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Acute regulation of hematocrit and acid–base balance in chicken embryos in response to severe intrinsic hypercapnic hypoxia

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ABSTRACT

The regulation of blood acid–base balance and hematology in day 15 chicken embryos in response to partial water submersion (with egg's air cell in air) and complete submersion producing severe intrinsic hypercapnic hypoxia and recovery in air was studied. The acid–base disturbance during submersion was characterized by initial rapid respiratory changes and then superseded by metabolic processes, resulting in a large progressive hysteresis. Throughout submersion and recovery, blood lactate concentration changed swiftly along with the changes in bicarbonate concentration ($[HCO_3^-]$), indicating that anaerobic glycolysis determined overall acid–base disturbances. Both partial and complete submersion produced large, rapid increases in hematocrit through proportional increases in mean corpuscular volume and red blood cell concentration. Death ensued once the internal pool of O_2 was exhausted and/or the acid–base disturbance became too severe for survival (i.e., $[HCO_3^-]_a < \sim 10\ mmol\ L^{-1}$). However, embryos recovered from acid–base and hematological disturbances within 120 min recovery in air after short bouts of complete (20 min) or partial (60 min) submersion, suggesting that shorter severe intrinsic hypercapnic hypoxia does not compromise viability of embryos.

1. Introduction

In chicken embryos both pure hypoxia and hypercapnic hypoxia induce acid–base disruption and change hematocrit (Hct) and red blood cell concentration ([RBC]) (e.g., Ackerman, 1970; Tazawa et al., 1971, 1981, 2012; Tazawa, 1982; Baumann et al., 1983; Nakazawa and Tazawa, 1988; Dzialowski et al., 2002; Andrewartha et al., 2011; Burggren et al., 2012; Mueller et al., 2013). Hct increases in response to decreasing O_2 concentration ($[O_2]$). Whether or not CO_2 was present, day 15 (d15) embryos exposed to moderate (e.g., 15% O_2) extrinsic hypoxia for 2–6 h increased Hct due to an increase in red blood cell volume (mean corpuscular volume, MCV) only. However, by 24 h Hct was elevated due to an increase in both MCV and [RBC] (Burggren et al., 2012; Mueller et al., 2013). Exposure to more severe extrinsic hypoxia (10% O_2) or hypercapnic hypoxia (5% CO_2, 10% O_2) increased Hct though MCV alone, with [RBC] remaining unchanged in d15 embryos (Tazawa et al., 2012). Creating moderate intrinsic hypoxia by covering partially the eggshell over the air cell for 24 h increased Hct through increasing MCV in d16 embryos. However, the increase in Hct in d19 embryos was due to an increase in both MCV and [RBC] (Tazawa et al., 1988).

In embryos exposed to these altered gaseous environments, acid–base balance is influenced by both O_2 and CO_2 in a dose- (concentration-) dependent fashion. Increased lactate (La^-) production accompanied by uncompensated metabolic acidosis resulted from extrinsic severe hypoxia (10% O_2), whereas severely hypercapnic hypoxic embryos additionally underwent an uncompensated respiratory acidosis (Tazawa et al., 2012). Time plays an important role in the progression of these acid–base disturbances. For example, d15 embryos experienced respiratory acidosis after 2 h of moderate hypercapnic hypoxia (5% CO_2, 15% O_2) exposure. By 6 h the acidosis was partially compensated by metabolic alkalosis. However, at 24 h the partial metabolic compensation had discontinued and $[La^-]$ decreased (Burggren et al., 2012; Mueller et al., 2013). Similarly, respiratory acidosis resulted in d16 embryos 10 min after intrinsic hypoxia had been induced by covering ~¼ of their eggshells with a gas impermeable material (Tazawa, 1981). By 1–6 h the respiratory acidosis was partially compensated by a metabolic alkalosis that continued for more than 24 h, in contrast to embryos exposed to extrinsic hypercapnic hypoxia (Burggren et al., 2012; Mueller et al., 2013).

Collectively, these experiments show that the different blood acid–base and hematological responses observed depend upon the severity and length of the hypercapnic hypoxia and whether it is produced intrinsically or extrinsically. The physiological effects of reducing gas exchange across the entire eggshell have not yet been examined and there are no data on embryos facing prolonged, severe intrinsic hypercapnic hypoxia. Water submersion

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greatly reduces gas exchange across the eggshell as a result of much lower O_2 capacitance and lower gas diffusion coefficient of water compared with air. This creates a severe, intrinsically hypoxic and hypercapnic environment for the embryo, with the CO_2 accumulation inside the egg disrupting acid–base balance and blood hematology. Prolonged submersion of course results in death of the embryo, but during short-term submersion, it is likely that the air cell prolongs survival by enabling a limited amount of gas exchange with the air above the water line.

Although the eggs of domestic chickens are unlikely to be submersed, many avian embryos are potentially vulnerable to brief or long-term flooding due to being incubated in nests on the ground, along the shore-line or floating in water bodies, (e.g., Rounds et al., 2004; Wilson and Peach, 2006; Sotherland et al., 1984; Shriver et al., 2007). Further, water submersion provides a simple, effective method for disrupting gas exchange through the eggshell pores and producing an intrinsically hypoxic, hypercapnic environment. Thus, this study aims to characterize the physiological effects of severe intrinsic hypercapnic hypoxia in chicken embryos produced by water submersion, which is a potential stressor for many avian embryos. Specifically, we used partial or complete water submersion to characterize the dose-dependent nature and dynamics of partially or fully blocking gas exchange on the survival length and progression of the acid–base and blood hematology disturbance. We hypothesize that blood hematology and acid–base disturbances in embryos experiencing severe intrinsic hypercapnic hypoxia produced by water submersion, and the mechanisms used to partially mitigate these disturbances, will differ from the physiological disturbances resulting from exposure to severe extrinsic hypercapnic hypoxia produced by modification of the surrounding gaseous environment. We further hypothesize that partial submersion will extend survival time (cf. complete submersion) by partially mitigating these disturbances.

2. Materials and methods

2.1. Egg incubation

Eggs of the White Leghorn (mainly Lohmann) strain of chickens (*Gallus gallus*) were obtained weekly from Texas A&M University (College Station, Texas, USA). Eggs were weighed (±0.01 g) and then incubated at a temperature of 37.5 ± 0.1 °C and relative humidity of ~55% in a forced draught incubator (model 1502, G.Q.F. Manuf. Co., GA, USA). The eggs were placed vertically on an automatic turning tray rotating the eggs every 3 h. On d14 of incubation, the eggs were candled to locate the allantoic vein and a site over the vein was marked on the eggshell. The eggs were moved into a desk-top incubator (Hova-Bator 1590, G.Q.F Manuf.) at 37.5 °C on the following day.

2.2. Protocols

The effects of intrinsic hypercapnic hypoxia on acid–base and hematology regulation were investigated by blocking gas exchange across the eggshell via submerging eggs in water or by wrapping eggs in Parafilm® (Laboratory film M, American National Can). Embryos were subjected to either a short submersion (or wrapping) and recovery protocol that allowed 100% embryonic survival or a survival submersion protocol to determine how long embryos could tolerate intrinsic hypercapnic hypoxia. Both protocols were conducted at 37.5 °C to facilitate comparisons with previously published work with extrinsic hypoxia with or without CO_2 (e.g., Burggren et al., 2012; Tazawa et al., 2012; Mueller et al., 2013).

