



Dynamics of blood viscosity regulation during hypoxic challenges in the chicken embryo (*Gallus gallus domesticus*)



Zachary F. Kohl*, Dane A. Crossley II, Hiroshi Tazawa, Warren W. Burggren

Developmental and Integrative Biology, Department of Biological Sciences, University of North Texas, 1155 Union Circle #305220, Denton, TX 76203, USA

ARTICLE INFO

Article history:

Received 7 July 2015

Received in revised form 30 July 2015

Accepted 31 July 2015

Available online 8 August 2015

Keywords:

Blood viscosity

Stress

Cardiovascular

Chick embryo

Chicken

Hematocrit

Hypoxia

Hypercapnia

Oxygen transport

Optimal hematocrit

ABSTRACT

Hypoxia in chicken embryos increases hematocrit (Hct), blood O₂ content, and blood viscosity. The latter may limit O₂ transport capacity (OTC) via increased peripheral resistance. Hct increase may result from increased nucleated red blood cell concentration ([RBC]) and mean corpuscular volume (MCV) or reduced plasma volume. We hypothesized changes in Hct, hemoglobin concentration ([Hb]), [RBC] and MCV and their effects on viscosity would reduce OTC. Five experimental treatments that increase Hct were conducted on day 15 embryos: 60 min water submergence with 60 min recovery in air; exposure to 15% O₂ with or without 5% CO₂ for 24 h with 6 h recovery; or exposure to 10% O₂ with or without 5% CO₂ for 120 min with 120 min recovery. Control Hct, [Hb], [RBC], MCV, and viscosity were approximately 26%, 9 g%, 2.0 10⁶ μL⁻¹, 130 μm³, and 1.6 mPa s, respectively. All manipulations increased Hct and blood viscosity without changing blood osmolality (276 mmol kg⁻¹). Increased viscosity was attributed to increased [RBC] and MCV in submerged embryos, but solely MCV in embryos experiencing 10% O₂ regardless of CO₂. Blood viscosity in embryos exposed to 15% O₂ increased via increased MCV alone, and viscosity was constant during recovery despite increased [RBC]. Consequently, blood viscosity was governed by MCV and [RBC] during submergence, while MCV was the strongest determinant of blood viscosity in extrinsic hypoxia with or without hypercapnia. Increased Hct and blood O₂ content did not compensate for the effect of increased viscosity on OTC during these challenges.

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1. Introduction

Oxygen delivery to systemic tissues in vertebrates is governed by several factors, including blood flow and the O₂ content of blood. Blood flow is inversely proportional to viscosity while O₂ content is proportional to hemoglobin concentration ([Hb]) and, by extension, hematocrit (Hct), the prime determinant of blood viscosity. O₂ delivery will be optimized when the Hct is neither too high (increasing viscosity), nor too low (impairing O₂ transport). Therefore, an optimal Hct can be predicted that minimizes convective transport costs while maximizing O₂ transport capacity (OTC) (Crowell and Smith, 1967). Predicted optimal Hcts agree with in vivo Hcts during exercise in many mammals (Schuler et al., 2010; Stark and Schuster, 2012), the cane toad (Hillman et al., 1985), tarpon (Wells et al., 2003), and embryonic and adult marine green turtles (Wells and Baldwin, 1994). However exceptions exist, such as deep diving marine mammals, where Hct is much greater than the predicted optimum and appears to be optimized for O₂

storage rather than delivery (Hedrick and Duffield, 1991; Castellini et al., 2006), or rainbow trout (Wells and Weber, 1991).

Hct elevation can result from any combination of increase in red blood cell concentration ([RBC]), increase in mean corpuscular volume (MCV), and decrease in plasma volume. Avian red blood cells are nucleated, potentially affecting the distensibility of the cell and its apparent viscosity (for an entry in to the literature see Beaufrière et al., 2013; Götting and Nikinmaa, 2015). Consistent with other vertebrates, in vitro blood viscosity in embryonic and adult chicken increases nonlinearly with Hct (Rand et al., 1964; Kohl et al., 2012). Blood hyperviscosity is widely assumed to increase the cost of O₂ transport in an already O₂ limiting environment (see original discussion by Crowell and Smith, 1967; Fan et al., 1980), possibly even offsetting the advantages of increased blood oxygen carrying capacity. Yet “blood doping” embryos late in incubation, day 15 (d15) to d17, to increase Hct from control values of 27% up to 38%, without affecting MCV, had no effect on embryonic oxygen consumption (Khorrami et al., 2008). Their results suggest that increases in Hct, and the attendant viscosity increases, may need to be large before they affect blood oxygen transport. In any event, the potentially complex relationship between blood viscosity and how it is driven by integrated changes in Hct, MCV and [RBC] in embryonic chickens is unknown.

Prior studies of embryonic chickens in response to hypoxic stress have focused on the changes in size and concentration of

Abbreviations: Hct, hematocrit; [Hb], hemoglobin concentration; MCV, mean corpuscular volume; [MCHb], mean corpuscular hemoglobin concentration; η , blood viscosity; Osm, osmolality; OTC, O₂ transport capacity.

* Corresponding author. Tel.: +1 940 565 3591; fax: +1 940 565 3821.

E-mail address: zac.f.kohl@gmail.com (Z.F. Kohl).

the red blood cells. In d15 embryos, 24 h exposure to moderate hypoxia (e.g., 15% O₂), with or without 5% CO₂, elevates Hct through increases in both [RBC] and MCV (Burggren et al., 2012). However [RBC] remained unchanged at 2 and 6 h of exposure to 15% O₂ with 5% CO₂ while Hct increases, indicating an increase in MCV alone (Mueller et al., 2013). Embryonic chickens at d15 respond quickly to acute exposure to severe hypoxia (e.g., 10% O₂) with or without 5% CO₂, by increasing MCV and thus Hct (Tazawa et al., 2012). Complete submergence of chicken eggs in water for one hour also increases Hct significantly through an equal contribution of MCV and [RBC] (Andrewartha et al., 2014). While it was speculated that these changes might increase oxygen carrying capacity or delivery, the potentially negative impact of increased Hct on the physical properties of the blood has not been assessed.

We hypothesized that the elevations of Hct, MCV, and [Hb] during hypoxia could not compensate for the increased viscosity expected when OTC was calculated. We identified the effect of Hct on blood viscosity and quantified the contribution of [RBC] and MCV to changes in blood viscosity using submergence and acute and chronic hypoxia, with or without hypercapnia, as tools to induce specific hematologic responses. Experiments were designed to determine concurrent, time-specific changes in blood viscosity and hematological respiratory variables (e.g. Hct, [RBC], MCV) with blood osmolality (Osm) in d15 embryos subjected to various regimes of hypoxia and hypercapnia and additionally to assess how changes in Hct and blood viscosity affected O₂ transport capacity.

