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Hematocrit and blood osmolality in developing chicken embryos (*Gallus gallus*): *In vivo* and *in vitro* regulation

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ABSTRACT

Hematocrit (Hct) regulation is a complex process involving potentially many factors. How such regulation develops in vertebrate embryos is still poorly understood. Thus, we investigated the role of blood pH in the regulation of Hct across developmental time in chicken embryos. We hypothesized that blood pH alterations *in vitro* (i.e., in a test tube) would affect Hct far more than *in vivo* because of *in vivo* compensatory regulatory processes for Hct. Large changes in Hct (through mean corpuscular volume (MCV)) and blood osmolality (Osm) occur when the blood was exposed to varying ambient temperatures (T_a's) and P_{CO_2} *in vitro* alongside an experimentally induced blood pH change from ~7.3 to 8.2. However, homeostatic regulatory mechanisms apparently limited these alterations *in vivo*. Changes in blood pH *in vitro* were accompanied by hydration or dehydration of red blood cells depending on embryonic age, resulting in changes in Hct that also were specific to developmental stage, due likely to initial blood gas and $[HCO_3^-]_v$ values. Significant linear relationships between Hct and pH ($Hct/\Delta pH = -21.4\%/(pH\ unit)$), Hct and $[HCO_3^-]$ ($\Delta Hct/\Delta[HCO_3^-] = 1.6\%/(mEq\ L^{-1})$) and the mean buffer value ($\Delta[HCO_3^-]/\Delta pH = -13.4\ (mEq\ L^{-1})/(pH\ unit)$) demonstrate that both pH and $[HCO_3^-]$ likely play a role in the regulation of Hct through MCV at least *in vitro*. Low T_a (24 °C) resulted in relatively large changes in pH with small changes in Hct and Osm *in vitro* with increased T_a (42 °C) conversely resulting in larger changes in both Hct and Osm. *In vivo* exposure to altered T_a caused age-dependent changes in Hct, demonstrating a trend towards increased Hct at higher T_a. Further, exposing embryos to a gas mixture where $P_{CO_2} = 5.1$ kPa for >4 h period at T_a of 37 or 42 °C also did not elicit a change in Hct or Osm. Presumably, homeostatic mechanisms ensured that *in vivo* Hct was stable during a 4–6 h temperature and/or hypercapnic stress. Thus, although blood pH decreases (induced by acute T_a increase and exposure to CO_2) increase MCV and, consequently, Hct *in vitro*, homeostatic mechanisms operating *in vivo* are adequate to ensure that such environmental perturbations have little effect *in vivo*.

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1. Introduction

Hematocrit (Hct) is a key factor influencing blood rheology and blood distribution within the various vascular beds of the circulation. Thus, regulation of Hct is of critical importance in the ultimate effectiveness of the perfusion and oxygenation of tissues. How Hct changes in response to *in vivo* blood chemistry perturbations is currently poorly understood in adult vertebrates. Further, how Hct regulation first appears and matures in the embryo, particularly during ontogeny when other interrelated systems (e.g., blood acid–base, metabolic, respiratory) are still developing, is largely unknown.

Hct is a product of mean corpuscular volume (MCV) and red blood cell concentration ([RBC]). Changes in blood pH and $[HCO_3^-]$ can alter MCV (and hence, Hct) by influencing Donnan equilibrium mechanisms and Na^+, K^+-ATPase activity (see Hoffmann et al., 2009; Nikinmaa, 1992 for review). In the ectothermic bird embryo, blood pH can be altered indirectly via changes in ambient temperature (T_a) or P_{CO_2} (Tazawa, 1973, 1982; Tazawa et al., 1981; Tazawa and Ono, 1974; Wangensteen et al., 1970/1971; Wangensteen and Weibel, 1982). With decreased blood pH (respiratory acidosis) Hct (measured at 24 h) increased in day 17 (d17) chicken embryos through increased MCV rather than increased erythropoiesis, demonstrating that embryonic Hct potentially responds to changes in P_{CO_2} (and thus pH) through changes in MCV (Tazawa et al., 1988). Day 19 chicken embryos additionally increase erythropoiesis concomitant with respiratory acidosis and deficiency of oxygen (Tazawa et al., 1988) suggesting that the response of Hct to altered P_{CO_2} (pH) in chicken embryos differs according to developmental stage, an observation driving the current investigation.

