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# 'Blood-doping' effects on hematocrit regulation and oxygen consumption in late-stage chicken embryos (*Gallus gallus*)

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## SUMMARY

**The extent to which hematocrit (Hct) is regulated and the impact of altered Hct on blood oxygen transport in avian embryos are largely unknown. Consequently, we investigated how acute blood removal or Ringer solution injection modified Hct in day 15 embryos, and how 'blood doping' with erythrocyte-enriched whole blood influenced $O_2$ consumption in day 15–17 chicken embryos. Mean Hct (±s.e.m.) at day 15, 16 and 17 was 26.7±0.6%, 28.0±0.4% and 30.7±0.5%, respectively. Blood withdrawal (19 increments of 125 μl each, separated by 30 min) caused a progressive fall in Hct to ~12% at day 15. Hct decline was strictly proportional to the extent of blood withdrawal. Incremental Ringer solution injection over an 8 h period, transiently increasing blood volume up to 85% over initial values, did not decrease Hct, indicating that injected Ringer solution rapidly left the circulating blood compartment. Blood doping with erythrocyte-enriched whole blood artificially elevated Hct from 27% to 38%, but caused no significant change in routine $O_2$ consumption (0.35–0.39 ml $O_2$ $min^{-1}$ $egg^{-1}$) at any point over the subsequent 6 h period in day 15–17 embryos. We conclude that Hct is not protected acutely in day 15 chicken embryos, with no evidence of erythrocyte sequestration or release. Additionally, at day 15–17, Hct increases of ~10% do not enhance embryonic oxygen consumption, suggesting that blood oxygen carrying capacity *per se* is not limiting to oxygen consumption.**

Key words: hematocrit, oxygen consumption, blood volume, development, embryo.

## INTRODUCTION

Gas exchange in bird eggs occurs across the porous shell and underlying chorioallantoic membrane, and is generally regarded as having a large diffusion limitation (Wangensteen and Rahn, 1970/71; Wangensteen et al., 1970/71; Wangensteen et al., 1974; Erasmus and Rahn, 1976; Ar et al., 1980; Ar et al., 1991; Paganelli, 1980; Tazawa et al., 1981; Rahn and Paganelli, 1982; Wakayama and Tazawa, 1988; Tazawa and Whittow, 2000; Wagner-Amos and Seymour, 2002; Wagner-Amos and Seymour, 2003). Yet, even in the heavily diffusion-limited respiratory systems of bird eggs, effective gas exchange ultimately depends upon all components of the 'oxygen cascade', including blood perfusion and the blood's ability to transport $O_2$ to the embryo's metabolically active tissues. Blood $O_2$ transporting capacity, in turn, depends upon the number of red blood cells and the concentration and character of the avian embryonic hemoglobins.

The fundamental role of the red blood cell in oxygen transport in all but the youngest avian embryos [where direct diffusion suffices – see Burggren (Burggren, 2005)] has led to several investigations of erythropoiesis (see Dieterlen-Lievre, 1997; Dragon and Baumann, 2003; Maina, 2004). Changes in hematocrit (Hct) and hemoglobin concentration ([Hb]; and thus changes in blood $O_2$ transporting capacity) are inducible well before hatching in bird embryos. Hypoxia (either through high altitude or experimental ambient hypoxia) is a potent erythropoietic stimulus in chicken embryos, though the response is not universal to all birds (see Monge and Leon-Velarde, 1991; Dragon and Baumann, 2003; Baumann and Dragon, 2005; Chan and Burggren, 2005). Presumably, the enhanced $O_2$ carrying capacity associated with stimulated erythropoiesis assists the chicken embryo in maintaining normal levels of blood $O_2$ transport. In adult vertebrates, at least, limitations in blood $O_2$-carrying capacity can generally be partially or fully compensated for by increases in cardiac output or tissue oxygen extraction (for a review, see Calbet et al., 2006). Avian embryos exhibit relatively complex chemoreceptor reflexes by at least the last third of embryonic development (see Burggren and Crossley, 2002; Crossley et al., 2003a; Crossley et al., 2003b; Khandoker et al., 2003), and arterial hypoxia may additionally stimulate an increased cardiac output and redistribution of blood flow within the tissues. Either increased cardiac output or, in the longer term, enhanced erythropoiesis raising Hct – or certainly both in concert – could ameliorate the negative effects of hypoxia and maintain tissue oxygenation.