2. Materials and methods

2.1. Egg incubation

Fertile eggs of Hyline White Leghorn chickens (*Gallus gallus*) (N = 536) were obtained weekly from a hatchery at Texas A&M University (College Station, TX, USA) to the laboratory at University of North Texas (Denton, TX, USA). The eggs were lightly washed in water with a sponge to remove extraneous material. On the day of incubation, the eggs were weighed (± 0.01 g) on an electronic balance and placed in a forced draught incubator (model 1502, G.Q.F. Manuf. Co., GA, USA) at 12:00 h. The eggs were placed vertically on an automatic turning tray rotating every 3 h and incubated at a temperature of 37.5 ± 0.1 °C and relative humidity of ~55%. On the day prior to experiment (day 13 or 14 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 randomly divided into “control” eggs and “treatment (water submerged or gas exposed)” eggs and moved into a desktop incubator (Hova-Bator 1590, G.Q.F. Manuf.) maintained at 37.5 °C. All studies were carried out at the University of North Texas in accordance with the approved institutional animal care and use committee protocol #11007 on embryos at d15.

2.2. Experimental protocols and their justifications

Changes in key blood properties in avian embryos can be experimentally induced by acute (<24 h) hypoxia, short-term obstruction of gas exchange through the eggshell covered by a gas impermeable material (<24 h), or water submergence of the egg (Tazawa et al., 1988, 2012; Burggren et al., 2012; Mueller et al., 2013; Andrewartha et al., 2014). Hct in particular increases in response to the above conditions. These stresses are well characterized in d15 embryos and were chosen to challenge embryonic O₂ transport in a predictable fashion, rather than mimic a natural (and thus variable) environmental stressor. Exposure protocols used for this study have been described in detail elsewhere (Burggren et al., 2012; Tazawa et al., 2012; Mueller et al., 2013; Mueller et al., 2014; Andrewartha et al., 2014) but are outlined briefly below.

2.3. Partial water submersion

Egg-shell gas exchange was severely compromised by partially submerging eggs in water for 60 min with 60 min recovery in air (Andrewartha et al., 2014). Treatment eggs were placed into the water bath (Model 3545, Lab-Line Instrument, USA) at 37.5 °C with the air cell up and part of the eggshell protruding from the water and exposed to air. Embryonic blood was sampled following 10, 30 or 60 min submergence, after which the embryos were sacrificed. Embryos in the recovery protocol were first submerged for 60 min and then returned to air for blood collection at 10, 30 or 60 min. Blood was collected from control eggs incubated in air 37.5 °C. A minimum of 13 embryos were sampled at each time point. This procedure provided intrinsic O₂ deficiency (hypoxemia) accompanying CO₂ accumulation with recovery from hypoxemia and hypercapnia.

2.4. Exposure to gas mixtures

Abrupt extrinsic hypoxia was provided by exposing eggs to 10% O₂ or 15% O₂ with or without 5% CO₂ with the accompanying recovery protocol in air. Eggs were placed on a cardboard egg stand in a 3.78 L air-tight plastic Ziploc® with inlet and outlet conduits at diagonally opposite corners. The bag was ventilated with humidified hypoxic gas mixtures supplied by a gas mixer (Qubit Systems, Ontario, Canada) at a rate of 1 L min⁻¹.

Embryos were exposed to 15% O₂ with or without 5% CO₂ for 24 h. On d14 of incubation, a subset of eggs were transferred into the gas exposure bag 24 h prior to blood collection. On d15, remaining eggs were moved into the gas exposure bag for blood collection at 0, 2, and 6 h of exposure. In the recovery protocol, the d15 eggs were removed from the gas exposure bag and blood was collected following 2 and 6 h in air. A minimum of 10 embryos were sampled at each time point.

Additional embryos were exposed to 10% O₂ with or without 5% CO₂ for 120 min with recovery in air for 120 min. Blood was collected prior to exposure (0 min or control), at 10, 30, 60, 90 and 120 min of exposure, and after 10, 30, 60, and 120 min recovery. A minimum of 12 embryos were sampled at each time point.

2.5. Blood collection and analysis

A small region of the eggshell previously marked over the site of the allantoic vein was removed and the vein was gently lifted by forceps through the hole. Blood of ≥ 0.6 ml was collected through a 25-gauge needle mounted on a 1 mL plastic syringe flushed in advance with heparinized saline. Blood was placed into a 1.5 mL plastic vial and well stirred. [RBC] (10⁶ cells μL^{-1}) and hemoglobin concentration ([Hb], g%) were determined on ~0.01 mL of blood using a hematology analyzer (Coulter analyzer, A^c · 10 T, Beckman, USA) and Osm (mmol kg⁻¹) was measured with a vapor pressure osmometer (Vapro 5520, Wescor, USA). Duplicate preparations of ~0.06 mL of blood were moved into hematocrit tubes, sealed and centrifuged for 4 min at 10,000 rpm and two determinations were averaged for a value of Hct in individual embryos ($\pm 0.1\%$, Readacrit Centrifuge, Becton Dickinson, USA). [RBC] determined by the Coulter analyzer was modified by an expression previously derived from a relation with [RBC] determined by a hematometer (Tazawa et al., 2011). MCV (μm^3), mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration ([MCHb], g%) were calculated from Hct, [RBC] and [Hb] (equations from Tazawa et al., 2011).

Viscosity was determined immediately after hematological measurements. The remaining blood was well stirred to prevent sedimentation and 0.5 mL of blood was placed into the sample cup of a Wells-Brookfield Viscometer (Model LVTD-CP, Middleboro, MA,

USA) and maintained at 38.0 ± 0.1 °C with an Isotemp 1006S recirculating water bath (Fisher Scientific, Waltham, MA, USA).

2.6. O₂ transport capacity

OTC was calculated with the following equation:

$$\text{OTC} = 1.3 \text{ ml O}_2 \times [\text{Hb}]/\eta \quad (1)$$

where η , blood viscosity, and [Hb] are specific to a given Hct (Hedrick et al., 1986). Complete OTC curves for control and following 60 min of partial submersion were calculated by determining the [Hb] at each Hct in 5% intervals from 5 to 60% and using the viscosity–Hct relationship over the same range to determine the Hct specific viscosity. The following equation describes the relationship between embryonic blood viscosity and Hct (Kohl et al., 2012):

$$\text{Viscosity} = 0.96^{(0.039 \times \text{Hct})}; r^2 = 0.97 \quad (2)$$

The *in vivo* OTC value, rather than the complete curve, was calculated for all treatments at each time-point.