2.2.1. Parafilm® wrapping

Eggs were wrapped in a sheet of Parafilm® and returned to the desk-top incubator at 37.5 °C (except eggs sampled at time 0). Blood was collected from embryos prior (control), immediately after (time 0: sampled within 2 min), and 10, 30 and 60 min after Parafilm® wrapping (Table 1). Blood samples were then analyzed for blood gas variables (Pco_2, pH, $[HCO_3^-]$), Hct and osmolality (Osm, mmol kg^{-1}) (see Section 2.3). Embryos in the recovery protocol were wrapped in Parafilm® for 60 min and the same variables were then measured during recovery (eggs unwrapped) at time 0, 10, 30, 60 and 120 min (Table 1).

Blood gas variables, Hct and Osm were additionally measured from embryos in the survival protocol which were sampled every 10 min during 60–140 min Parafilm®-wrapping (Table 1).

2.2.2. Partial water submersion (air cell at the water's surface)

Eggs placed into the water bath (Model 3545, Lab-Line Instrument, USA) at 37.5 °C naturally floated with the air cell up and part of the eggshell protruding into the air above the water bath. At the time of blood collection, the egg was removed from the water and, while still moist, the entire egg minus the area underlying the air cell was immediately wrapped with Parafilm® to maintain the internal environmental conditions during the brief (<2 min) blood sampling process. During the short submersion protocol the following variables were measured: blood gas variables (Pco_2, pH, $[HCO_3^-]$), hematological respiratory variables with mean corpuscular indices (Hct, [RBC]), hemoglobin concentration ([Hb], g%), MCV, mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration ([MCHb], g%), Osm and lactate concentration ($[La^-]$, mmol L^{-1}). All of these variables were measured prior to (control) and 10, 20, 30 and 60 min following partial water submersion (Table 1). Embryos in the recovery protocol were partially submersed for 60 min, placed back in air in the desk-top incubator and the same variables were then measured during recovery in air at 0, 10, 30, 60 and 120 min (Table 1).

Blood gas variables, hematological respiratory variables, Osm and $[La^-]$ were additionally measured from embryos in the survival protocol which were sampled every 10 min during 60–140 min partial submersion (Table 1).

2.2.3. Complete water submersion

Eggs were completely submersed to block gas exchange across the entire eggshell. A plastic sheet and weights were used to hold the eggs completely underwater. The partial submersion protocol (Section 2.2.2) was then followed, but the entire surface of the egg (including the air cell) was wrapped with Parafilm® immediately after removal from the water bath, to maintain experimental conditions during blood sampling.

During the short submersion protocol the blood gas variables, hematological respiratory variables, Osm and $[La^-]$ were measured prior to (control) and after 10, 20 and 30 min complete water submersion (Table 1). Embryos in the recovery protocol were completely submersed for 20 min, placed back in air in the desk-top incubator and the same variables were then measured during recovery at 0, 10, 30, 60 and 120 min (Table 1).

Blood gas variables, hematological respiratory variables, Osm and $[La^-]$ were additionally measured from embryos in the survival protocol which were sampled every 10 min during 20–70 min complete submersion (Table 1) based on preliminary embryonic survival data.

2.3. Blood collection and analysis

Blood was collected following previously published procedures (Burggren et al., 2012; Tazawa et al., 2012). Briefly, a 6–8 mm diameter region of the eggshell was removed and an underlying allantoic

vein gently lifted by forceps through the hole in the eggshell. Immediate filling of the vein with blood indicated that the heart was still beating and the embryo was alive. If the retracted vein failed to fill, blood was not sampled from the embryos and it was recorded as deceased.

Approximately 0.4 mL of blood was sampled from the vein using a 25 gauge needle mounted on a 1 mL plastic syringe flushed in advance with heparinized saline. Sampled blood was gently transferred into a 2 mL plastic vial, and the pH, Pco_2 and [HCO_3^-] were immediately (within 2 min of eggs being removed from incubator/water) determined on ~0.12 mL of blood with a blood gas analyzer (ABL5, Radiometer Medical A/S, Copenhagen, Denmark) at 37 °C. Blood gas variables (pH, [HCO_3^-], Pco_2) were referred to as "arterialized values"; e.g., pH_a, as previously defined (Piiper et al., 1980; Tazawa et al., 2011). The vial was then inverted several times to ensure thorough blood mixing. [RBC] and [Hb] were determined on ~0.01 mL of blood using a blood cell counter (Coulter analyzer, $A^c{\cdot}10$ T, Beckman, USA). Osm was determined on ~0.01 mL using a vapor pressure osmometer (Vapro 5520, Wescor, USA). [La^-] was determined on one drop of blood using a lactate meter (Nova Lactate Plus Meter, Nova Biomedical, MA, USA). Duplicate preparations of ~0.06 mL of blood were transferred into hematocrit tubes, sealed and centrifuged for 4 min at 10,000 rpm and the mean Hct determined (±0.1%, Readacrit Centrifuge, Becton Dickinson, USA). The mean corpuscular indices (MCV, MCH and [MCHb]) were calculated by the equations outlined previously (Tazawa et al., 2011).

2.4. Embryo mass

After blood collection, embryos were euthanized by putting eggs in a refrigerator. The yolk and extra-embryonic membranes were removed, and the embryo's wet body mass determined (±0.01 g) with an electronic balance.

2.5. Statistical analysis

The data were examined for normality and equal variance. Differences between multiple-group means were tested by one-way ANOVA with post hoc multiple comparison analysis to determine differences between individual group means. Two group means consisting of several subsets individually were examined by two-way ANOVA for the significance of difference between two group means and between individual subsets. Significance was assumed at $P < 0.05$. All data were presented as means ± 1 S.E.M.

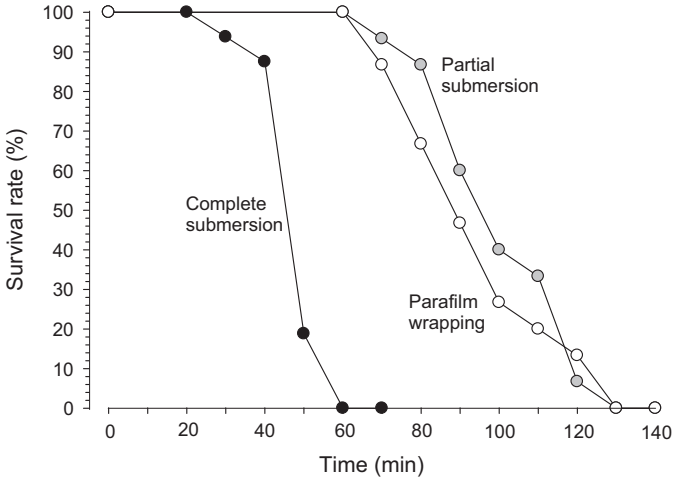

Fig. 1. Survival rate (%) of d15 chicken embryos during 140 min of Parafilm®-wrapping (open symbols), 140 min of partial water submersion (shaded symbols) or 70 min of complete water submersion (closed symbols).

3. Results

3.1. Embryo survival during Parafilm®-wrapping and prolonged water submersion

Mortality of completely submersed embryos began to occur at 30 min of submersion, with no survival past 60 min (Fig. 1). In comparison, all embryos survived 60 min partial water submersion and Parafilm®-wrapping. Mortality of partially submersed and Parafilm®-wrapped embryos was delayed until 70 min, increasing until mortality reached 100% at 130 min. Fifty percent embryonic survival occurred at ~45, 95 and 90 min in completely and partially submersed and Parafilm®-wrapped embryos, respectively.

3.2. Egg mass and embryo mass

3.2.1. Parafilm® wrapped embryos

Ninety-two embryos were divided into one control, four Parafilm® wrapped groups and five unwrapped (recovery) groups (i.e., 10 groups in total – Table 1). There was no difference in mean egg (58.20 ± 0.36 g) or body mass (13.06 ± 0.13 g) of embryos between the 10 groups ($P = 0.092$ and $P = 0.055$, respectively).