To investigate the ability of the late chicken embryo to regulate Hct, we have investigated whether experimental modifications in Hct through hemorrhage and Ringer solution infusion are compensated for acutely in day 15–17 chicken embryos. To investigate the specific role of Hct in the normal functioning of the oxygen cascade from environment to tissues, we increased Hct through red blood cell infusions [blood 'doping' or 'boosting' (e.g. Ekblom, 2000; Schumacher and Ashenden, 2004)] along with concurrent $O_2$ consumption measurements to understand how alterations in blood carrying capacity affect oxygen consumption.

## MATERIALS AND METHODS

### Egg source and incubation

Eggs of fertilized White Leghorn chickens (*Gallus gallus* L.) were shipped from Texas A&M University (College Station, TX, USA) to the University of North Texas (Denton, TX, USA), where they were incubated in commercial incubators at 38°C and a relative humidity of 60%. Eggs were turned automatically every hour. A total of 287 eggs were used in this study. Egg mass ranged from 51.07 to 77.33 g (mean ± 1 s.e.m., 58.7±0.37 g).

### Venous cannulation for Hct manipulation

For withdrawal or infusion of blood or Ringer solution, a vein in the chorioallantoic membrane (CAM) was cannulated with a method adapted from that of Tazawa et al. (Tazawa et al., 1980). Briefly, each egg was candled to find the largest CAM vein at the egg's blunt end. The egg was then half-buried in a sand bath set at 38°C to maintain egg temperature throughout surgery. A piece of eggshell (~4 mm in diameter) above the selected vein was removed. The inner eggshell membrane was carefully removed to reveal the underlying vein, which was then non-occlusively cannulated in a downstream direction. The cannula comprised a 30 gauge needle, bent at 90° approximately 2 mm from the tip, which was glued into 100 mm of PE 10 tubing, which in turn was glued into 100 mm of PE 50 tubing. Prior to its insertion, the cannula was filled with heparinized (100 units $ml^{-1}$) Ringer solution (commercial lactate Ringer USP; 130 mequiv $l^{-1}$ $Na^+$, 4 mequiv $l^{-1}$ $K^+$, 110 mequiv $l^{-1}$ $Cl^-$, 28 mequiv $l^{-1}$ lactate and 3 mequiv $l^{-1}$ $Ca^{2+}$; osmolality ~275 mosmol $kg^{-1}$ $H_2O$). The cannula was secured in place in the vessel with cyanoacrylate glue. The open end of the cannula was then closed with a small stainless steel pin. The egg was returned to the incubator (at 38°C) immediately after cannula implantation.

### Hct determination

Hct was determined on 25 μl of undiluted blood drawn into a Hamilton syringe through the implanted cannula. Blood volume in day 15 embryos is ~2.5 ml (see Tazawa and Whittow, 2000), so the volume of this blood sample represents ~1% of blood volume in day 15–17 chicken embryos. Sampled blood was transferred to a capillary tube, which was then sealed and centrifuged for 5 min in a mircocentrifuge (ACCU-STAT MP Readacrit; Pittsburgh, PA, USA) before Hct was determined.

### Protocols for Hct manipulation

In the first series of experiments on day 15 embryos, controlled blood withdrawal was used to reduce Hct. Every 30 min, 125 μl of blood (~5% of control estimated blood volume) was withdrawn from the CAM vein cannula into a Hamilton syringe. In the second set of experiments on different day 15 embryos, hypervolemia was induced in an attempt to reduce Hct. Every 30 min, 150 μl of heparinized Ringer solution was injected into the CAM vein. Then, 10 min after each Ringer solution injection, 25 μl of blood was withdrawn for Hct determination, resulting in an acute net blood volume increase of 125 μl for each such injection cycle.