Accordingly, we hypothesize that *in vivo* Hct is regulated acutely by altered T_a and P_{CO_2} (and consequently pH) due to changes in MCV. Additionally, we hypothesize that the response of Hct to altered T_a may be age-dependent and associated with changes in

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blood osmotic pressure or osmolality (Osm). This study is designed to elucidate both the *in vivo* and *in vitro* effects and underlying mechanisms of altered P_{CO_2} and T_a on pH, Osm, and Hct of blood collected from developing chicken embryos. Homeostatic mechanisms *in vivo* should tightly regulate MCV so that changes in Hct are minimal as RBC's are circulated around the body, whereas outside the body the RBC's are directly susceptible to alterations in pH. It is thus expected that inducing pH changes via CO_2 exposure *in vitro* will have a greater effect on Hct than *in vivo*.

2. Materials and methods

2.1. Incubation of eggs and blood collection

Fertile eggs of the domestic fowl (*Gallus gallus domesticus*, layer strain) were obtained from Texas A & M University (College Station, TX, USA). Eggs were weighed (±0.01 g), then incubated at a temperature of 37.5 ± 0.1 °C and relative humidity of ~55% (Incubator: 1502, G.Q.F. Manuf. Co., USA). Individual embryos were removed from the incubator on d11 through 18, and their hematological variables measured as described below. On the target developmental day (one of d11, 13, 15, 17, 18), blood was collected from the allantoic artery, which had been located by candling one day previously. A 6–8 mm diameter region of the eggshell was removed and the underlying allantoic artery gently lifted by forceps through the hole in the eggshell. The blood vessel was punctured for sampling by a 25 gauge needle mounted on a 1 mL sampling syringe that had been flushed with heparinized saline (100 mg in 100 mL saline). A mean volume of approximately 0.27, 0.47 and 0.70 mL of blood could be collected from d11, d13 and d15–18 embryos, respectively. Blood from two d11 embryos and in some cases two d13 embryos was pooled to meet the required volume of at least 0.5 mL of blood necessary for two blood analyses with intervening tonometry. Sampled eggs were then euthanized via exposure to a cold and anoxic environment for 2 h and the embryos were removed from their eggshell, following confirmation of cardiac arrest. The yolk and extra-embryonic membranes were then removed, and the embryo's body mass measured (±0.01 g) with an electronic balance. When blood from two individuals was pooled, the egg and body masses of the same embryos were similarly averaged and treated as a single datapoint.

2.2. In vitro effects of altered P_{CO_2} and T_a on Hct

Each hermetically sealed syringe containing sampled blood was inverted several times to ensure sample mixing. Then 0.12 mL of blood was immediately measured for pH, P_{CO_2} and $[HCO_3^-]$ (calculated by the analyzer from pH and P_{CO_2}) with a blood gas analyzer (ABL5, Radiometer Medical A/S) at 37 °C. Duplicate preparations of 0.06 mL of blood were transferred into sealed hematocrit tubes and centrifuged for 4 min at 10,000 rpm and the mean % Hct determined (±0.1%, READACRIT Centrifuge, Becton Dickinson). The pH, P_{CO_2}, $[HCO_3^-]$ and Hct determined at 37 °C immediately after blood collection from the allantoic artery were identified as "venous values" of individual parameters; i.e., pH_v, Pv_{CO_2}, $[HCO_3^-]_v$ and Hct_v (Note that allantoic arterial values represent mixed venous values, since an allantoic artery is analogous to an artery in the pulmonary arterial circulation) (Piiper et al., 1980).