3.2.2. Partially water-submersed embryos

Embryos in the partially water-submersed protocol did not differ in mean egg mass (57.31 ± 0.29 g) or body mass (13.35 ± 0.12 g) amongst the 10 groups (Table 1) ($P = 0.951$ and $P = 0.570$ for egg

Table 1
Number of eggs examined in short water submersion (or Parafilm® wrapping) and recovery or longer survival protocols.

		Time (min)														
		Control	0	10	20	30	40	50	60	70	80	90	100	110	120	130	140
Parafilm	Wrapped	19	9	8		7			9								
	Recovery*		7	8		7			9						9		
	Survival	22							15/15	13/15	10/15	7/15	4/15	3/15	2/15	0/15	0/15
Partial	Submersion	20		11	10	10			22								
	Recovery*			10		10			10			10			20		
	Survival	21							15/15	14/15	13/15	9/15	6/15	5/15	1/15	0/15	0/15
Complete	Submersion	12		12	12	17											
	Recovery#			11		10			10			10			10		
	Survival	18			16/16	15/16	14/16	3/16	0/16	0/16							

Survival *N* is presented as (number of embryos that survived)/(number of embryos examined). All embryos survived the wrapped/submersion and recovery protocols. Recovery*: embryos were recovered following 60 min Parafilm® wrapping or partial submersion. Recovery#: embryos were recovered following 20 min complete submersion.

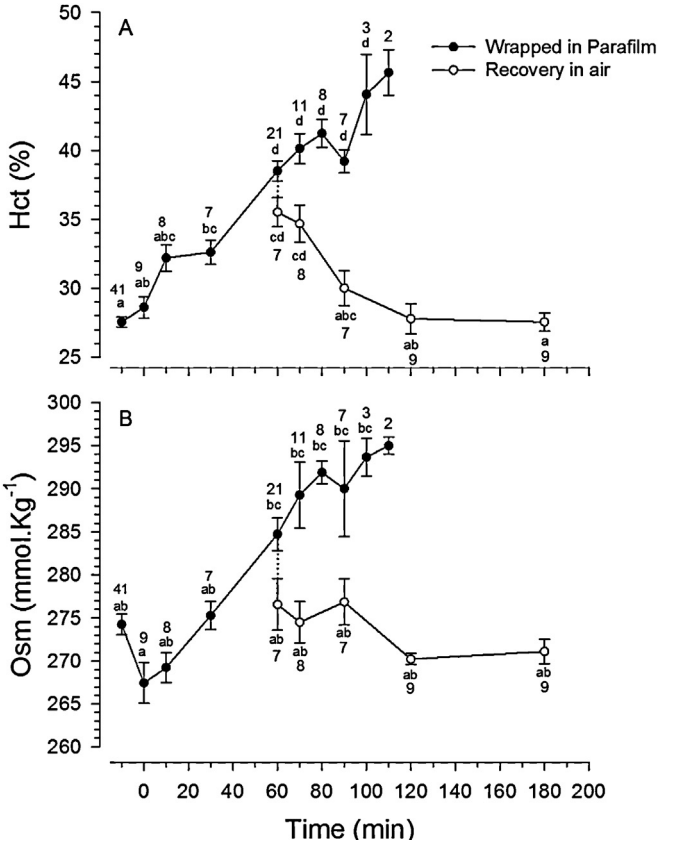

Fig. 2. Responses of (A) hematocrit (Hct) and (B) osmolality (Osm) in d15 embryos to Parafilm®-wrapping (closed symbols) and recovery following 60 min wrapping (open symbols). Values are means ± 1 S.E.M. and numerical figures indicate *N* values. Different lowercase letters indicate statistically significant means for a given variable.

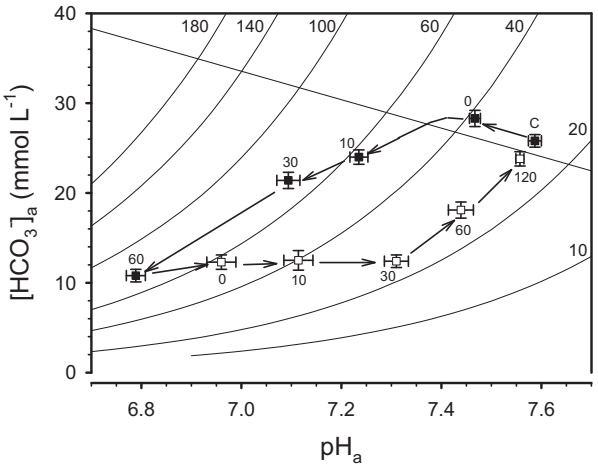

Fig. 3. Davenport (pH-[HCO_3^-]) diagram illustrating the time-course of the acid–base disturbance of d15 embryos wrapped in Parafilm® for 60 min (closed symbols) with 120 min recovery in air (open symbols). C is control and numerical figures indicate time (in min) following experimental treatment. Numbered curves show the Pco_2 isopleths (mmHg). The solid regression is a model buffer line with the determined mean slope of −16 mmol L^{-1} pH^{-1}, indicating the status of the acid–base disturbances (Burggren et al., 2012). Values are means ± 1 S.E.M. and *N* values are as in Fig. 2.

mass and body mass, respectively). All embryos ($N = 123$, including 20 control) survived 60 min partial submersion and the subsequent 120 min recovery in air.

3.2.3. *Completely water-submersed embryos*

Embryos used in the completely water-submersed protocol did not differ in mean egg mass (56.32 ± 0.36 g) or body mass (12.82 ± 0.12 g) amongst the 9 groups (Table 1) ($P = 0.254$ and $P = 0.570$ for egg mass and body mass, respectively). All embryos ($N = 87$, including 12 control embryos) survived 20 min submersion and subsequent recovery.

3.3. *Physiological effects of intrinsic hypercapnia and hypoxia*

3.3.1. *Parafilm®-wrapped embryos*

Hct in the control population was 27.6 ± 0.4%. Hct began to increase after embryos were wrapped in Parafilm® – for 10 min and increased significantly by 30 min to 32.6 ± 0.9% (Fig. 2A). A maximal Hct of 44.1 ± 2.9% was reached after embryos had been wrapped for 100 min. When embryos were unwrapped and allowed to recover in air, Hct began to decrease, reaching values similar to control within 30 min (30.0 ± 1.3%) and remaining at control levels throughout the remainder of the recovery period.

Blood osmolality (control = 274 ± 1 mmol kg^{-1}) also increased when the eggs were wrapped in Parafilm®, with a significant increase at 60 min (285 ± 2 mmol kg^{-1}) (Fig. 2B). During recovery, Osm immediately decreased to control levels (277 ± 2 mmol kg^{-1}).

$P_a co_2$ (control = 27 ± 1 mmHg) increased markedly to 40 ± 1 mmHg with a significant decrease in pH_a (control = 7.59 ± 0.01) to 7.47 ± 0.01 10 min after Parafilm®-wrapping (Fig. 3). $P_a co_2$ further increased during the first 30 min (74 ± 3 mmHg) with a subsequent small increase during the next 30 min (78 ± 3 mmHg at 60 min). Blood pH_a continuously decreased across the 60 min wrapped period, dropping by 0.8 pH units to 6.79 ± 0.02 at 60 min. Concurrently, $[HCO_3^-]_a$ decreased from a control value of 25.8 ± 0.7 mmol L^{-1} to less than half (10.8 ± 0.7 mmol L^{-1}) at 60 min. Once the eggs were unwrapped, pH_a continuously increased and returned to the control level after 120 min recovery, whereas $[HCO_3^-]_a$ remained unchanged during the first 30 min of recovery and increased along mean Pco_2 isopleth of 26 mmHg across the next 90 min period (Fig. 3).