### Artificial erythrocythemia

Artificial erythrocythemia (blood doping) was used to acutely increase Hct. Within 1 h prior to experiments, approximately 1.5 ml of blood was collected by chorioallantoic venipuncture into a heparinized syringe from each of 15, 2–3 day donor embryos, which were subsequently killed. Collected blood was then pooled. Preliminary experiments revealed no obvious agglutination or similar reactions within the pooled blood sample. Immediately after pooling, donor blood was centrifuged to separate erythrocytes from plasma. Approximately 700 μl of plasma was removed from this pooled sample, and the erythrocytes were then re-suspended in the remaining plasma. The reconstituted blood sample was then stirred in a vortex mixer for 20 s to ensure complete mixing. This procedure yielded whole blood with a Hct of approximately 50–65%, which was approximately 20–30% higher than in controls. The blood sample was visually observed for color change induced by aeration during re-suspension, to ensure the blood could still be re-oxygenated. This sample of high Hct blood was then used immediately in blood doping experiments.

The cannula inserted into a CAM vein served as the site for injection of 400 μl of the high Hct blood sample into a recipient embryo. Injection of donor blood into a recipient embryo was always well tolerated, with no obvious *in vivo* clotting or impairment of the microcirculation, even after multiple injections over several hours. Blood doping resulted in a net, acute blood volume elevation of 300 μl (400 μl injection with 100 μl withdrawal for Hct determination), which represents an increase of ~12–14% for day 15–17 embryos.

Embryos were then sampled for Hct and subjected to oxygen consumption measurements, as described below.

### Oxygen consumption measurements

Routine oxygen consumption was measured on individuals within sealed, flow-through respirometers (volume, 296 ml). Air warmed to 38°C flowed at 70 ml $min^{-1}$ through a port into the bottom of the respirometer and out of a port at its top, ensuring continual replenishment of the gas in the respirometer. The gas stream exiting the chamber passed initially through soda lime (to remove $CO_2$) and then through Drierite (to remove $H_2O$) before entering an eight-channel oxygen analyzer (model FC-1B, Sable Systems Inc., Las Vegas, NV, USA). A second minor stream of gas tapped off the inflow stream to the respirometer was scrubbed for $CO_2$ and water vapor and also sent to the analyzer for analysis of the inflow $O_2$ level. Gas flow through the respirometer was controlled with a Sable Systems gas analyzer sub-sampler (version 2.0), and was adjusted so that the $O_2$ differential between in-flowing and out-flowing gas was ~0.4–0.6% throughout the experiment. Prior to beginning the oxygen consumption ($\dot{V}_{O_2}$) measurements, each respirometer containing an egg to be measured was completely submerged for a minimum of 30 min in a water bath (Fisher ISOTEMP 1028P, Pittsburgh, PA, USA) thermostatically held at 38°C to ensure thermal equilibrium.

$\dot{V}_{O_2}$ of each egg was calculated by Sable Systems data analysis software after appropriate entry of variables. Three separate respirometers were run concurrently, with duplicate measurements made for each egg. All $\dot{V}_{O_2}$ values were calculated on a per egg basis.

The protocol for the $\dot{V}_{O_2}$ measurement was started by placing a completely intact, non-cannulated egg into a ventilated respirometer and letting it thermally equilibrate for 30 min, after which a baseline (pre-cannulation) level of $\dot{V}_{O_2}$ was determined. The egg was then removed from the respirometer and a CAM vein cannulated as described above. The embryo was allowed to recover in an incubator (38°C) for 1 h following cannulation before being returned to its respirometer for the remainder of the experiment. After a second 30 min period in the respirometer following cannulation, another $\dot{V}_{O_2}$ measurement was taken, immediately followed by the first Hct determinations. The embryo was then blood doped, as described above, and the attendant increase in Hct documented. $\dot{V}_{O_2}$ was determined every 30 min over a course of 6 h after blood doping, followed by a third and final Hct determination.

### Statistical analysis

All $\dot{V}_{O_2}$ and Hct data for each stage were tested for normality and equality of variances. Hct data for blood volume change was non-parametric, resulting in the use of a Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks to determine statistical significance. Significance between different groups was tested for using Dunn's method. A one-way ANOVA was utilized to determine significance between control Hct data at each stage, followed by a Holm–Sidak pairwise multiple comparison test. Hct values determined during the experimental procedure described above were tested for significance with either Student's paired *t*-test or a Mann–Whitney ranked-sums test, depending on normality. $\dot{V}_{O_2}$ data were analyzed using a Kruskal–Wallis ANOVA on ranks to determine statistical differences between each treatment group, followed by a two-way repeated measures ANOVA to determine treatment and stage effects. SigmaStat version 3.0 (Systat Software, Inc., San Jose, CA, USA) was used to conduct all statistical analyses. All statistical decisions were made using a 0.05 level of significance. All averages are presented as means ± 1 s.e.m.

