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

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

Keywords: Blood viscosity, stress, cardiovascular, chick embryo, chicken, hematocrit, hypoxia, hypercapnia, oxygen transport, optimal hematocrit.

27 **Abstract**

28 Hypoxia in chicken embryos increases hematocrit (Hct), blood O₂ content, and blood viscosity.
29 The latter may limit O₂ transport capacity (OTC) via increased peripheral resistance. Hct increase may
30 result from increases in the concentration of nucleated red blood cells ([RBC]) or mean corpuscular
31 volume (MCV). We hypothesized that changes in Hct, hemoglobin concentration ([Hb]), [RBC] and
32 MCV and their effects on viscosity would reduce OTC. Five experimental treatments that increase
33 hematocrit were conducted on day 15 embryos: 60 min water submergence with 60 min recovery in air;
34 exposure to 10% O₂ with or without 5% CO₂ for 120 min with 120 min recovery; or exposure to 15% O₂
35 with or without 5% CO₂ for 24 h with 6 h recovery. Control Hct, [Hb], [RBC], MCV, and viscosity were
36 approximately 26%, 9 g%, $2.0 \times 10^6 \mu\text{L}^{-1}$, $130 \mu\text{m}^3$, and 1.6 mPa•s, respectively. All manipulations
37 increased Hct and blood viscosity without changing blood osmolality (276 mmol kg⁻¹). Increased
38 viscosity was attributed to increases in [RBC] and MCV in submerged embryos, but solely MCV in
39 embryos experiencing 10% O₂ with or without CO₂. Blood viscosity in embryos exposed to 15% O₂
40 increased via increased MCV alone, and viscosity was constant during recovery despite increased [RBC].
41 Consequently, blood viscosity was governed by MCV and [RBC] during submergence, while MCV was
42 the strongest determinant of blood viscosity in extrinsic hypoxia with or without hypercapnia. Increased
43 Hct and blood O₂ content did not compensate for the effect of increased viscosity on OTC during these
44 challenges.

45

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).

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 Beaufrère, 2013; Götting and Nikinmaa, 2015). Consistent with other vertebrates, *in vitro* blood viscosity in embryonic and adult chicken increases nonlinearly with Hct (Kohl et al., 2012; Rand et al., 1964). 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 (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), suggesting 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 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] during (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 have 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 (Hct, [RBC], MCV) with blood osmolality (Osm) in d15 embryos subjected to various regimes of hypoxia and hypercapnia and additionally predicted how changes in Hct and blood viscosity interact 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 $37.5 \pm 0.1^\circ\text{C}$ and relative humidity of $\sim 55\%$. On a 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 #11-007 on embryos at day 15 (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 (Andrewartha et al., 2014; Burggren et al., 2012; Mueller et al., 2013; Tazawa et al., 1988; Tazawa et al., 2012). Hct in particular increases in response to the above conditions. These stresses are well characterized in d15 embryos and were chosen to challenge embryonic O_2 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 (Andrewartha et al., 2014; Burggren et al., 2012; Mueller et al., 2013; Tazawa et al., 2012) 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 eggshell protruding from water 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 mixture supplied by a gas mixer (Qubit Systems, Ontario, Canada) at a rate of 1 L min⁻¹.

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.

Additional 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.

2.5 Blood collection and analysis

A small region of the eggshell previously marked over the site of the allantoic vein was removed