3.3.2. *Partially water-submersed embryos*

Hct (control = 27.4 ± 0.4%) increased significantly within 10 min (30.9 ± 0.6%) and further increased during 20 min (35.0 ± 0.9%) to 100 min (38.9 ± 0.9%) partial water submersion (Fig. 5A). These increases corresponded to proportional increases in Hct from control (Hct_c), as defined previously by $\Delta Hct = 100 \times (Hct - Hct_c)/Hct_c$ (Andrewartha et al., 2011), of ~12, 27 and 42% at 10, 20 and 100 min, respectively. During recovery after 60 min submersion, Hct decreased (although less rapidly than the increase during partial submersion) returning to control values after 60 min (26.6 ± 0.9%) in air.

[RBC] (control = 2.05 ± 0.02 μL^{-1}) increased significantly from 20 min partial submersion (2.26 ± 0.06 × 10^6 μL^{-1}) and peaked at 60 min (2.30 ± 0.03 × 10^6 μL^{-1}) partial submersion corresponding proportional increases from control of ~10 and 12% at 20 and 60 min, respectively (Fig. 5C). [RBC] began to decrease within 10 min (2.02 ± 0.05 × 10^6 μL^{-1}) in air to control levels. MCV (control = 135 ± 1 μ^3) increased rapidly, within 10 min (145 ± 2 μ^3), reaching a maximum at 90 min partial submersion (173 ± 3 μ^3) (Fig. 5E). The proportional increase from control was ~8 and 28% at 10 and 90 min, respectively. MCV returned to values similar to control within 30 min of recovery in air (158 ± 2 μ^3).

Reflecting changes in [RBC], [Hb] (control = 8.9 ± 0.2 g%) increased rapidly to reach ~10 g% during partial submersion (9.8 ± 0.2 to 10.2 ± 0.3 g% at 20 and 60 min, respectively) (Fig. 6A). During recovery following 60 min partial submersion, [Hb] decreased rapidly to control values (9.0 ± 0.2 and 8.3 ± 0.2 g%

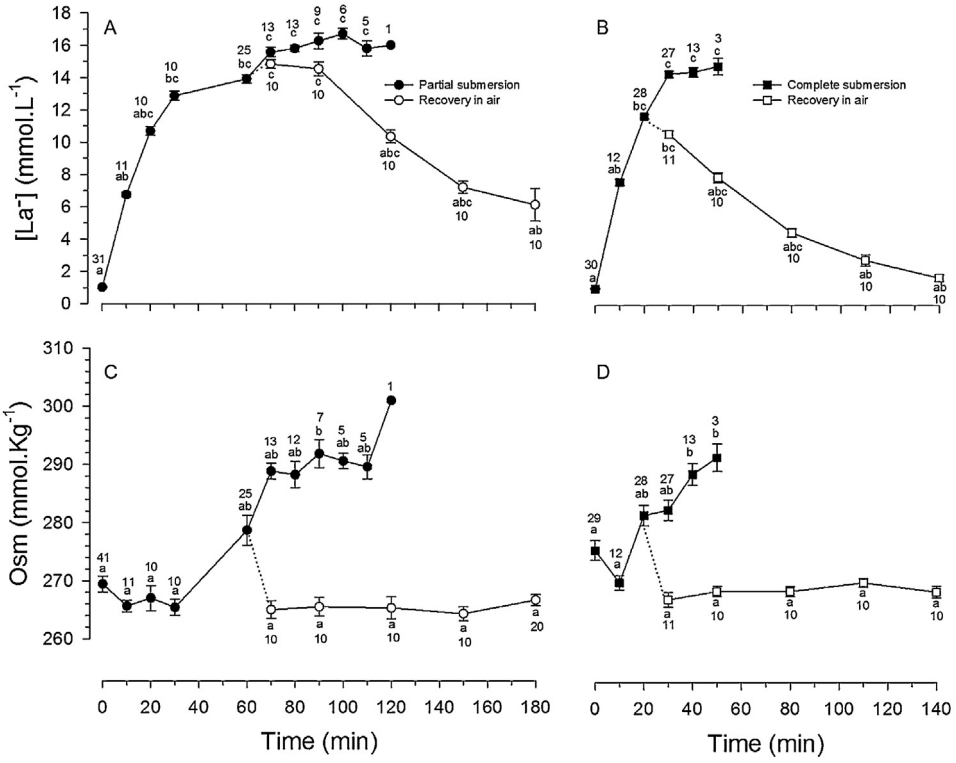

Fig. 4. Responses of (A and B) lactate concentration ([La$^-$]) and (C and D) osmolality (Osm) in d15 embryos to partial (closed round symbols) or complete (closed square symbols) submersion and recovery in air (open round symbols) (open square symbols). Control values prior to submersion were plotted at time 0. Partially and completely submersed embryos were returned to air after 60 and 20 min submersion, respectively. Dotted lines connect the data points where embryos were returned to air and the first data determined after recovery. Values are means ± 1 S.E.M. and *N* values are indicated by numerical figures. Different lowercase letters indicate statistically significant means for a given variable and protocol.

at 10 and 60 min recovery, respectively) in air. MCH remained unchanged, compared with control, throughout partial submersion and recovery in air with a mean value of 43.9 ± 0.2 pg (P = 0.079) (Fig. 6C). [MCHb] (control = 32.3 ± 0.5 g%) decreased during submersion becoming significant from 30 min (26.9 ± 0.5 g%) onwards (Fig. 6E). [MCHb] remained unchanged during the first 30 min recovery in air (~27.5 g%), and increased to control levels at 60 min (31.3 ± 0.4 g%).

Lactate concentration ([La$^-$]) (control = 1.0 ± 0.1 mmol L^{-1}) increased significantly by 30 min partial submersion (12.9 ± 0.3 mmol L^{-1}), reaching a maximal value at 100 min (16.7 ± 0.3 mmol L^{-1}) (Fig. 4A). During recovery following 60 min partial submersion, [La$^-$] slowly decreased and returned to control levels after 60 min in air (10.4 ± 0.4 mmol L^{-1}).

Osm (control = 269 ± 1 mmol kg^{-1}) increased during submersion, reaching a maximal value at 90 min (292 ± 1 mmol kg^{-1}) (Fig. 4C). During recovery in air, Osm decreased rapidly, reaching values similar to control within 10 min (265 ± 2 mmol kg^{-1}).

$P_a co_2$ (control = 31 ± 1 mmHg) doubled within 10 min of partial water submersion (62 ± 3 mmHg) and continued to increase through 30 min (90 ± 3 mmHg). $P_a co_2$ then remained elevated at 60 min (85 ± 3 mmHg) (Fig. 7A). During recovery in air, $P_a co_2$ rapidly decreased by more than half at 10 min (38 ± 2 mmHg) and by 30 min reached a value (29 ± 1 mmHg) similar to control.

The control pH_a of 7.56 ± 0.01 decreased markedly within 10 min to 7.22 ± 0.01 and continued to decrease to 6.84 ± 0.01 until 60 min partial submersion. During recovery in air, pH_a increased to 7.12 ± 0.02 within 10 min and reached control level of 7.55 ± 0.01 within 120 min.