## Results

### Hct and normal development

Hct at day 15, 16 and 17 was 26.7±0.6% ($N$=89), 28.0±0.4% ($N$=71) and 30.7±0.5% ($N$=75), respectively (Fig. 1). The increase with development over this 2 day period was highly significant (ANOVA, $P$<0.01). Embryos at day 15 and 16 and embryos at day 16 and 17 were not significantly different, but Hct at day 17 was significantly higher than that at day 15 (Holm–Sidak method, $P$=0.006). The variation in Hct was quite large in all three examined stages, with a range of ~20% in Hct evident on days 15 and 16, and of ~15% on day 17.

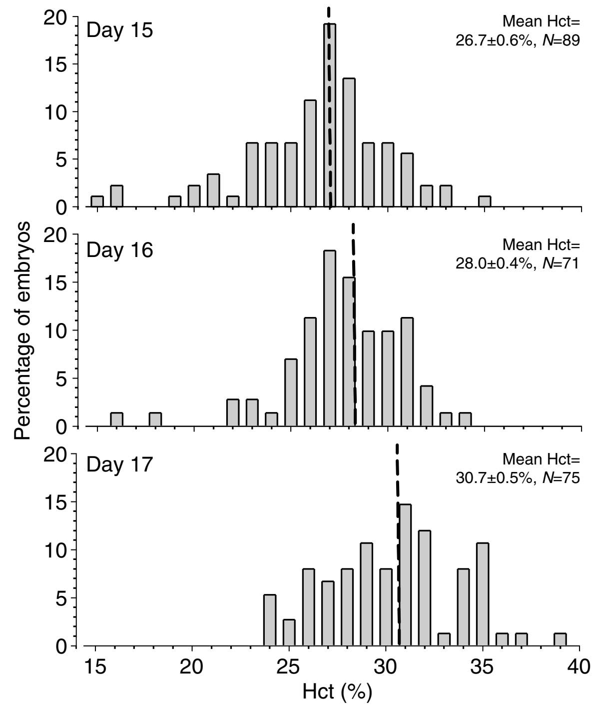


Fig. 1. Frequency distribution and mean values (±1 s.e.m.) of hematocrit (Hct) at various stages. Dashed line represents median Hct for each stage.

### Blood volume change and Hct

Acute blood removal (5% of initial volume every 30 min) in day 15 embryos ($N$=10) resulted in a significant (Kruskal–Wallis, $P$<0.001) and progressive decrease in Hct (Fig. 2A). The decrease in Hct became significant upon a blood volume loss of ⩾40% (Dunn's method, $P$<0.05).

A second group of day 15 embryos ($N$=5) was injected with 150 μl Ringer solution (equivalent to ~5% increase in blood volume) every 30 min for approximately 6–12 h (Fig. 2B). Despite repeated injections of Ringer solution that in some cases totaled up to more than double the estimated initial total embryo blood volume, there was no significant change in Hct from control measurements (Kruskal–Wallis, $P$>0.1).

### Artificial erythrocythemia

Embryos at day 15, 16 and 17 all showed a significant 10–15% increase in Hct immediately following injection of erythrocyte-enriched blood (Kruskal–Wallis one-way ANOVA on ranks, $P$<0.001; Fig. 3A). This erythrocythemia persisted for at least 6 h following injection in all three populations. In day 15 and 17 embryos, there was no significant change in Hct during the 6 h post-injection period (one-way repeated measures ANOVA). In day 16 embryos, however, there was a significant but small decrease in Hct back towards control values (one-way repeated measures ANOVA, $P$<0.05).