2.7. Statistical analysis

All data were examined for normality and equal variance. Parametric ANOVA or ANOVA on ranks were used where appropriate. Differences in egg mass, embryo body mass, Osm and hematological variables across times of experimental procedures (submersion or hypoxic gas exposures) or at each time of experimental procedures were examined for significance using a one-way ANOVA with all pairwise multiple comparisons by the Tukey test or Dunn's method in SigmaStat 3.5 (Systat Software Inc., Chicago, IL, USA). Differences in mean values of variables across gas exposure times and between different gas treatments were examined using a two-way ANOVA with all pairwise multiple comparisons by the Holm–Sidak test. The relation between viscosity and hematological respiratory variables (Hct, [RBC], MCV) was expressed by a linear equation and the significance of correlation coefficient was examined by *t*-test. The significant level was $P < 0.05$. All data were presented as mean \pm 1 S.E.M.

3. Results

3.1. Fresh egg mass and body mass

Measurements were carried out on a total of 536 chicken embryos across partial water submersion and four hypoxic treatments with recovery in air: 10% O₂ with or without 5% CO₂ and 15% O₂ with or without 5% CO₂. There were no differences in fresh egg mass or body mass between groups of eggs examined for the time course of responses to submersion and hypoxic exposures.

3.2. Osmolality

Submersion of eggs had no significant effect ($P > 0.05$) on Osm until 60 min of submersion, at which point Osm was significantly higher than control eggs (282 ± 0.1 mmol/kg; $N = 16$; $P = 0.011$). Mean embryonic Osm was 276.3 ± 0.3 mmol/kg ($N = 436$) for all other time-points and treatments.

3.3. Partial submersion

Viscosity under control conditions was 1.57 ± 0.03 mPa s. Viscosity began to increase after 10 min of submersion and was significantly greater than control at 30 min (2.06 ± 0.05 mPa s), peaking at 60 min (2.30 ± 0.06 mPa s) ($P < 0.001$) (Fig. 1A). Viscosity remained significantly

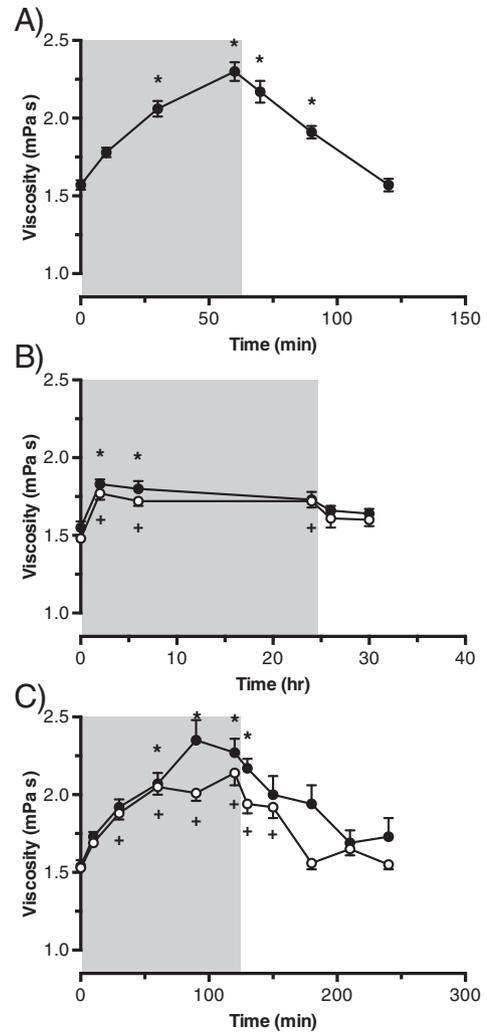


Fig. 1. Time course of blood viscosity in d15 embryos that were (A) partially submerged, (B) exposed to 15% O₂ with 5% CO₂ (filled circle) and without CO₂ (open circle), and (C) exposed to 10% O₂ with 5% CO₂ (filled circle) and without CO₂ (open circle). Exposure time indicated by gray background and recovery is white. Significant differences from control are indicated with a cross (10 and 15% O₂ with 5% CO₂) and asterisk (10 and 15% O₂). Sample size of each mean value with S.E.M. (bar) is shown in Tables 1–3.

elevated during the first 30 min of recovery, but returned to control values at 60 min.

Hct under control conditions was $26.7 \pm 0.8\%$. Hct increased significantly within 10 min peaking at 60 min ($35.7 \pm 0.8\%$) ($P < 0.001$) (Fig. 2A). Hct remained significantly elevated during the first 30 min of recovery, but returned to control values at 60 min.

[RBC] under control conditions was $2.01 \pm 0.05 \times 10^6 \mu\text{L}^{-1}$. This value was significantly elevated at 30 min ($2.33 \pm 0.05 \times 10^6 \mu\text{L}^{-1}$) and 60 min of submersion ($2.25 \pm 0.04 \times 10^6 \mu\text{L}^{-1}$) and after 10 min of recovery ($2.20 \pm 0.05 \times 10^6 \mu\text{L}^{-1}$), returning to control values during the last 30 min of recovery ($P < 0.001$) (Table 1).

MCV under control conditions was $133 \pm 2 \mu\text{m}^3$. This value increased significantly at 10 min ($146 \pm 2 \mu\text{m}^3$), peaked at 60 min of submersion ($159 \pm 2 \mu\text{m}^3$) and remained elevated at 10 min of recovery ($160 \pm 2 \mu\text{m}^3$). MCV then returned to control values after 60 min of recovery ($P < 0.001$) (Table 1).

[Hb] under control conditions was 9.0 ± 0.2 g%. Attendant with changes in [RBC], [Hb] increased significantly from control to 10.0 ± 0.3 g% at 30 min of submersion and returned to control after 30 min of recovery ($P < 0.001$) (Table 1). Since MCH remained unchanged during submersion and recovery ($P = 0.691$), [MCHb] response mirrored inversely the change in MCV ($P < 0.001$) (Table 1).