$[HCO_3^-]_a$ (control = 27.4 ± 0.7 mmol L^{-1}) also decreased during the initial 10 min partial submersion (24.3 ± 0.9 mmol L^{-1}) and continued to decrease, reaching a minimum by 60 min (13.7 ± 0.3 mmol L^{-1}). A further small decrease in $[HCO_3^-]_a$ initially occurred during the first 10 min of recovery in air (11.8 ± 0.5 mmol L^{-1}) before $[HCO_3^-]_a$ began to increase at 30 min (13.5 ± 1.1 mmol L^{-1}). $[HCO_3^-]_a$ then markedly increased to 20.9 ± 0.8 mmol L^{-1} at 60 min and returned to control values at 120 min (24.6 ± 0.8 mmol L^{-1}).

3.3.3. *Completely water-submersed embryos*

Control Hct (27.9 ± 0.5%) responded rapidly to complete submersion by increasing significantly by 20 min (36.7 ± 0.4%) before reaching a maximal level of ~40% during 30–50 min (Fig. 5B). During recovery after 20 min complete submersion, Hct decreased to control levels of 31.4 ± 0.4% by 30 min in air. [RBC] (control = 2.02 ± 0.02 × 10^6 μL^{-1}) rapidly increased by 20 min (2.31 ± 0.03 × 10^6 μL^{-1}) and 30 min (2.41 ± 0.04 × 10^6 μL^{-1}) submersion corresponding to proportional increases of ~14 and 19% at 20 and 30 min of submersion, respectively (Fig. 5D). [RBC] returned to control levels after 30 min recovery in air (2.14 ± 0.03 × 10^6 L^{-1}). MCV (control = 138 ± 1 μ^3) increased rapidly at 10 min (153 ± 2 μ^3) with a further increase at 20 min (159 ± 1 μ^3) and reaching a maximum at 50 min (167 ± 3 μ^3) complete submersion (Fig. 5F). These increases corresponded to a proportional increase of ~11, 15 and 21% at 10, 20 and 50 min, respectively. MCV remained unchanged during the first 10 min of recovery in air (160 ± 2 μ^3) and returned to control levels from 60 min (140 ± 2 μ^3) onwards.

Reflecting the changes in [RBC], [Hb] (control = 8.9 ± 0.1 g%) increased significantly by 20 min (10.2 ± 0.1 g%) and remained elevated (~10 g%) for 20–50 min complete water submersion (Fig. 6B). [Hb] returned to control levels within 10 min recovery in air (9.8 ± 0.2 g%). MCH remained unchanged throughout both complete submersion and recovery in air, with mean value of 44.1 ± 0.3 pg (P = 0.836) (Fig. 6D). [MCHb] (control = 31.9 ± 0.2 g%)

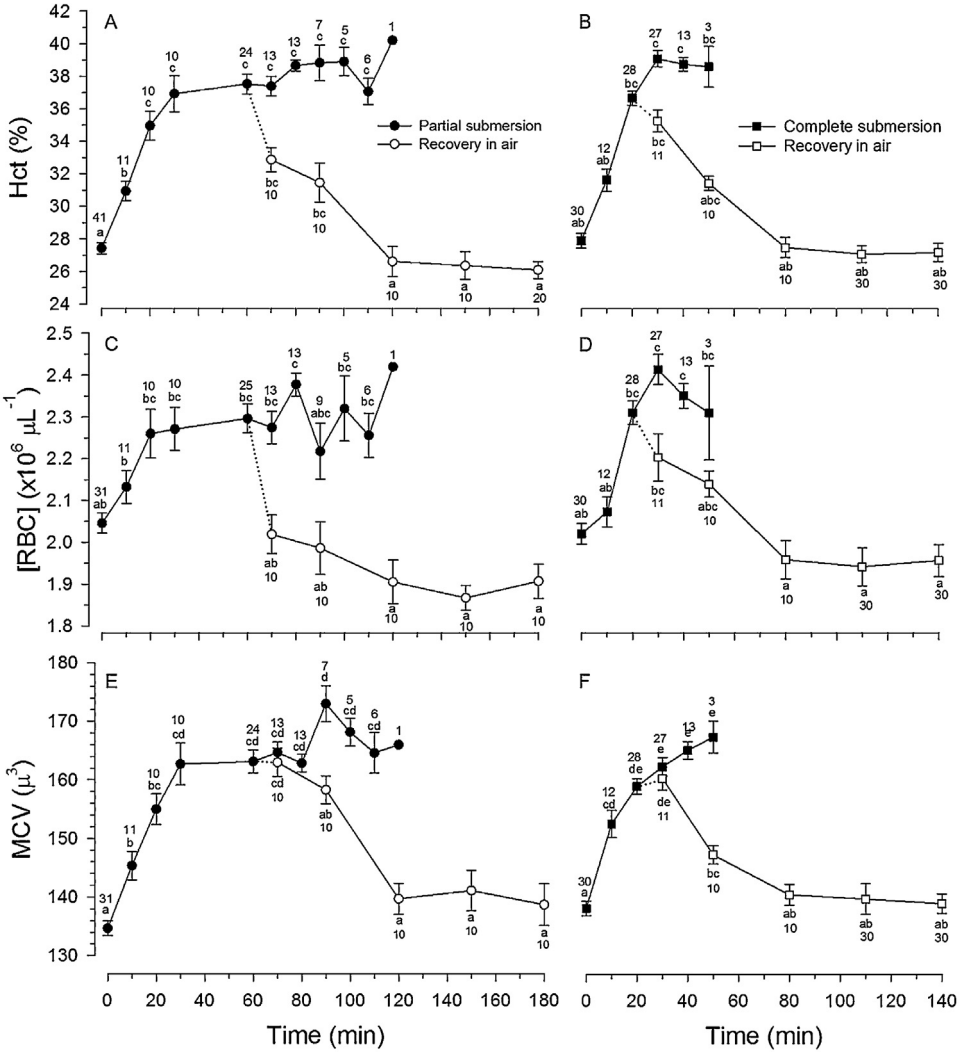

Fig. 5. Responses of (A and B) hematocrit (Hct), (C and D) red blood cell concentration ([RBC]) and (E and F) mean corpuscular volume (MCV) in d15 embryos to partial (closed round symbols) or complete (closed square symbols) submersion and recovery in air (open round symbols) (open square symbols). The control values determined prior to submersion were plotted at time 0. Partially and completely submersed embryos were returned to air after 60 and 20 min submersion, respectively. Values are means ± 1 S.E.M. and *N* values are indicated by numerical figures. Dotted lines and different lowercase letters are the same as in Fig. 4.

decreased significantly at 20 min (27.8 ± 0.2 g%) complete submersion with ~26 g% onward (Fig. 6F). [MCHb] returned to control levels after 30 min recovery in air (30.2 ± 0.3 g%).

$[La^-]$ (control = 1.0 ± 0.1 mmol L^{-1}) increased significantly by 20 min complete water submersion (11.7 ± 0.1 mmol L^{-1}) with a maximal value reached at 50 min (14.8 ± 0.5 mmol L^{-1}) (Fig. 4B). During recovery in air, $[La^-]$ decreased within 30 min (7.9 ± 0.3 mmol L^{-1}) reaching control values. Osm (control = 271 ± 2 mmol kg^{-1}) increased during complete water submersion to 287 ± 9 mmol kg^{-1} at 40 min (Fig. 4D). During recovery, Osm decreased to control values within 10 min (261 ± 2 mmol kg^{-1}).