149 and the vein was gently lifted by forceps through the hole. Blood of ≥ 0.6 ml was collected through a
150 25-gauge needle mounted on a 1 mL plastic syringe flushed in advance with heparinized saline. Blood
151 was placed into a 1.5 mL plastic vial and well stirred. [RBC] (10^6 cells μL^{-1}) and hemoglobin
152 concentration ([Hb], g%) were determined on ~ 0.01 mL of blood using a hematology analyzer (Coulter
153 analyzer, A^c-10T, Beckman, USA) and Osm (mmol kg^{-1}) was measured with a vapor pressure
154 osmometer (Vapro 5520, Wescor, USA). Duplicate preparations of ~ 0.06 mL of blood were moved into
155 hematocrit tubes, sealed and centrifuged for 4 min at 10,000 rpm and two determinations were averaged
156 for a value of Hct in individual embryos ($\pm 0.1\%$, Readacrit Centrifuge, Becton Dickinson, USA).
157 [RBC] determined by the Coulter analyzer was modified by an expression previously derived from a
158 relation with [RBC] determined by a hematometer (Tazawa et al., 2011). MCV (μm^3), mean corpuscular
159 hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration ([MCHb], g%) were calculated
160 from Hct, [RBC] and [Hb] (equations from Tazawa et al., 2011).

161 Viscosity was determined immediately after hematological measurements. The remaining blood
162 was well stirred to prevent sedimentation and 0.5 mL of blood was placed into the sample cup of a
163 Wells-Brookfield Viscometer (Model LVTD-CP, Middleboro, MA, USA) and maintained at $38.0 \pm 0.1^\circ\text{C}$
164 with an Isotemp 1006S recirculating water bath (Fisher Scientific, Waltham, MA, USA).

165

166 *2.6 O₂ transport capacity*

167 OTC was calculated with the following equation:

$$168 \quad \text{OTC} = 1.3 \text{ ml O}_2 \times [\text{Hb}] / \eta \quad (1)$$

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

$$174 \quad \text{Viscosity} = 0.96^{(0.039 \cdot \text{Hct})}, r^2 = 0.97 \quad (2)$$

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

177

178

179 2.7 Statistical analysis

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

191

192 3. Results

193

194 3.1 Fresh egg mass and body mass

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

199

200 3.2 Osmolality

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

204

205 3.3 Partial submersion

206 Viscosity under control conditions was 1.57 ± 0.03 mPa•s. Viscosity began to increase after 10 min
207 of submersion and was significantly greater than control at 30 min (2.06 ± 0.05 mPa•s), peaking at 60 min
208 (2.30 ± 0.06 mPa•s) ($P<0.001$) (Fig. 1A). Viscosity remained significantly elevated during the first 30 min
209 of recovery, but returned to control values at 60 min.

210 Hct under control conditions was 26.7 ± 0.8 %. Hct increased significantly within 10 min peaking at
211 60 min (35.7 ± 0.8 %) ($P<0.001$) (Fig. 2A). Hct remained significantly elevated during the first 30 min of
212 recovery, but returned to control values at 60 min.

213 [RBC] under control conditions was 2.01 ± 0.05 10^6 μL^{-1} . This value was significantly elevated at
214 30 min (2.33 ± 0.05 10^6 μL^{-1}) and 60 min of submersion (2.25 ± 0.04 10^6 μL^{-1}) and after 10 min of
215 recovery (2.20 ± 0.05 10^6 μL^{-1}), returning to control values during the last 30 min of recovery ($P<0.001$)
216 (Table 1).

217 MCV under control conditions was 133 ± 2 μm^3 . This value increased significantly at 10 min
218 (146 ± 2 μm^3), peaked at 60 min of submersion (159 ± 2 μm^3) and remained elevated at 10 min of recovery
219 (160 ± 2 μm^3). MCV then returned to control values after 60 min of recovery ($P<0.001$) (Table 1).

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

224

225 3.4 Moderate hypoxia with hypercapnia

226 In response to 24 h exposure to 15% O_2 with 5% CO_2 , 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.

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 $2.29 \pm 0.03 \times 10^6 \mu\text{L}^{-1}$ ($P < 0.001$; Table 2).