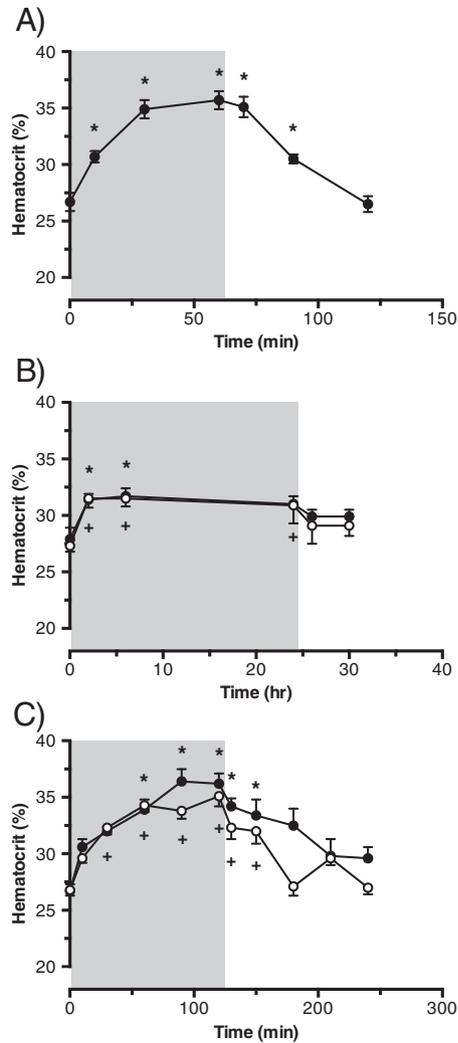


Fig. 2. Time course of hematocrit in d15 embryos that were (A) partially submerged, (B) exposed to 15% O₂ with 5% CO₂ (filled circle) and without CO₂ (open circle), and (C) exposed to 10% O₂ with 5% CO₂ (filled circle) and without CO₂ (open circle). Exposure time indicated by gray background and recovery is white. Significant differences from control are indicated with a cross (10 and 15% O₂ with 5% CO₂) and asterisk (10 and 15% O₂). Sample size of each mean value with S.E.M. (bar) is shown in Tables 1–3.

3.4. Moderate hypoxia with hypercapnia

In response to 24 h exposure to 15% O₂ with 5% CO₂, viscosity significantly increased from control to 2 h (1.83 ± 0.03 mPa s), but then slightly decreased at 6 h and returned to control at 24 h (P < 0.001; Fig. 1B, filled circle). During recovery in air, blood viscosity returned to control within 2 h, and was maintained at that value through 6 h of recovery.

Table 1

Hematological and rheological variables in the blood of the chicken embryo, *Gallus gallus domesticus*, during partial submersion and recovery (preceded by R). Means (± SEM) within the same column with different superscript letters are significantly different (P < 0.05).

Time (min)	N	Hct (%)	[RBC] (10 ⁶ μL ⁻¹)	MCV (μm ³)	[Hb] (g%)	MCH (pg)	[MCHb] (g%)	Viscosity (mPa s)
0	15	26.7 ± 0.8 ^a	2.01 ± 0.05 ^{ab}	133 ± 1.9 ^a	9.0 ± 0.2 ^{ab}	44.9 ± 1.0	33.8 ± 0.6 ^a	1.57 ± 0.03 ^a
10	15	30.7 ± 0.5 ^b	2.11 ± 0.04 ^{bc}	146 ± 1.6 ^{bc}	9.5 ± 0.1 ^{bc}	45.1 ± 0.8	30.9 ± 0.5 ^{ab}	1.78 ± 0.03 ^{ab}
30	14	34.9 ± 0.8 ^c	2.33 ± 0.06 ^d	150 ± 1.9 ^c	10.0 ± 0.3 ^c	43.1 ± 0.7	28.7 ± 0.4 ^{bc}	2.06 ± 0.05 ^{bc}
60	16	35.7 ± 0.8 ^c	2.25 ± 0.04 ^{cd}	159 ± 2.1 ^d	9.8 ± 0.2 ^c	43.7 ± 0.8	27.5 ± 0.4 ^c	2.30 ± 0.06 ^c
R10	13	35.1 ± 0.9 ^c	2.20 ± 0.05 ^{bcd}	160 ± 1.7 ^d	9.8 ± 0.4 ^{bc}	44.4 ± 1.1	27.8 ± 0.5 ^c	2.17 ± 0.07 ^c
R30	13	30.5 ± 0.4 ^b	2.01 ± 0.03 ^{ab}	153 ± 2.4 ^{cd}	8.8 ± 0.1 ^{ab}	43.8 ± 0.8	28.7 ± 0.5 ^{bc}	1.91 ± 0.04 ^{bc}
R60	14	26.5 ± 0.7 ^a	1.90 ± 0.04 ^a	140 ± 1.8 ^{ab}	8.3 ± 0.2 ^a	43.6 ± 1.1	31.3 ± 0.6 ^{ab}	1.57 ± 0.04 ^a

Hct significantly increased from control during exposure to 15% O₂ with 5% CO₂, even after 24 h (31.0 ± 0.7%), and returned to control during recovery in air (P < 0.001; Fig. 2B, filled circle).

[RBC] remained unchanged from control during exposure to 15% O₂ with 5% CO₂ however increased significantly during recovery in air, averaging 2.29 ± 0.03 10⁶ μL⁻¹ (P < 0.001; Table 2).

MCV significantly increased during exposure, and remained so through the end of exposure at 24 h (144 ± 2 μm³; P < 0.001; Table 2). During recovery in air, MCV decreased below the control at 2 h (129 ± 2 μm³), finally returning to controls level at 6 h.

As with [RBC] during exposure to 15% O₂ with 5% CO₂, [Hb] remained unchanged (P = 0.986) during exposure, but increased significantly during recovery in air (P < 0.001). MCH remained unchanged (P = 0.057) and thus [MCHb] mirrored inversely the change in MCV (P < 0.001; Table 2).

3.5. Moderate hypoxia without hypercapnia

Blood viscosity significantly increased from control to 1.77 ± 0.04 mPa s at 2 h, and remained elevated through 24 h (1.72 ± 0.04 mPa s; P < 0.001; Fig. 1B, open circle). During recovery in air, viscosity returned to control levels at 2 h, which was maintained for at least 6 h.

Hct significantly increased during exposure, remaining elevated until 24 h (30.9 ± 0.7%) and returned to control level during recovery in air (P < 0.001; Fig. 2B, open circle).

[RBC] remained unchanged until 24 h of exposure (2.15 ± 0.05 10⁶ μL⁻¹; Table 2). [RBC] increased significantly at 2 h (2.31 ± 0.13 10⁶ μL⁻¹) of recovery in air (P = 0.015).

MCV significantly increased during exposure to 144 ± 2 μm³ at 24 h (P < 0.001). During recovery in air, MCV decreased to a stable value no different than control levels by 2 h (Table 2).

Similarly to the [RBC] response to 10% O₂ without 5% CO₂, [Hb] remained unchanged during exposure, but it increased significantly during recovery in air (P = 0.037). MCH remained unchanged and thus [MCHb] mirrored inversely the change in MCV (Table 2).

3.6. Severe hypoxia with hypercapnia

In response to 10% O₂ with 5% CO₂, viscosity increased significantly after 60 min (2.07 ± 0.07 mPa s) and remained elevated throughout further exposure, plateauing at 90 min (2.35 ± 0.13 mPa s) and 120 min (2.27 ± 0.09 mPa s) (P < 0.001; Fig. 1C, filled circle). Viscosity remained elevated during the first 10 min of recovery (2.17 ± 0.06 mPa s) and subsequently began to decrease, returning to control values after 30 min.