P_aco_2 (control = 32 ± 1 mmHg) more than doubled within 10 min (69 ± 2 mmHg) and continued to increase at 20 min (86 ± 2 mmHg) and 30 min complete submersion (98 ± 3 mmHg) (Fig. 7B). During recovery in air, P_aco_2 decreased rapidly during the first 10 min (40 ± 2 mmHg) with a further decrease at 30 min (33 ± 2 mmHg) of recovery to control levels. pH_a (control = 7.55 ± 0.01) decreased markedly within 10 min (7.16 ± 0.01) and continued to decrease until 30 min complete submersion (6.85 ± 0.2) (Fig. 7B). A similar rapid change in pH_a occurred when the embryos were recovered in air with pH_a increasing within 10 min (7.25 ± 0.01) and reaching control values from 60 min (7.55 ± 0.01) onward. $[HCO_3^-]_a$ (control = 28.0 ± 0.6 mmol L^{-1}) also decreased soon after complete submersion (23.3 ± 0.5 mmol L^{-1} at 10 min) and continued decreasing at 20 min (19.8 ± 0.6 mmol L^{-1}) reaching a minimal level at 30 min (16.3 ± 0.8 mmol L^{-1}). $[HCO_3^-]_a$ initially decreased further during the first 10 min recovery in air (17.0 ± 0.5 mmol L^{-1}) before increasing at 30 min (21.2 ± 0.7 mmol L^{-1}) and returning to control values from 60 min (27.9 ± 0.7 mmol L^{-1}) onward.

4. Discussion

When respiratory gas exchange through the eggshell pores is blocked by water submersion, embryos experience a progressive intrinsic hypoxia as they deplete the O_2 stores within the egg. Concurrently, CO_2 accumulates, until ultimately death results. Oxygen uptake is greatly reduced during water submersion due to the lower capacitance of water for O_2 compared with air (Dejours, 1975). Further, water absorption has been reported in submersed eggs (Sotherland et al., 1984) and it is plausible that gas exchange may be further hindered by water physically blocking the eggshell pores. The well-studied chicken embryo is ideal for exploring acute

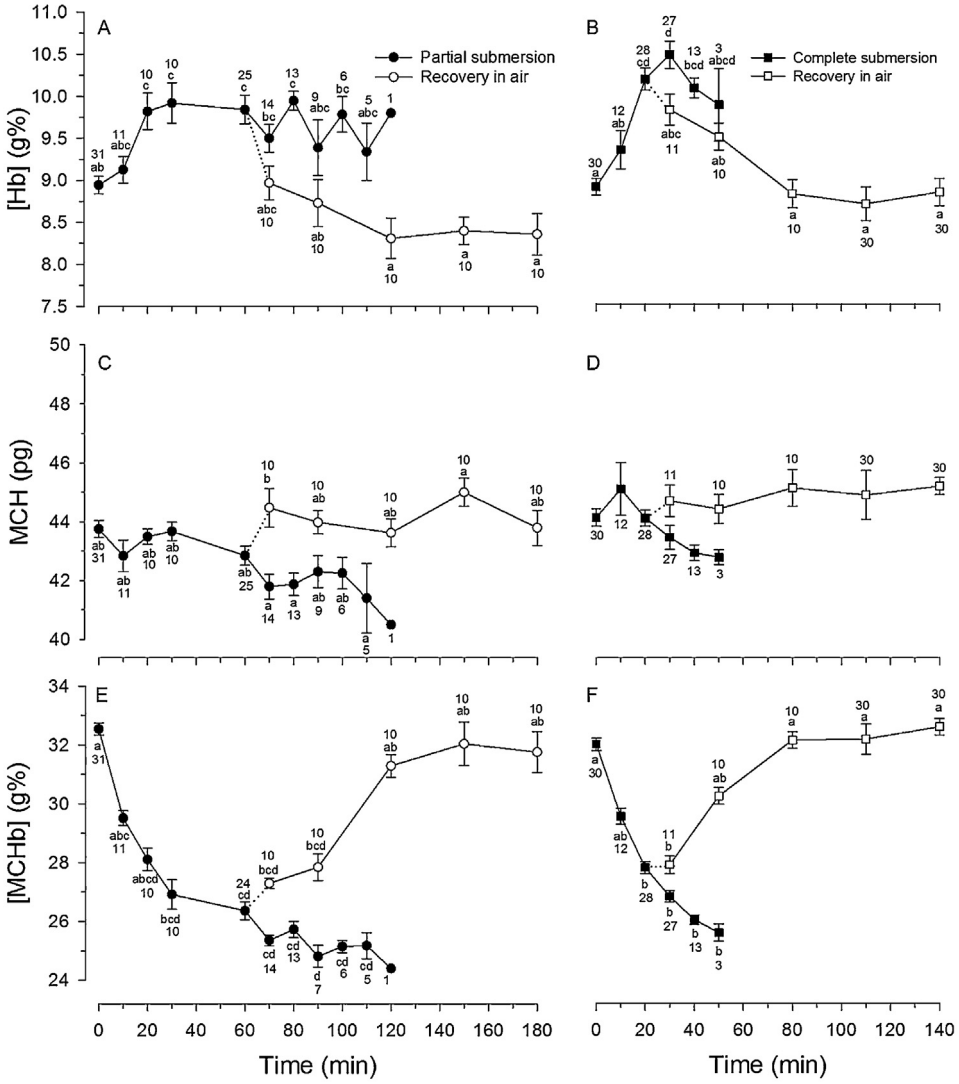

Fig. 6. Responses of (A and B) hemoglobin concentration ([Hb]), (C and D) mean corpuscular hemoglobin (MCH) and (E and F) mean corpuscular hemoglobin concentration ([MCHb]) in d15 embryos to partial (closed round symbols) or complete (closed square symbols) water submersion and recovery in air (open round symbols) (open square symbols). Values are means ± 1 S.E.M. and *N* values are indicated by numerical figures. Dotted lines and different lowercase letters are the same as in Fig. 4.

physiological changes during intrinsic hypercapnic hypoxia due to the wealth of literature examining the effects of extrinsic hypoxia and hypercapnic hypoxia (see Burggren et al., 2012; Tazawa et al., 2012; Mueller et al., 2013 for an entry into the literature).

4.1. Submersion and embryonic survival

No embryos survived complete water submersion for 60 min – the most severe intrinsic hypoxic hypercapnia treatment. However, survival was increased in the partially submersed embryos through the small amount but significant gas exchange through the air cell at the water's surface. All embryos survived 60 min and ~60% of embryos survived 90 min partial submersion. This is similar to the ~60% survival of d15 embryos exposed to 120 min severe extrinsic hypercapnic hypoxia (5% CO_2, 10% O_2) (Fig. 1; Tazawa et al., 2012). From these survival data and the speed of the hematological and acid–base disturbance (see below), it can be inferred that the intrinsic hypercapnic hypoxia created by water submersion (both complete and partial) in this study is more physiologically challenging than severe extrinsic hypercapnic hypoxia (5% CO_2, 10% O_2) (e.g. Tazawa et al., 2012).