MCV significantly increased during exposure, and remained so through the end of exposure at 24 h ($144 \pm 2 \mu\text{m}^3$; $P < 0.001$; Table 2). During recovery in air, MCV decreased below the control at 2 h ($129 \pm 2 \mu\text{m}^3$), 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 2h, 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 ($2.15 \pm 0.05 \times 10^6 \mu\text{L}^{-1}$; Table 2). [RBC] increased significantly at 2 h ($2.31 \pm 0.13 \times 10^6 \mu\text{L}^{-1}$) of recovery in air ($P = 0.015$).

MCV significantly increased during exposure to $144 \pm 2 \mu\text{m}^3$ at 24 h ($P < 0.001$; Table 5). 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

253 and thus [MCHb] mirrored inversely the change in MCV (Table 2).

254

255 3.6 Severe hypoxia with hypercapnia

256 In response to 10% O₂ with 5% CO₂, viscosity increased significantly after 60 min (2.07 ± 0.07
257 mPa•s) and remained elevated throughout further exposure, plateauing at 90 min (2.35 ± 0.13 mPa•s) and
258 120 min (2.27 ± 0.09 mPa•s) ($P < 0.001$; Fig. 1C, filled circle). Viscosity remained elevated during the first
259 10 min of recovery (2.17 ± 0.06 mPa•s) and subsequently began to decrease, returning to control values
260 after 30 min.

261 Hct also increased significantly from control after 60 min (33.9 ± 0.9 %) and remained high
262 throughout further exposure, plateauing at 90 min (36.4 ± 1.1 %) and 120 min (36.2 ± 0.9 %) ($P < 0.001$; Fig.
263 2C, filled circle). During recovery in air, Hct remained high during the first 10 min (34.2 ± 0.7 %) and
264 subsequently began to decrease (33.4 ± 1.4 % at 30 min) and by 60 min had returned to control levels.

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

266 MCV increased significantly at 30 min (150 ± 2 μm^3) and remained elevated throughout exposure,
267 peaking at 120 min (167 ± 2 μm^3). During recovery, MCV began to decrease within 30 min (155 ± 3
268 μm^3) and by 60 min had returned to control levels ($P < 0.001$) (Table 3).

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

272

273 3.7 Severe hypoxia without hypercapnia

274 In response to 10% O₂, control blood viscosity increased significantly after 30 min to 1.88 ± 0.04
275 mPa•s and remained elevated throughout further exposure, peaking at 120 min (2.14 ± 0.08 mPa•s)
276 ($P < 0.001$; Fig. 1C, open circle). Viscosity remained elevated during the first 30 min (1.92 ± 0.07 mPa•s)
277 of recovery and subsequently decreased, returning to control levels after 60 min.

278 Hct also increased significantly after 30 min (32.2 ± 0.6 %) and remained elevated throughout

279 further hypoxic exposure, peaking at 120 min (35.1 ± 0.9 %) ($P < 0.001$; Fig. 2C, open circle). During
280 recovery in air, Hct remained elevated during the first 30 min (32.0 ± 1.1 %) and subsequently decreased,
281 returning to control levels at 60 min.

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

283 MCV increased significantly during hypoxic exposure from control at 10 min (142 ± 2 μm^3) and
284 further increased with time to a peak at 120 min (168 ± 3 μm^3 ; $P < 0.001$). MCV decreased significantly
285 from the peak value within 30 min of recovery (157 ± 3 μm^3) and by 60 min had returned to control levels
286 (Table 3).

287 As with [RBC], [Hb] remained unchanged during exposure to 10% O₂ without 5% CO₂ and during
288 recovery in air ($P = 0.051$). MCH remained unchanged ($P = 0.538$) and thus [MCHb] mirrored inversely
289 the change in MCV ($P < 0.001$; Table 3).