Hct also increased significantly from control after 60 min (33.9 ± 0.9%) and remained high throughout further exposure, plateauing at 90 min (36.4 ± 1.1%) and 120 min (36.2 ± 0.9%) (P < 0.001; Fig. 2C, filled circle). During recovery in air, Hct remained high during the first 10 min (34.2 ± 0.7%) and subsequently began to decrease (33.4 ± 1.4% at 30 min) and by 60 min had returned to control levels.

Table 2

Hematological and rheological variables in the blood of the chicken embryo, *Gallus gallus domesticus*, during exposure to 15% O₂ with or without 5% CO₂ and recovery (preceded by R). Means (\pm SEM) within the same column with different superscript letters are significantly different ($P < 0.05$).

Time (h)	N	Hct (%)	[RBC] ($10^6 \mu\text{L}^{-1}$)	MCV (μm^3)	[Hb] (g%)	MCH (pg)	[MCHb] (g%)	Viscosity (mPa s)
<i>With 5% CO₂</i>								
0	12	27.9 \pm 1.0 ^a	2.01 \pm 0.06 ^a	138 \pm 1.9 ^{bc}	8.4 \pm 0.2	42.0 \pm 0.4	30.4 \pm 0.4 ^a	1.55 \pm 0.04 ^a
2	13	31.4 \pm 0.5 ^c	2.00 \pm 0.04 ^a	158 \pm 2.0 ^d	8.0 \pm 0.1	40.4 \pm 0.6	25.6 \pm 0.3 ^c	1.83 \pm 0.03 ^c
6	14	31.7 \pm 0.7 ^c	2.06 \pm 0.04 ^a	154 \pm 2.5 ^d	8.4 \pm 0.2	40.6 \pm 0.4	26.4 \pm 0.3 ^c	1.80 \pm 0.05 ^{bc}
24	13	31.0 \pm 0.7 ^{bc}	2.15 \pm 0.04 ^{ab}	144 \pm 1.5 ^c	8.6 \pm 0.2	40.1 \pm 0.4	27.9 \pm 0.4 ^b	1.73 \pm 0.05 ^{abc}
R2	12	29.9 \pm 0.6 ^{abc}	2.31 \pm 0.04 ^b	129 \pm 1.6 ^a	9.2 \pm 0.2	39.8 \pm 0.4	30.8 \pm 0.4 ^a	1.66 \pm 0.03 ^{ab}
R6	12	29.9 \pm 0.6 ^{abc}	2.29 \pm 0.06 ^b	130 \pm 1.5 ^{ab}	9.3 \pm 0.2	40.6 \pm 0.3	31.1 \pm 0.3 ^a	1.64 \pm 0.03 ^{ab}
<i>Without CO₂</i>								
0	12	27.3 \pm 0.5 ^a	2.05 \pm 0.04 ^a	134 \pm 2.0 ^{ab}	8.5 \pm 0.2	41.3 \pm 0.3	31.0 \pm 0.4 ^a	1.48 \pm 0.02 ^a
2	13	31.5 \pm 0.8 ^b	2.11 \pm 0.04 ^{ab}	149 \pm 1.9 ^c	8.5 \pm 0.1	40.5 \pm 0.2	27.2 \pm 0.4 ^b	1.77 \pm 0.04 ^c
6	12	31.5 \pm 0.7 ^b	2.15 \pm 0.05 ^{ab}	147 \pm 1.9 ^c	8.5 \pm 0.2	38.9 \pm 1.1	27.1 \pm 0.2 ^b	1.72 \pm 0.03 ^{bc}
24	12	30.9 \pm 0.7 ^b	2.15 \pm 0.05 ^{ab}	144 \pm 2.4 ^{bc}	8.5 \pm 0.2	39.8 \pm 0.7	27.6 \pm 0.4 ^b	1.72 \pm 0.04 ^{bc}
R2	10	29.1 \pm 1.6 ^{ab}	2.31 \pm 0.31 ^b	126 \pm 1.5 ^a	8.9 \pm 0.5	38.4 \pm 0.6	30.5 \pm 0.3 ^a	1.61 \pm 0.06 ^{ab}
R6	11	29.1 \pm 0.9 ^{ab}	2.24 \pm 0.05 ^b	130 \pm 1.0 ^a	8.8 \pm 0.3	39.1 \pm 0.4	30.2 \pm 0.3 ^a	1.60 \pm 0.04 ^{ab}

[RBC] was not significantly affected by hypercapnic hypoxia exposure ($P = 0.52$; Table 3).

MCV increased significantly at 30 min ($150 \pm 2 \mu\text{m}^3$) and remained elevated throughout exposure, peaking at 120 min ($167 \pm 2 \mu\text{m}^3$). During recovery, MCV began to decrease within 30 min ($155 \pm 3 \mu\text{m}^3$) and by 60 min had returned to control levels ($P < 0.001$) (Table 3).

[Hb], like [RBC], remained unchanged during exposure to severe hypoxia with hypercapnia and during recovery in air ($P = 0.304$). MCH also remained unchanged ($P = 0.264$) and thus [MCHb] mirrored inversely the change in MCV ($P < 0.001$) (Table 3).

3.7. Severe hypoxia without hypercapnia

In response to 10% O₂, control blood viscosity increased significantly after 30 min to 1.88 ± 0.04 mPa s and remained elevated throughout further exposure, peaking at 120 min (2.14 ± 0.08 mPa s) ($P < 0.001$; Fig. 1C, open circle). Viscosity remained elevated during the first

30 min (1.92 ± 0.07 mPa s) of recovery and subsequently decreased, returning to control levels after 60 min.

Hct also increased significantly after 30 min ($32.2 \pm 0.6\%$) and remained elevated throughout further hypoxic exposure, peaking at 120 min ($35.1 \pm 0.9\%$) ($P < 0.001$; Fig. 2C, open circle). During recovery in air, Hct remained elevated during the first 30 min ($32.0 \pm 1.1\%$) and subsequently decreased, returning to control levels at 60 min.

[RBC] did not change during 10% O₂, 0% CO₂ exposure and recovery ($P = 0.084$; Table 3).

MCV increased significantly during hypoxic exposure from control at 10 min ($142 \pm 2 \mu\text{m}^3$) and further increased with time to a peak at 120 min ($168 \pm 3 \mu\text{m}^3$; $P < 0.001$). MCV decreased significantly from the peak value within 30 min of recovery ($157 \pm 3 \mu\text{m}^3$) and by 60 min had returned to control levels (Table 3).

As with [RBC], [Hb] remained unchanged during exposure to 10% O₂ without 5% CO₂ and during recovery in air ($P = 0.051$). MCH remained

Table 3

Hematological and rheological variables in the blood of the chicken embryo, *Gallus gallus domesticus*, during exposure to 10% O₂ with or without 5% CO₂ and recovery (preceded by R). Means (\pm SEM) within the same column with different superscript letters are significantly different ($P < 0.05$).