After ensuring the blood sample was well mixed, the remainder of each sample was then transferred into a tonometer consisting of a glass, concave-ended vial (5.5 cm height, 2.8 cm diameter) with inlet and outlet conduits to allow gas mixtures to pass through the sample. The tonometer was sealed with a rubber lid, and placed on the lab bench at ~24 °C until the blood of 5–6 embryos had been collected and the initial Hct and blood gas variables determined, a process requiring ~1 h. The blood in 5–6 separate tonometers was then equilibrated with one of the following gas mixtures: air (P_{CO_2} = 0.7 kPa), P_{CO_2} = 2.9 kPa or P_{CO_2} = 5.1 kPa (with 20.6 kPa O_2, N_2 balance) at 24 °C, 37 °C or 42 °C. For equilibration with P_{CO_2} = 2.9 and 5.1 kPa gas mixtures (supplied by pre-mixed gas cylinders; Air Liquide), tonometers were vented with the gas mixtures for 5 min in the waterbath. They were then sealed to avoid water evaporation from the blood and the samples were shaken within the tonometers ensuring that a thin layer of blood coated the walls allowing for equilibration for the remaining 85 min. Preliminary data collected using blood samples from embryos on d11, 13, 15, 17 and 18 of incubation (N = 10–12 for each group) demonstrated that changes in Hct occurred after 30 min to 1 h of equilibration and reached plateau after 1 h at each measured T_a (24 °C, 37 °C and 42 °C), thus, values are reported after an equilibration time of 1.5 h. After 1.5 h, the tonometer was removed from the waterbath and the blood immediately assessed for pH, P_{CO_2} and $[HCO_3^-]$ at 37 °C; referred to as pH_{eq}, $P_{CO_2(eq)}$ and $[HCO_3^-]_{eq}$ and Hct_{eq} (again measured in duplicate and the mean determined). Further, values were expressed as ΔpH ($= pH_{eq} - pH_v$) and $\Delta[HCO_3^-]$ ($= [HCO_3^-]_{eq} - [HCO_3^-]_v$). In presenting changes in Hct (ΔHct), the *in vitro* change from *in vivo* arterial value ($Hct_{eq} - Hct_v$) was normalized by individual Hct_v and expressed by percent change, $\Delta Hct = 100 \times (Hct_{eq} - Hct_v)/Hct_v$), to emphasize even small changes in Hct. The absolute changes in Hct can be obtained by multiplying the fractional Hct_v value. The blood equilibrated at 24 °C or 42 °C was corrected for pH determined at 37 °C by the factor of −0.016 pH unit/°C (Dejours, 1981; Howell et al., 1970); i.e., +0.21 for 24 °C blood and −0.08 for 42 °C blood. Blood samples from 12 embryos were measured at each T_a (24 °C, 37 °C and 42 °C), at gas mixture (P_{CO_2} = 0.7, 2.9 and 5.1 kPa) and developmental stage (d11, 13, 15, 17 and 18). This protocol resulted in 9 different treatment groups on 5 development days with a total of 540 embryos.

Osm was measured for the blood samples of an additional 148 embryos in total on d13, 15 and 17 of incubation. Collected blood was measured for blood gas variables and equilibrated with one of three gas mixtures (P_{CO_2} = 0.7, 2.9 and 5.1 kPa) at one of three T_a's (24 °C, 37 °C and 42 °C) with the tonometer. Following subsequent determination of blood gas variables and Hct, Osm was determined on 0.01 mL of blood using a vapor pressure osmometer (Vapro 5520, Wescor).

2.3. In vivo responses of Hct to altered T_a and P_{CO_2}

The *in vivo* responses of Hct to altered T_a were examined in d11, 13, 15, 17 and 18 embryos. Preliminary experiments indicated that egg temperature equilibrated with T_a after approximately 3–5 h and the protocol was designed ensuring that embryo body temperature had reached steady state. Thus, embryos were exposed to the experimental conditions for at least 4 h with no exposure time exceeding 6 h. On the experimental day, embryos were divided into 4 treatment groups:

- group 1: control embryos incubated continuously at T_a of 37.5 °C,
- group 2: embryos exposed to T_a of 24 °C for >4 h,
- group 3: embryos exposed to T_a of 42 °C for >4 h, and
- group 4: embryos exposed to T_a of 42 °C for 4-h with subsequent exposure to 37.5 °C for another >4 h.

Eggs in group 2 were transferred from a 37.5 °C-incubator to room temperature of ~24 °C. Eggs in groups 3 and 4 were placed into an incubator at 42 °C and half of them (group 4 eggs) were returned to the 37.5 °C-incubator (after 4 h of 42 °C exposure) for a further >4 h. After exposure to altered or control T_a, approximately 0.2 mL of blood was collected and Hct determined as outlined for

the *in vitro* protocol. Eleven to 36 eggs in each group were examined for each day of development (453 embryos in total). In supplementary experiments, blood Osm and pH were determined in addition to Hct for 16 embryos subjected to group 1 (exposure to 37.5 °C), group 2 (24 °C) or group 3 (42 °C) protocols on d13, 15 and 17 of incubation (144 embryos in total).