290

291 3.8 O₂ transport capacity

292 OTC curves were generated using values of [Hb] at control and 60 min of submergence, ~ 9 and 9.8
293 g%, respectively, and Hct-viscosity relationship, were calculated in d15 embryos using Eqns. 1 and 2
294 (Fig. 3). Optima were predicted to occur at an Hct of $\sim 27\%$ in both cases, though only control Hct was
295 observed at this value *in vivo*. Exposure to severe hypoxia, with or without 5% CO₂, and water
296 submersion increased Hct 31-36% (ΔHct) and viscosity 40-47% ($\Delta\text{viscosity}$) over control values. These
297 adjustments moved Hct away from the optimum Hct for O₂ delivery in chicken embryos. As a result, *in*
298 *vivo* OTC values, rather than complete curves, decreased significantly by 22-25%% at 60 min for all
299 three treatments and 28% at 120 min of both hypoxia exposures (Fig. 4A, 4C). When extrinsic hypoxia
300 was moderate (e.g., 15%), with or without CO₂, OTC decreased 15 and 13%, respectively (Fig. 4B).

301

302 4. Discussion

303

304 Changes in blood viscosity during O₂ and CO₂ 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 stimuli (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 CO₂ 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 ~ 110 μL , assuming an averaged MCV during submersion/exposure of ~ 150 μm^3 . RBC release did not occur during severe hypoxia exposure, but did during recovery from moderate hypoxia. This suggests that RBC release was not related to enhancement of O₂ transport, but might be related to duration of hypoxia and, partially, severity of hypoxia. That is, 24 h 15% hypoxia exposure caused an increase in [RBC] after exposure, but 2 h 10% hypoxia treatment 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 as a potential reservoir of

331 non-circulating RBCs may be an interesting avenue for future investigations (Andrewartha et al., 2014).
332 Further exploration of the sites for RBC sequestration and release in embryonic birds is highly
333 warranted.

334 In contrast to submergence and moderate hypoxia, severe extrinsic hypoxia, with or without CO₂,
335 increased Hct and viscosity through an increase in MCV only (Figs. 1C and 2C; Table 3). These changes
336 in MCV to extrinsic severe hypoxia were consistent with previous findings for Day 15 embryos (Tazawa
337 et al., 2012). Viscosity changes were related to those in MCV only and independent of [RBC], which
338 remained unchanged even during severe hypoxia with hypercapnia, unlike submersion, which produced
339 progressive intrinsic hypercapnic hypoxia.

340 Although the maximal value of Hct in response to moderate hypoxia was lower than that in
341 response to severe hypoxia and the response took 24 h versus 1 – 2 h in severe hypoxia exposure,
342 viscosity similarly changed in parallel with changes in Hct in moderate hypoxia exposures (Figs. 1B and
343 1C). Consequently, the changes in viscosity occur in proportion to changes in Hct irrespective of the
344 cause and duration of hypoxia.

345 .

346 4.2 Blood viscosity and relative oxygen transport capacity in embryonic chickens

347 Hct and blood viscosity are positively related at a constant temperature. The relationship is
348 exponential when determined *in vitro* in vertebrate blood with red cells (Al-Roubaie et al., 2011; Trevan,
349 1918), and is effectively linear over the range observed in this study on d15 chicken embryos (Kohl et al.,
350 2012). Blood viscosity accounts for the majority of resistance to flow in the cardiovascular system of
351 amphibians, with the parallel arrangement of the gas exchange and systemic circulations minimizing the
352 resistance associated with the vessels themselves (Kohl et al., 2013). Embryonic and extra-embryonic
353 circulations resemble, on a basic level, the parallel arrangement found in amphibians. Therefore
354 viscosity may also be the dominant contributor to resistance to blood flow in the embryonic chicken
355 (Tazawa and Takenaka, 1985; Tazawa and Johansen, 1987), although this has not been tested. Our
356 results support our hypothesis that increased viscosity outweighs the increased blood O₂ carrying

357 capacity accompanying intrinsic and extrinsic hypoxia, likely adding an additional challenge to the
358 cardiovascular system during these periods of stress. However, it is possible that metabolic depression, a
359 common phenomenon in vertebrates exposed to hypoxia, reduces O₂ demand to a point where the greater
360 transport costs of more viscous blood have been neutralized. D16 chicken embryos exposed to 11 and
361 17% O₂ for 40 minutes reduced their metabolism and incurred no subsequent O₂ debt (Mortola and
362 Besterman, 2007). Our protocols employed substantially longer exposure times (60 min, 120 min, and 24
363 hr in partial submersion, 10% O₂, and 15% O₂, respectively) that led to metabolic acidosis, suggesting
364 metabolic depression was not sufficient to maintain aerobic metabolism during the extended exposures
365 (Burggren et al., 2012; Tazawa et al., 2012; Mueller et al., 2013).