### $\dot{V}_{O_2}$ and Hct level

Values of routine $\dot{V}_{O_2}$ measured in day 15–17 embryos before and after cannulation are presented in Table 1, which also provides egg mass and $N$ values for data presented in Fig. 3B. Pre-cannulation $\dot{V}_{O_2}$ values in day 15–17 embryos ranged from 0.35 to 0.38 ml $O_2$ min$^{-1}$ egg$^{-1}$, and were not significantly different between developmental days ($P$>0.05). Vein cannulation had no significant effect on $\dot{V}_{O_2}$ of embryos at day 15 and 17 (Table 1). In day 16 embryos, $\dot{V}_{O_2}$ decreased significantly (Student's paired *t*-test, $P$=0.01) but only slightly to 0.31±0.01 ml $O_2$ min$^{-1}$ egg$^{-1}$.

Large, artificially induced increases in Hct through blood doping caused no significant difference in $\dot{V}_{O_2}$ at any point over the 6 h post-injection period in the three populations (Kruskal–Wallis, $P$⩾0.985, 0.328 and 0.946; Fig. 3B). There were also no significant interactions between stage and treatment ($P$>0.05 two-way ANOVA on ranks).

## DISCUSSION

### Regulation of Hct in chicken embryos

Regulation of Hct in adult vertebrates is important in maintaining blood $O_2$ transport homeostasis, and involves numerous factors. Chronically, Hct is impacted by the balance between the rate of erythropoiesis and the rate of removal of aging erythrocytes. In adults, the kidney functions as a 'critmeter', regulating Hct *via* erythropoietin secretion (for a review, see Donnelly, 2003). Acutely, rapid changes in Hct can result from fluid fluxes between the circulating blood volume and non-vascular compartments. Erythrocytes are also sequestered and released by the spleen. In many vertebrates, splenic contractions release stored erythrocytes, a catecholamine-mediated response especially prone during exercise, during decreases in blood volume, or upon exposure to toxins (Hughes et al., 1984; Jensen, 1987; Yamamoto, 1987; Ojiri et al., 2002; Stewart and McKenzie, 2002; Marques et al., 2006; Shah, 2006).

Normal circulating Hct appears overall to be relatively well regulated in the late chicken embryo, since even the normal rapid increase in Hct of >4% in just 2 days appears to be a consistent feature across studies (Fig. 4). Mean Hct values from these studies increased from 28.0±0.7% (day 15) to 30.6±1.0% (day 16) to 32.3±1.1% (day 17), with little variation between studies. However, as evident in our present study, at each developmental stage there

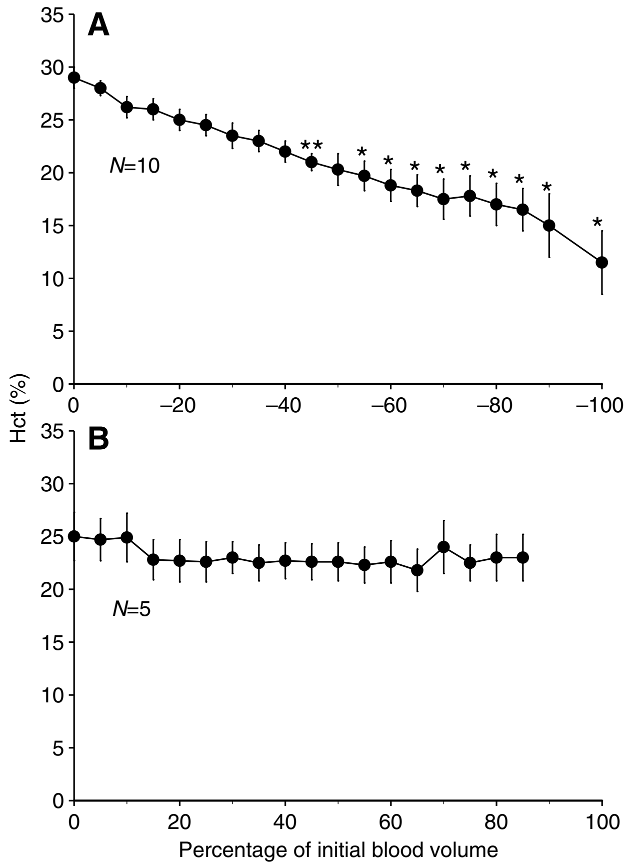


Fig. 2. Hct changes in response to graded Ringer solution addition or blood removal in day 15 embryos. (A) Effect of whole blood removal on Hct. Asterisks indicate values significantly different from control Hct. (B) Effect of repeated Ringer solution injection on Hct in day 15 embryos. Despite acute blood volume increases by up to 115%, Hct did not change significantly from control. Mean values ± 1 s.e.m.