Time (min)	N	Hct (%)	[RBC] ($10^6 \mu\text{L}^{-1}$)	MCV (μm^3)	[Hb] (g%)	MCH (pg)	[MCHb] (g%)	Viscosity (mPa s)
<i>With 5% CO₂</i>								
0	12	26.7 \pm 0.6 ^a	2.03 \pm 0.03	131 \pm 1.5 ^a	8.3 \pm 0.2	40.6 \pm 0.7	31.0 \pm 0.7 ^a	1.54 \pm 0.04 ^a
10	13	30.6 \pm 0.7 ^{abc}	2.18 \pm 0.04	140 \pm 1.9 ^{ab}	8.8 \pm 0.2	40.4 \pm 0.3	28.8 \pm 0.3 ^a	1.73 \pm 0.03 ^{ab}
30	13	32.0 \pm 0.4 ^{abc}	2.13 \pm 0.04	150 \pm 1.6 ^{bc}	8.8 \pm 0.1	41.2 \pm 0.3	27.4 \pm 0.3 ^{ab}	1.92 \pm 0.05 ^{abc}
60	13	33.9 \pm 0.9 ^{bc}	2.13 \pm 0.01	159 \pm 2.2 ^c	8.5 \pm 0.2	40.1 \pm 0.4	25.2 \pm 0.4 ^b	2.07 \pm 0.07 ^{bc}
90	14	36.4 \pm 1.1 ^c	2.23 \pm 0.06	164 \pm 2.7 ^c	9.1 \pm 0.3	40.6 \pm 0.5	24.9 \pm 0.2 ^b	2.35 \pm 0.13 ^c
120	13	36.2 \pm 0.9 ^c	2.17 \pm 0.05	167 \pm 2.3 ^c	8.7 \pm 0.2	40.0 \pm 0.5	24.1 \pm 0.4 ^b	2.27 \pm 0.09 ^c
R10	13	34.2 \pm 0.7 ^{bc}	2.12 \pm 0.05	162 \pm 2.3 ^c	8.3 \pm 0.2	39.4 \pm 0.6	24.4 \pm 0.4 ^b	2.17 \pm 0.06 ^c
R30	13	33.4 \pm 1.4 ^{bc}	2.15 \pm 0.06	155 \pm 3.0 ^{bc}	8.4 \pm 0.3	39.1 \pm 0.6	25.3 \pm 0.4 ^b	2.00 \pm 0.12 ^{abc}
R60	14	32.5 \pm 1.5 ^{abc}	2.17 \pm 0.06	149 \pm 3.4 ^{abc}	8.7 \pm 0.3	40.2 \pm 0.5	27.1 \pm 0.6 ^a	1.94 \pm 0.12 ^{abc}
R90	13	29.8 \pm 1.5 ^{ab}	2.17 \pm 0.08	136 \pm 2.0 ^{ab}	8.5 \pm 0.2	39.4 \pm 0.6	28.9 \pm 0.6 ^a	1.69 \pm 0.08 ^{ab}
R120	14	29.6 \pm 1.0 ^{ab}	2.15 \pm 0.04	137 \pm 2.8 ^{ab}	8.6 \pm 0.2	39.8 \pm 0.5	29.2 \pm 0.6 ^a	1.73 \pm 0.12 ^{ab}
<i>Without CO₂</i>								
0	13	26.8 \pm 0.5 ^a	2.07 \pm 0.06	130 \pm 2.0 ^a	9.0 \pm 0.2	43.7 \pm 0.7	33.6 \pm 0.6 ^a	1.53 \pm 0.02 ^a
10	13	29.6 \pm 0.4 ^{ab}	2.08 \pm 0.03	142 \pm 1.8 ^b	9.0 \pm 0.2	43.6 \pm 0.9	30.6 \pm 0.5 ^b	1.69 \pm 0.02 ^{ab}
30	13	32.3 \pm 0.6 ^{bc}	2.11 \pm 0.03	153 \pm 0.0 ^c	9.2 \pm 0.2	43.7 \pm 0.9	28.6 \pm 0.5 ^{bc}	1.88 \pm 0.04 ^{bc}
60	13	34.3 \pm 0.6 ^{bc}	2.14 \pm 0.03	160 \pm 2.1 ^{cd}	9.3 \pm 0.2	43.4 \pm 0.7	27.2 \pm 0.5 ^{cd}	2.05 \pm 0.05 ^c
90	13	33.8 \pm 0.7 ^c	2.07 \pm 0.05	164 \pm 1.5 ^d	8.7 \pm 0.2	42.2 \pm 0.9	25.8 \pm 0.5 ^d	2.01 \pm 0.05 ^{bc}
120	14	35.1 \pm 0.9 ^c	2.10 \pm 0.04	168 \pm 2.8 ^d	8.9 \pm 0.1	42.7 \pm 0.7	25.6 \pm 0.5 ^d	2.14 \pm 0.08 ^c
R10	13	32.3 \pm 1.0 ^c	2.00 \pm 0.04	161 \pm 2.3 ^{cd}	8.5 \pm 0.2	42.3 \pm 0.7	26.3 \pm 0.6 ^{cd}	1.94 \pm 0.06 ^{bc}
R30	13	32.0 \pm 1.1 ^{bc}	2.04 \pm 0.04	157 \pm 2.5 ^c	8.6 \pm 0.3	42.4 \pm 0.8	27.1 \pm 0.5 ^{cd}	1.92 \pm 0.07 ^{bc}
R60	14	27.1 \pm 0.8 ^{bc}	1.97 \pm 0.03	138 \pm 2.3 ^{ab}	8.4 \pm 0.2	41.8 \pm 0.8	30.5 \pm 0.8 ^b	1.56 \pm 0.04 ^a
R90	13	29.6 \pm 0.6 ^{ab}	2.15 \pm 0.05	138 \pm 2.6 ^{ab}	9.1 \pm 0.2	42.4 \pm 0.8	30.8 \pm 0.7 ^b	1.65 \pm 0.04 ^{ab}
R120	13	27.0 \pm 0.6 ^a	2.05 \pm 0.03	132 \pm 1.7 ^a	8.6 \pm 0.2	42.1 \pm 0.6	31.7 \pm 0.8 ^{ab}	1.55 \pm 0.03 ^a

unchanged ($P = 0.538$) and thus [MCHb] mirrored inversely the change in MCV ($P < 0.001$; Table 3).