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

379 The method of hypoxia induction resulted in clear differences in the mode of Hct change, via
380 changes in [RBC] and/or MCV, and the severity of accompanying viscosity changes. Since blood
381 viscosity changes proportionally with changes in Hct during submersion and hypoxia, equivalent
382 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, 4, and 5). 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 favoring 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.

401

402 **5. Summary**

403

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.

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 504

505 **Figure legends**

506 Fig. 1. Time course of blood viscosity in d15 embryos that were (A) partially submerged, (B) exposed to
507 15% O₂ with 5% CO₂ (filled circle) and without CO₂ (open circle), and (C) exposed to 10% O₂ with 5%
508 CO₂ (filled circle) and without CO₂ (open circle). Exposure time indicated by gray background and
509 recovery is white. Significant differences from control are indicated with a cross (10 and 15% O₂ with
510 5% CO₂) and asterisk (10 and 15% O₂). Sample size of each mean value with S.E.M. (bar) is shown in
511 Tables 1-3.

512

513 Fig. 2. Time course of hematocrit in d15 embryos that were (A) partially submerged, (B) exposed to
514 15% O₂ with 5% CO₂ (filled circle) and without CO₂ (open circle), and (C) exposed to 10% O₂ with 5%
515 CO₂ (filled circle) and without CO₂ (open circle). Exposure time indicated by gray background and
516 recovery is white. Significant differences from control are indicated with a cross (10 and 15% O₂ with
517 5% CO₂) and asterisk (10 and 15% O₂). Sample size of each mean value with S.E.M. (bar) is shown in
518 Tables 1-3.

519

520 Fig. 3. O₂ transport curves calculated using Eqn 1 in control (solid line) and after 60 min of submergence
521 (dashed line) for d15 embryos. Calculated with specific hematologic variables using Eqn 1 (Table 1).
522 Solid arrow indicates control Hct and the grey arrows indicate OTC at a Hct of 38% when only [RBC]
523 changed , the value achieved in Khorrami et al. (2008; see discussion) and at a Hct of 36%, achieved
524 with a combination of increased MCV and [RBC], after 60 min of partial submersion. Blood viscosity is
525 represented by η .

526

527 Fig. 4. Time course of O₂ transport capacity in d15 embryos that were (A) partially submerged, (B)
528 exposed to 15% O₂ with 5% CO₂ (filled circle) and without CO₂ (open circle), and (C) exposed to 10%
529 O₂ with 5% CO₂ (filled circle) and without CO₂ (open circle). Exposure time indicated by gray
530 background and recovery is white. Significant differences from control are indicated with a cross (10 and

531 15% O₂ with 5% CO₂) and asterisk (10 and 15% O₂). OTC and S.E.M. (bar) calculated from values in
532 Tables 1-3. Blood viscosity is represented by η .

533 Figure 1.

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535

538 Figure 3.

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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 ($\pm$ 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)
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

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 μ L ⁻¹)	MCV (μ m ³)	[Hb] (g%)	MCH (pg)	[MCHb] (g%)	Viscosity (mPa•s)
With 5% CO ₂								
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}
Without CO ₂								
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}

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 μ L ⁻¹)	MCV (μ m ³)	[Hb] (g%)	MCH (pg)	[MCHb] (g%)	Viscosity (mPa•s)
With 5% CO ₂								
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}
Without CO ₂								
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