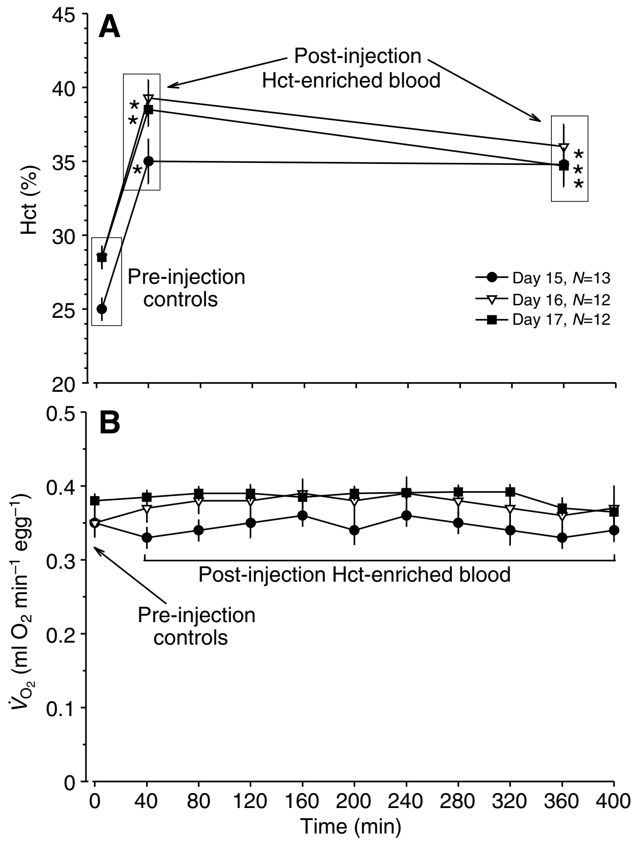


Fig. 3. Time course of the effect of injection of erythrocyte-enriched blood on Hct and $\dot{V}_{O_2}$ in chicken embryos. Injection occurred 5 min after measurement of pre-injection values. (A) Increases in Hct following injection of 400 µl of erythrocyte-enriched blood. Asterisks indicate values significantly different from control Hct at each day of development. (B) Effects of Hct elevation on $\dot{V}_{O_2}$. No post-injection measurements differed significantly from control at any developmental stage. Mean values ± 1 s.e.m.

are outliers with considerably higher or especially lower Hct (Fig. 1). Presumably, in late incubation the erythropoietic mechanisms evident in juvenile and adult birds (Luger, 2003) begin to assert themselves. Indeed, environmental hypoxia begins to trigger erythropoiesis between day 14 and 18 in chicken embryos (Tazawa et al., 1988; Camm et al., 2004). However, the reflex arcs that control embryonic erythropoiesis, and ultimately regulate Hct, remain enigmatic.

Erythrocyte sequestration and release as a mechanism for Hct regulation presumably occurs in adult birds as it does in mammals, but has received little attention in birds of any developmental stage. In the present study, day 15 chicken embryos experiencing graded blood removal were unable to maintain Hct at pre-intervention levels even transiently, with Hct falling progressively with each blood withdrawal (Fig. 2A). Tazawa (Tazawa, 1982) similarly observed a decline in Hct caused by four repetitive samplings in day 16 embryos. Based on these findings, day 15–16 chicken embryos apparently do not release sequestered erythrocytes, at least not in sufficient numbers to offset red blood cell loss from even the initial mild hemorrhage.