4.2. Acid–base balance regulation during submersion and Parafilm®-wrapping

4.2.1. Characteristics of acid–base balance during intrinsic hypercapnic hypoxia and recovery

Water submersion (partial or complete) or Parafilm®-wrapping produced a rapid, severe acid–base disturbance with an associated rapid increase in Hct. The magnitude and speed of these changes were greater than in embryos exposed to extrinsic moderate or severe hypoxia or hypercapnic hypoxia (Burggren et al., 2012; Tazawa et al., 2012; Mueller et al., 2013). Partially submersed and Parafilm®-wrapped embryos showed similar physiological responses. The acid–base disturbance was characterized by a progressive respiratory acidosis followed by metabolic acidosis which became more prominent during the latter half of submersion/wrapping (evident from a decrease in pH_a and $[HCO_3^-]_a$ in parallel to the P_aco_2 isopleths) (Figs. 3 and 7A). Embryonic death resulted when $[HCO_3^-]_a$ fell below ~10 mmol L^{-1} during prolonged partial submersion or Parafilm®-wrapping. If embryos were allowed to recover in air from submersion or Parafilm®-wrapping, then rapid respiratory compensation was the first mechanism for acid–base changes due to rapidly restored gas

exchange. Accumulated CO_2 was released from the egg, resulting in increased pH_a with little change in $[HCO_3^-]_a$. A progressive respiratory alkalosis accompanied by a yet unknown regulatory, metabolic mechanism appeared to preserve $[HCO_3^-]_a$ above the critical level (~10 mmol L^{-1} in d15 embryos; Tazawa et al., 2012). In Parafilm®-wrapped embryos, after 30 min of recovery following unwrapping, the respiratory alkalosis was surpassed by metabolic compensation (i.e., $[HCO_3^-]_a$ increased in parallel with the P_aco_2 isopleths) (Fig. 3). While the gas exchange of the Parafilm®-wrapped embryos was decreased across the entire eggshell surface, the partially submersed eggs could exchange gases with the ambient air through the air cell. Although this area for gas exchange was substantially smaller than normal, the small degree of gas exchange through the air cell somewhat mitigated the decrease in $[HCO_3^-]_a$ during the final phase of water submersion (Fig. 7A). The gas exchange across the air cell was ultimately insufficient to support the O_2 demands and CO_2 elimination requirements of the embryos, which then led to an increase in P_aco_2 similar to the increase observed in Parafilm®-wrapped embryos.

The acid–base disturbance of the completely submersed embryos proceeded faster than the partially submersed embryos, because the covered air cell further prohibited gas exchange (i.e. accelerated CO_2 accumulation and O_2 depletion in the egg) (Fig. 7). However, the timing of the hematological respiratory response was similar with the hematological respiratory variables of embryos at 30 min complete submersion progressing to a similar stage of disturbance as embryos that had been partially submersed for 30 min (Figs. 5 and 6). Despite the faster progression in acid–base disturbances, embryos recovered from complete submersion (within 60 min) faster than embryos recovering from partial submersion, likely due to their shorter exposure time (Fig. 7).

The increased survival time in the partially (cf. completely) submersed embryos was likely due to the small amount of gas exchange allowed across the air-cell slowing the acid–base and hematological disturbances (Fig. 1). Although decreased survival occurred beyond 20 min complete and 60 min partial water submersion, embryos submersed for these maximal times recovered acid–base balance within the 120 min recovery period in air (Figs. 3 and 7). Air cell contact with the environmental air (i.e., partial submersion) did indeed prolong survival in water by ~40–60 min by slowing down the acid–base disturbances and preserving $[HCO_3^-]$ above the lethal level, i.e., ~10 mmol L^{-1}.

4.2.2. Glycolysis and acid–base regulation during submersion-induced intrinsic hypoxia

$[La^-]$ is relatively low in chicken embryos until d18 (Høiby et al., 1987) and previous studies have demonstrated a decrease in overall chicken embryo metabolism during hypoxia rather than increase in glycolysis (Ar et al., 1991; Mortola and Besterman, 2007). Embryos did increase $[La^-]$ (~0.8 mmol L^{-1}) when exposed to mild hypoxia (15% O_2, 0% CO_2), but the much larger increases during severe hypoxia (7–10%) (~15 mmol L^{-1}) and at 60 min submersion (~14 mmol L^{-1}) indicate that glycolysis may play a more important role at lower oxygen concentrations (Bjønnes et al., 1987; Burggren et al., 2012; Tazawa et al., 2012; present study).

A build-up of lactic acid produced by glycolytic pathways during hypoxia appears to be largely responsible for the metabolic acidosis during submersion. In contrast, the compensatory metabolic alkalosis and recovery of acid–base balance are likely restored by a progressive decrease in $[La^-]$ production during recovery in air (Figs. 4 and 7). As O_2 was progressively depleted during submersion, $[La^-]$ increased concurrent with a reduction in $[HCO_3^-]_a$ (Fig. 8). The opposite occurred during recovery in air such that at any time-point throughout submersion and recovery changes in $[La^-]$ were matched by reciprocal changes in $[HCO_3^-]_a$. A similar pattern also occurred in embryos exposed to severe extrinsic

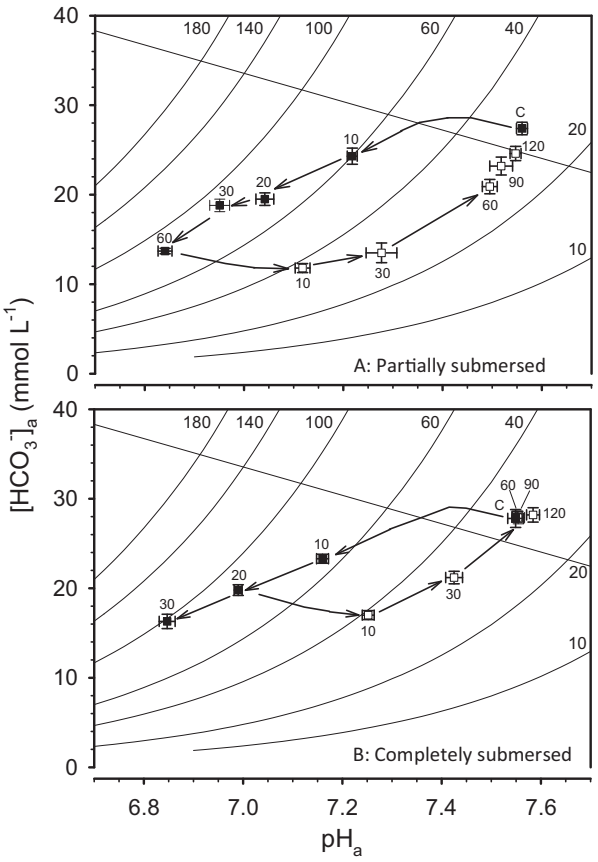

Fig. 7. Davenport diagram illustrating the time-course of the acid–base disturbance of d15 embryos (A) partially submersed for 60 min or (B) completely submersed for 30 min in water (closed symbols) with 120 min recovery in air (open symbols). The completely submersed embryos were returned to air after 20 min submersion. Values are means ± 1 S.E.M. and *N* values are as in Fig. 4.

hypoxia (10% O_2 with or without 5% CO_2) (Tazawa et al., 2012). Several authors have proposed that HCO_3^- transfers across the chorioallantoic membrane (CAM) between the blood in the allantoic circulation and the allantoic fluid (e.g. Boutilier et al., 1977; Gabrielli and Accili, 2010; Everaert et al., 2011 for review). Administering $NaHCO_3$ or HCl into embryonic allantoic fluid produces metabolic alkalosis or acidosis, respectively, in the allantoic blood supporting this mechanism (H. Tazawa, unpublished data). Accordingly, we infer that the metabolic acidosis during submersion results from HCO_3^- moving out of the blood across the CAM into the allantoic fluid in response to increased $[La^-]$. When embryonic gas exchange is restored and aerobic respiration reinitiated, $[La^-]$ production decreases and HCO_3^- moves into blood, increasing $[HCO_3^-]_a$ back to control levels.

