) difference between both the determinations (ΔHct) was $-2.5 \pm 0.8\%$ (see Fig. A2). The time-specific changes in Hct in embryos exposed to milder hyperoxia (5% CO_2, 40% O_2) and normoxia (5% CO_2, 20% O_2) were not different between

the Coulter analyzer and centrifugation ($P = 0.731$) (Fig. A2). The relative difference of Hct (ΔHct) between both the determinations was $-0.6 \pm 1.1\%$ and $-1.1 \pm 0.7\%$ for embryos exposed to hyperoxia (5% CO_2, 40% O_2) and normoxia (5% CO_2, 20% O_2) for 24 h, respectively (Fig. A2). Previous studies, in which Hct was determined in embryos exposed to normoxia or hyperoxia in the presence or absence of CO_2, also indicated no significant difference between the Coulter analyzer and centrifuge methods (Burggren et al., 2012; Tazawa et al., 2012; Fig. A2). However, Hct determined for d15 embryos partially submerged in water for 60 min was underestimated by -13% (ΔHct) by the Coulter (H. Tazawa, unpublished data). Similarly, underestimation of Hct by the Coulter (-12%) also occurred after complete submergence for 30 min. These results are in accordance with the underestimation of Hct under hypoxia in this study. Consequently, Hct of embryos exposed to normoxia or hyperoxia with or without CO_2 can be determined equally by both the Coulter analyzer and the centrifuge. However, Hct of embryos exposed to hypoxia with or without CO_2 or embryos suffered intrinsic hypoxemia are underestimated by the Coulter analyzer.

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