### Blood volume regulation in avian embryos

Regulation of blood volume in adults has been extensively studied in most vertebrate taxa (for a review, see Takei, 2000), and involves a highly integrated and very complex suite of mechanisms embodied in the Guyton model of mammalian circulation (Simanonok et al.,

Table 1. Routine oxygen consumption in control (pre-cannulation) and cannulated day 15–17 chicken embryos

| | | | $\dot{V}_{O_2}$ (ml $O_2$ min$^{-1}$ egg$^{-1}$) | |
|---|---|---|---|---|
| Developmental stage | *N* | Egg mass (g) | Control (pre-cannulation) | Post-cannulation |
| Day 15 | 13 | 57.55±2.18 | 0.35±0.02 | 0.35±0.02 |
| Day 16 | 12 | 59.40±1.12 | 0.35±0.01 | 0.31±0.01* |
| Day 17 | 12 | 59.51±1.95 | 0.38±0.02 | 0.38±0.02 |

Values are means ± s.e.m. *Significantly different from control (pre-cannulation) value.

1994). As is the case for Hct regulation, however, very little is known about the ontogeny of blood volume regulation generally, let alone the underlying mechanisms. In the present study, day 15 chicken embryos receiving repeated injections of Ringer solution showed no decrease in Hct, which would be evidence of lasting hemodilution, despite dramatic increases in blood plasma volume. Increased capillary permeability or blood pressure (or both) would facilitate rapid ultrafiltration of fluid out of the circulating blood compartment. In this scenario, blood volume actually would not increase, and Hct would be maintained at pre-injection levels. Interestingly, our observations are in contrast to earlier observations of hypervolemic hemodilution (i.e. decreased Hct) by Tazawa (Tazawa, 1982) in day 16 embryos.

Blood pressure associated with volume loading has been measured in very early chicken embryos (e.g. Wagman et al., 1990; Yoshigi et al., 1997), but not in more advanced embryonic stages closer to hatching. Whether blood volume increases actually increase blood pressure in avian embryos will depend in part on vascular compliance. Up until internal piping in the bird embryo, blood circulates through a very large chorioallantoic membrane lining the bird shell. The compliance of this membrane, how it changes during development, and whether this unique circulatory structure influences blood pressure and volume regulation requires additional experimentation. Altimiras and Crossley (Altimiras and Crossley, 2000) reported that baroreflex function in chicken embryos progressively matures beginning around day 18 of incubation, so the underpinnings of some form of physiological regulation of blood volume could be in place in day 15–17 embryos. Testing this hypothesis will require determination of blood pressure and direct measurement of blood volume during the course of graded blood removal or repeated Ringer solution injections in late-incubation chicken embryos.

To some extent, blood volume will be maintained by simple, passive mechanisms that drive water across capillaries in response to changing osmotic gradients. However, capillary permeability changes are also heavily implicated in blood volume regulation, and have received considerable attention in early chicken embryos (e.g. Cruz et al., 1997; Defouw and Defouw, 2001). Capillary permeability decreases during development in the chicken embryo, especially after day 10 (Ribatti et al., 1993). The physiological implications of these permeability changes at the system level in older embryos have yet be explored.

### Artificial erythrocythemia and $O_2$ consumption

Experimental adjustment of Hct (and thus of blood $O_2$ capacity) is a powerful way of manipulating potential systemic $O_2$ transport. Indeed, there is a general positive relationship between short-term blood $O_2$ capacity and $\dot{V}_{O_2}$ in a wide range of vertebrates (Tazawa et al., 1971; Hillman et al., 1985; Yahav et al., 1997; Tan and Lim, 2001; Gaudard et al., 2003), though the correlation is not inevitable (e.g. Wood et al., 1979; Cooper and Morris, 2004). In chicken embryos, total $\dot{V}_{O_2}$ is near its zenith by day 15–17 (see Tazawa and Whittow, 2000; Dzialowski et al., 2002), which would also argue for optimization of elements participating in $O_2$ transport between environment and tissues. However, in the present study, day 15–17 embryos showed no increase in routine $\dot{V}_{O_2}$ despite a 10–15% artificially induced increase in Hct. It is possible that Hct might have been influential in affecting oxygen consumption if the embryo was consuming oxygen at a far higher rate than that evident in our measurements. While typically there is a considerable difference between routine and maximal oxygen consumption in birds and other vertebrates, it is not clear to what