3.8. O_2 transport capacity

OTC curves were calculated using values of [Hb] at control and 60 min of submergence, ~9 and 9.8 g%, respectively, and the Hct–viscosity relationship in d15 embryos using Eqs. 1 and 2 (Fig. 3). Optima were predicted to occur at a Hct of ~27% in both cases, though only control Hct was observed at this value *in vivo*. Exposure to severe hypoxia, with or without 5% CO_2 , and water submersion increased Hct 31–36% (Δ Hct) and viscosity 40–47% (Δ viscosity) over control values. These adjustments moved Hct away from the optimum Hct for O_2 delivery in chicken embryos. As a result, *in vivo* OTC values, rather than complete curves, decreased significantly by 22–25% at 60 min for all three treatments and 28% at 120 min of both hypoxia exposures (Fig. 4A, 4C). When extrinsic hypoxia was moderate (e.g., 15%), with or without CO_2 , OTC decreased 15 and 13%, respectively (Fig. 4B).

4. Discussion

Changes in blood viscosity during O_2 and CO_2 stress reported in this study indicate viscosity to be an important component of the acute response to respiratory stress. Our results also indicate that the mechanisms associated with altered viscosity depend upon the type of stimulus (i.e. intrinsic/extrinsic hypoxia and hypercapnia).

4.1. Dynamics of time-specific responses of viscosity and hematology to intrinsic or extrinsic hypoxia

Embryos of similar developmental stage submerged partially with air cells protruding into air were previously found to survive for 60 min (Andrewartha et al., 2014) and all embryos in the current study survived 60 min of submersion. Viscosity, Hct, [Hb], [RBC], [MCHb], and MCV all increased during exposure and all variables returned to control values in recovery (Fig. 1). Increased blood viscosity was attributed to increased MCV and [RBC], which have been documented previously (Andrewartha et al., 2014). Moderate hypoxia, regardless of CO_2 level, was the only other condition to effect [RBC] (Table 2). The changes of $\sim 3 \times 10^5$ cells μL^{-1} of [RBC] in all three conditions correspond to $\sim 7.5 \times 10^8$ RBCs, assuming a total blood volume of ~ 2.5 mL in d15 embryos (Yosphe-Purer et al., 1953; Barnes and Jensen, 1959; Kind, 1975). This also corresponds to a RBC volume of $\sim 110 \mu L$, assuming an averaged MCV during submersion/exposure of $\sim 150 \mu m^3$. RBC release did not occur during severe hypoxia exposure, but did

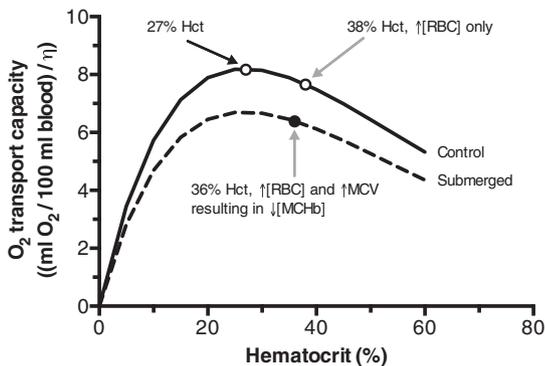


Fig. 3. O_2 transport curves calculated using Eq. 1 in control (solid line) and after 60 min of submergence (dashed line) for d15 embryos. Calculated with specific hematologic variables using Eq. 1 (Table 1). Solid arrow indicates control Hct and the grey arrows indicate OTC at a Hct of 38% when only [RBC] changed, the value achieved in Khorrami et al. (2008; see discussion), and at a Hct of 36%, achieved with a combination of increased MCV and [RBC], after 60 min of partial submersion. Blood viscosity is represented by η .

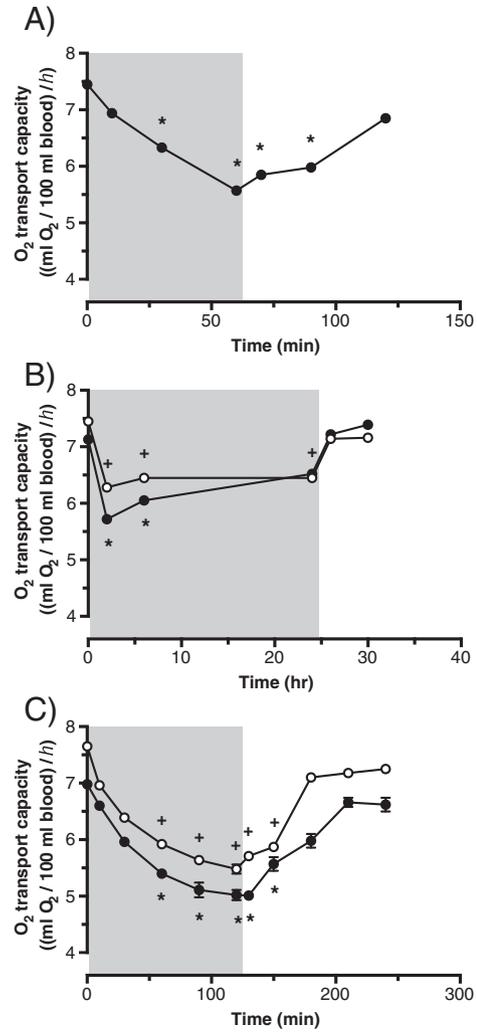


Fig. 4. Time course of O_2 transport capacity in d15 embryos that were (A) partially submerged, (B) exposed to 15% O_2 with 5% CO_2 (filled circle) and without CO_2 (open circle), and (C) exposed to 10% O_2 with 5% CO_2 (filled circle) and without CO_2 (open circle). Exposure time indicated by gray background and recovery is white. Significant differences from control are indicated with a cross (10 and 15% O_2 with 5% CO_2) and asterisk (10 and 15% O_2). OTC and S.E.M. (bar) calculated from values in Tables 1–3. Blood viscosity is represented by η .

during recovery from moderate hypoxia. This suggests that RBC release was not related to enhancement of O_2 transport, but might be related to duration of hypoxia and, partially, severity of hypoxia. That is, 24 h of 15% hypoxia caused an increase in [RBC] after exposure, but 2 h of 10% hypoxia caused no changes in [RBC]. A shift of fluid volume away from the vasculature may also explain the [RBC] component of Hct variation. However our methods did not allow further examination of this topic.

The spleen releases and sequesters RBCs in adult vertebrates (see Brendolan et al., 2007 for review). This organ may play a similar role in chicken embryos, though the role of and even the anatomy of the spleen in birds is poorly understood. However, the relatively large volume of released or sequestered RBCs estimated likely exceeds the capacity of the developing embryo spleen. An extra-embryonic tissue/organ such as the chorioallantoic membrane acting as a potential reservoir of non-circulating RBCs may be an interesting avenue for future investigations (Andrewartha et al., 2014). Further exploration of the sites for RBC sequestration and release in embryonic birds is highly warranted.