4.3. Regulation of Hct during submersion

4.3.1. Contributions of MCV and [RBC] during submersion

Rapid changes in Hct resulted from Parafilm®-wrapping, partial or complete submersion and recovery (Figs. 2A, 5A and B). [RBC] and MCV increased rapidly by similar magnitudes during the first 30 min partial and complete submersion contributing equally to the increase in Hct ($P = 0.741$ and $P = 0.558$, respectively; Fig. 5). For example, the proportional increase in submersed embryo Hct (ΔHct) of ~38% at 60 min was due to a proportional increase in [RBC] (Δ[RBC]) and MCV (ΔMCV) of ~18% (Fig. 5A, C, and E). There were similar equal contributions of [RBC] and MCV to ΔHct during recovery from complete submersion with Δ[RBC] of ~10% and

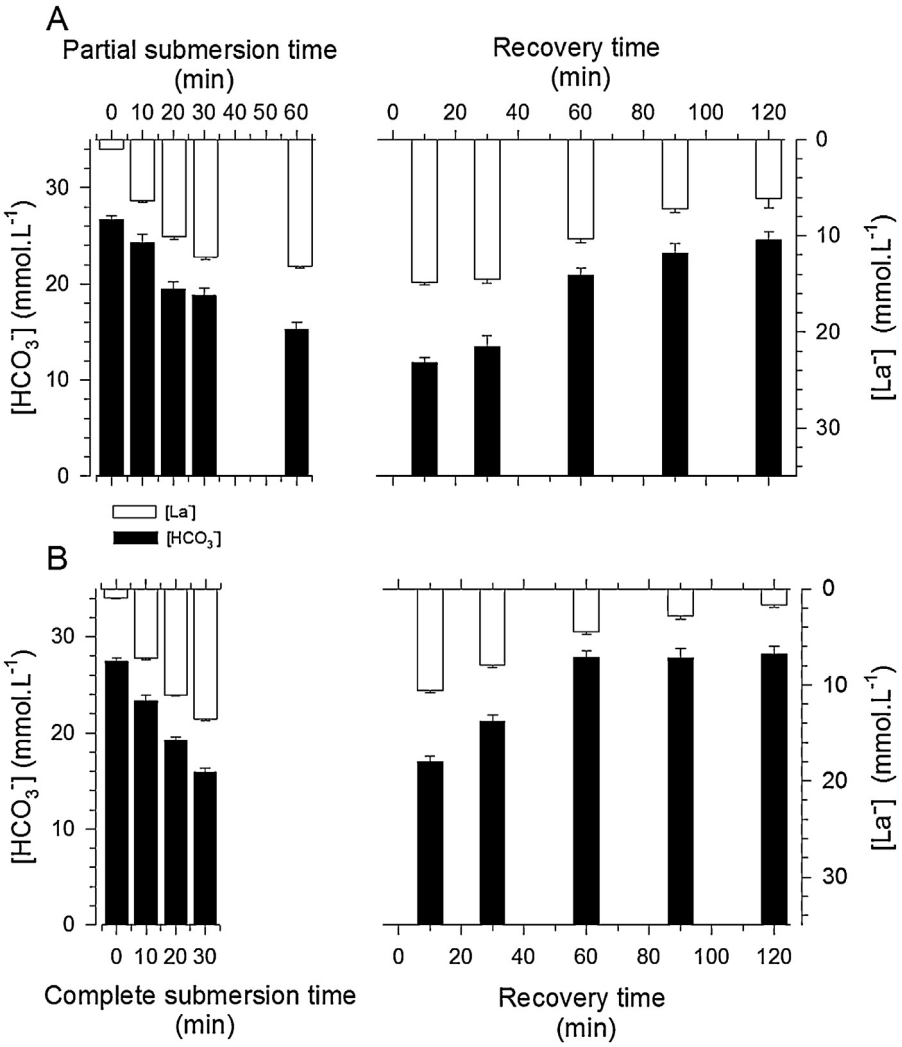

Fig. 8. Changes in bicarbonate ($[HCO_3^-]$) and lactate concentration ($[La^-]$) (A) during 60 min partial submersion and (B) 30 min complete submersion with 120 min recovery in air in d15 embryos. Partially and completely submersed embryos were returned to air after 60 min and 20 min submersion, respectively.

ΔMCV of ~13% resulting in a ΔHct of ~24% at 10 min (Figs. 5B, D, and F). However, changes in MCV were predominantly responsible for the decrease in Hct during recovery in partially submersed embryos. For example, ΔHct of ~20% at 10 min recovery was attributed to ΔMCV of ~18% with no corresponding change in Δ[RBC] (Figs. 5A, C, and E). Accordingly, alterations in [RBC] (likely through sequestration) proceeded faster than changes in MCV in partially submersed embryos (<10 min, Fig. 5C) and also in completely submersed embryos (<30 min, Fig. 5D) although ΔHct at 10 min recovery was attributed to both Δ[RBC] and ΔMCV, like due to delayed sequestration.

If embryos remained partially or completely submersed (during the survival protocol), Hct plateaued at ~40% (before death) due to an increase in both [RBC] (~2.4×10^6 μL^{-1}) and MCV (~170 μ^3) (Fig. 5). The ~12% increase in Hct from the control level of ~28 to ~40% after 30 min complete or 60 min partial submersion would have required ~1 billion or ~300 μL of RBC's to be released into the blood stream assuming a total blood volume of ~2.5 mL in d15 embryos (Yosphe-Purer et al., 1953; Kind, 1975). It is yet unknown where non-circulating RBCs are stored in chicken embryos. The spleen releases and sequesters RBCs in adult vertebrates (see Brendolan et al., 2007 for review), although details are very limited for adult birds in this respect. The spleen may play a similar role in chicken embryos, but the relatively large volume of RBCs both released and sequestered during intrinsic hypoxia likely exceeds the capacity of the still-developing spleen. Other sites, such as the CAM, may be important in red cell sequestration, but this hypothesis requires additional experimentation.

4.3.2. Comparison of Hct regulation with embryos exposed to extrinsic severe hypoxia

Hct increased almost immediately upon development of intrinsic hypercapnic hypoxia produced by submersion (Fig. 5) or by exposure to extrinsic hypoxia or hypercapnic hypoxia (e.g., Tazawa et al., 2012). However, the mechanism for this increase (increased MCV, [RBC] or both) was dependent upon the severity and length of the hypoxia and embryo developmental age. While intrinsic hypercapnic hypoxia increased both MCV and [RBC] (Fig. 5), severe extrinsic hypercapnic hypoxia (5% CO_2, 10% O_2) or hypoxia (10% O_2) increased Hct only through changes in MCV (Tazawa et al., 2012). Longer exposure (24 h) to moderate extrinsic hypercapnic hypoxia (5% CO_2, 15% O_2) similarly increased Hct through MCV in d15 and d17 embryos, although in d15 embryos the increase was due to an increase in both MCV and [RBC] (Burggren et al., 2012). Overall, changes in MCV (with some associated [RBC] release) are predominantly responsible for Hct regulation in advanced embryos during extrinsic hypercapnic hypoxia exposure (Burggren et al., 2012; Tazawa et al., 2012; Mueller et al., 2013) with increases in

[RBC] additionally boosting [Hb] levels during more physiologically challenging circumstances such as water submersion (i.e., intrinsic hypoxia) (Fig. 6). Consequently, embryonic O_2 transport is enhanced during water submersion. However, even with these enhancements, death eventually occurs once the constrained pool of O_2 has been used and/or the acid–base disturbance becomes too severe for survival (i.e., $[HCO_3^-]_a < \sim 10$ mmol L^{-1} in d15 embryos). Nevertheless, restoration of acid–base balance and the hematological respiratory variables within 120 min recovery in air following severe intrinsic or extrinsic hypercapnic hypoxia indicates that the viability of embryos facing imminent death was likely not compromised during short-term exposure. Thus, even in partially developed embryos, regulatory mechanisms have developed sufficiently to partially mitigate acute interruptions to gas transport. Extrapolating the present findings to wild avian embryos suggests that they may similarly survive partial water submersion during short bouts of nest flooding.

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