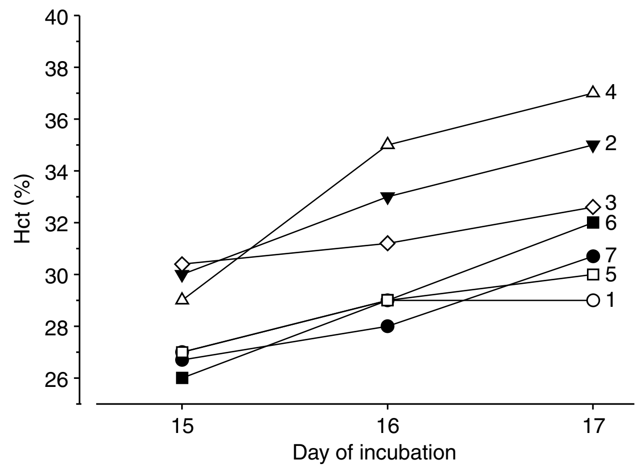


Fig. 4. Changes in Hct as a function of development in late-stage (day 15–17) chicken embryos. Data from seven studies, including the present one, are presented. 1 (Romanoff, 1967); 2 (Tazawa et al., 1971); 3 (Tazawa, 1971); 4 (Tazawa, 1972); 5 (Tazawa, 1980); 6 (Dziawolski et al., 2002); 7 present study.

extent routine oxygen consumption would increase in an embryo in its egg under normal conditions, given the limited opportunities for 'exercise' or even for being visually or mechanically stimulated.

That routine $\dot{V}_{O_2}$ was not increased by elevated Hct in these intermediate- to late-stage chicken embryos suggests that blood oxygen capacity, as a key element of the oxygen cascade from environment to tissues, is not a limiting factor. In this scenario, enhancement of blood oxygen capacity will not have nearly as large an effect as, for example, increasing oxygen diffusion across the shell and into the egg. The large diffusion barrier across the bird egg has been well documented (e.g. Pettit and Whittow, 1982; Rahn et al., 1987; Meir et al., 1999; Monge et al., 2000; Wagner-Amos and Seymour, 2002). Acute exposure to hyperoxia increases the $P_{O_2}$ gradient and subsequently increases $O_2$ diffusion across the egg shell. As a result, hyperoxic exposure also markedly increases $\dot{V}_{O_2}$ in day 16–18 chicken embryos (Tazawa et al., 1992). Collectively, these data suggest that oxygen diffusion into the shell may be a larger limiting factor in late embryonic gas exchange than blood $O_2$ carrying capacity, which could be circumvented by increases in tissue blood flow, for example.

No discussion of the effects of artificial erythrocythemia on oxygen consumption is complete without considering the blood viscosity effects accompanying increased Hct. Blood viscosity increases with increasing Hct in reptiles, birds and mammals (see Barshtein et al., 2007; Viscor et al., 2003). With increasing viscosity comes the specter of reduced blood flow, which could offset any potential advantage to blood $O_2$ transport associated with elevated Hct. Indeed, sharply elevated viscosity and the associated decrease in blood flow reduces the diffusive capacity of gas exchange organs (Piiper and Scheid, 1992). In fact, blood viscosity increase is one of the reasons why severe blood doping in human athletes is considered ineffective at best and dangerous at worst (Spivak, 2001). In the present study, increased Hct had no effect on routine $\dot{V}_{O_2}$, which theoretically could be explained by an increase in blood viscosity accompanying the acute experimentally induced increase in Hct. However, at least in humans, Hct generally needs to increase to values of greater than

~50% before negatively affecting tissue blood flow (see Lowe et al., 2002; El-Sayed et al., 2005). In our experiments the maximum induced Hct was ~40% (Fig. 3A), which suggests that viscosity effects were probably not limiting blood flow.

## CONCLUSIONS

Although day-specific values of mean Hct in chicken embryos show very similar increases in late development, there are outliers that deviate by as much as 15–20% from the mean for all 3 days examined (Fig. 1). If $\dot{V}_{O_2}$ was tightly linked to blood oxygen capacity, these variations in Hct could well have negative implications for individual embryos during the last critical stages of development. Yet, the clear independence of routine $\dot{V}_{O_2}$ from large variations in Hct in days 15–17 (Fig. 3) indicates that either (1) late embryonic routine $\dot{V}_{O_2}$ is not oxygen limited despite large variations in blood oxygen capacity, or (2) adjustments in tissue perfusion may compensate for disturbances to blood oxygen carrying capacity. Future experiments will test these ideas.

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