In contrast to submergence and moderate hypoxia, severe extrinsic hypoxia, with or without CO_2 , increased Hct and viscosity through an increase in MCV only (Figs. 1C and 2C; Table 3). These changes in

MCV to extrinsic severe hypoxia were consistent with previous findings for Day 15 embryos (Tazawa et al., 2012). Viscosity changes were related to those in MCV only and independent of [RBC], which remained unchanged even during severe hypoxia with hypercapnia, unlike submersion, which produced progressive intrinsic hypercapnic hypoxia.

Although the maximal value of Hct in response to moderate hypoxia was lower than that in response to severe hypoxia and the response took 24 h versus 1–2 h in severe hypoxia exposure, viscosity similarly changed in parallel with changes in Hct (Figs. 1B and 1C). Consequently, the changes in viscosity occur in proportion to changes in Hct irrespective of the cause and duration of hypoxia.

4.2. Blood viscosity and relative oxygen transport capacity in embryonic chickens

Hct and blood viscosity are positively related at a constant temperature. The relationship is exponential when determined *in vitro* in vertebrate blood with red cells (Trevan, 1918; Al-Roubaie et al., 2011), and is effectively linear over the range observed in this study on d15 chicken embryos (Kohl et al., 2012). Blood viscosity accounts for the majority of resistance to flow in the cardiovascular system of amphibians, with the parallel arrangement of the gas exchange and systemic circulations minimizing the resistance associated with the vessels themselves (Kohl et al., 2013). Embryonic and extra-embryonic circulations resemble, on a basic level, the parallel arrangement found in amphibians. Therefore viscosity may also be the dominant contributor to resistance to blood flow in the embryonic chicken (Tazawa and Takenaka, 1985; Tazawa and Johansen, 1987), although this has not been tested. Our results support our hypothesis that increased viscosity outweighs the increased blood O₂ carrying capacity accompanying intrinsic and extrinsic hypoxia, likely adding an additional challenge to the cardiovascular system during these periods of stress. However, it is possible that metabolic depression, a common phenomenon in vertebrates exposed to hypoxia, reduces O₂ demand to a point where the greater transport costs of more viscous blood have been neutralized. D16 chicken embryos exposed to 11 and 17% O₂ for 40 min reduced their metabolism and incurred no subsequent O₂ debt (Mortola and Besterman, 2007). Our protocols employed substantially longer exposure times (60 min, 120 min, and 24 hr in partial submersion, 10% O₂, and 15% O₂, respectively) that led to metabolic acidosis, suggesting metabolic depression was not sufficient to maintain aerobic metabolism during the extended exposures (Burggren et al., 2012; Tazawa et al., 2012; Mueller et al., 2013).

Increasing Hct to 35% by infusing normal RBCs collected from donor embryos has no effect on O₂ consumption in d15 chicken embryos (Khorrami et al., 2008). However, Khorrami et al.'s results do not contradict the conclusions of this study. Embryonic blood OTC would be only slightly shifted from optimum with a Hct of 35%, assuming normal MCV and MCH (Fig. 3). In this case, much of the increased convective transport costs associated with increased blood viscosity are probably offset by increased blood O₂ content and may not detectably influence O₂ consumption. It is also important to note that *in vivo* Hct does not always exactly match predicted optimal Hct—e.g. in the saltwater crocodile (Wells et al., 1991). The concept of optimal Hct is useful in assessing the status and demands on the cardiovascular system, but as an optimization may be representative of a balance between competing demands, not always dominated by O₂ delivery. Obviously, blood's responsibilities are not limited to just the transport of O₂, but also include CO₂ transport, O₂ storage, convective heat transport, ion transport, pH regulation, waste and nutrient transport, etc. These demands may easily shift the observed Hct away from the predicted Hct.

The method of hypoxia induction resulted in clear differences in the mode of Hct change, via changes in [RBC] and/or MCV, and the severity of accompanying viscosity changes. Since blood viscosity changes proportionally with changes in Hct during submersion and hypoxia,

equivalent changes in the ratio of [Hb] to Hct indicate that OTC responds identically to submersion and to both forms of hypoxia exposure. Time-specific patterns of OTC in response to intrinsic and extrinsic hypoxia exposures directly reflect changes in [MCHb], which are proportional to [Hb] and inversely proportional to Hct. Further, [MCHb] changes inversely with MCV because MCH remains unchanged during submersion and hypoxia exposures. Consequently, as submersion and hypoxia exposures progress, increased RBC hydration enlarges cell volume (MCV) and decreases [MCHb] and OTC (Fig. 4 and Tables 1–3). Although [Hb] increases progressively with time submerged, the increase is not large enough to reverse the trend in OTC in chicken embryos.

Changes in MCV may favor increased Hb oxygen affinity at the respiratory exchange surfaces through several mechanisms. As red blood cells swell, [MCHb] decreases. *In vivo* reductions in [MCHb] are associated with increased Hb oxygen affinity (Lykkeboe and Weber, 1978). Swelling of trout erythrocytes via water uptake due to adrenergic stimulation leads to increased cell volume and intracellular pH, both increasing Hb O₂ affinity (Nikinmaa, 1983). Bellingham et al. (1971) also demonstrated in humans that cell swelling increased Hb O₂ affinity via decreased [MCHb] and organic phosphate concentration. Therefore, it is possible the observed increased MCV and decreased [MCHb], leading to a concomitant reduction in organic phosphates and increased pH, favors increased Hb O₂ affinity during hypoxia (Tazawa and Mochizuki, 1976). Further investigation into responses of blood O₂ content and Hb O₂ affinity in submerged and hypoxic embryos would clarify O₂ transport and delivery modifications.

5. Summary

Embryonic chicken blood viscosity, Hct, [RBC], MCV, [Hb] and [MCHb] are all clearly affected by acute environmental stresses. Embryonic chickens respond to both intrinsic and extrinsic acute hypoxia with a reversible, increased Hct. However, the consequences of intrinsic vs. extrinsic hematologic responses vary, with intrinsic hypoxia resulting in true erythrocythemia (increased [RBC]) and increased MCV, while extrinsic (10% O₂ with and without 5% CO₂) increased Hct is achieved through increased MCV alone. Prolonged moderate extrinsic hypoxia, with and without 5% CO₂, results in increased [RBC] and MCV, similar to acute intrinsic hypoxia but to a lesser magnitude. Blood viscosity is proportional to Hct and changes rapidly during intrinsic and extrinsic hypoxic stresses. These results support our hypothesis that compensatory changes in Hct do not increase the OTC of embryonic chicken blood. Additionally, our results demonstrate blood viscosity is a rapidly dynamic variable that should be considered as an important component of the embryonic chicken hypoxic response.

Acknowledgments

We thank Dr. Stanley Hillman for use of the viscometer throughout the duration of the experiment. Support for this study was provided by NSF operating grant IOS-1025823 to Warren W. Burggren and NSF CAREER award IBN IOS-0845741 to Dane A. Crossley II.

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