In vivo responses of Hct to increased ambient CO_2 (P_{CO_2} = 5.1 kPa) were examined in additional d13, 15 and 17 embryos (83 embryos in total). On the target day, eggs were placed into an airtight 3.78 L (26.8 cm × 27.9 cm) vinyl bag in a 37.5 °C-incubator (after location of the allantoic artery on the previous day). Inlet and outlet conduits allowed a gas mixture (P_{CO_2} = 5.1 kPa) to be continuously introduced into the bag at a flow of ~600 mL min^{-1} for >4 h. The egg was quickly wrapped in foil to minimize environmental change during blood sampling; blood was then collected and measured for blood gas variables, Osm and Hct. Additionally, 12 d17 embryos were placed into a 42 °C-incubator and exposed to P_{CO_2} = 5.1 kPa for > 4 h and then blood was collected and measured for blood gas variables, Osm and Hct.

2.4. Statistical analyses

All Hct data for each incubation day and *in vivo* response data were tested for normality and equal variance. Differences between group means were examined by one way ANOVA with Tukey's post hoc tests. Differences of means between two groups were examined by paired or un-paired Student's *t*-tests with significance assumed at $P < 0.05$.

3. Results

3.1. Blood gas variables and Hct in developing embryos

Allantoic arterial blood was collected from a total of 540 embryos on d11, 13, 15, 17 and 18 of incubation and measured immediately for blood gas variables (pH, P_{CO_2} and $[HCO_3^-]$ at 37 °C) and Hct. As anticipated from previous studies, Pv_{CO_2} and $[HCO_3^-]_v$ increased with embryonic development (Table 1). These increases were significantly correlated with daily increases in Hct_v i.e., $Hct_v = 11.52 + 0.44\,Pv_{CO_2}$ ($r = 0.839$, $t = 15.896$, $P < 0.001$, $N = 540$) and $Hct_v = 8.61 + 0.72 \cdot [HCO_3^-]_v$ ($r = 0.841$, $t = 16.030$, $P < 0.001$, $N = 540$).

Table 1
Fresh egg and embryo body mass, blood gas variables (pHv, Pv_{CO_2} $[HCO_3^-]_v$), and hematocrit (Hct_v) of chicken embryos used for *in vitro* equilibration experiment.

Incubation day	11	13	15	17	18
Egg mass (g)	56.45 ±0.24	56.50 ±0.33	56.90 ±0.33	56.12 ±0.33	56.74 ±0.34
Body mass (g)	3.10 ±0.03	6.24 ±0.06	12.28 ±0.12	18.87 ±0.18	22.09 ±0.18
pH_v[a]	7.53 ±0.01	7.47 ±0.01	7.47 ±0.01	7.46 ±0.01	7.46 ±0.01
Pv_{CO_2} (kPa)[a]	2.7 ±0.1	4.0 ±0.1	5.1 ±0.1	5.7 ±0.1	5.8 ±0.1
$[HCO_3^-]_v$ (mEq L^{-1})[a]	16.6 ±0.2	21.3 ±0.2	27.3 ±0.3	31.8 ±0.4	31.1 ±0.3
Hct_v (%)	19.2 ±0.2	23.5 ±0.2	28.3 ±0.2	31.8 ±0.3	32.4 ±0.3

Values are mean ± 1 S.E.M. $N = 108$ for each developmental day and thus $N = 540$ in total.

[a] pH_v, Pv_{CO_2} and $[HCO_3^-]_v$ values were measured at 37 °C immediately after blood collection from an allantoic artery.

3.2. In vitro effects of altered P_{CO_2} and T_a on Hct

3.2.1. In vitro response of Hct to altered P_{CO_2} and T_a

In vitro equilibration to varying T_a's and CO_2 tensions produced different changes in equilibrated Hct (Hct_{eq}) when compared with the initial *in vivo* arterial Hct values (Hct_v). Comparison was made by percent changes in Hct, i.e., $\Delta Hct = 100 \times (Hct_{eq} - Hct_v)/Hct_v$. Importantly, these changes between equilibrated and arterial Hct's were quite different at different stages of development (Fig. 1). In response to equilibration of the blood to air (which contains 0.3% CO_2 or P_{CO_2} = 0.7 kPa; open boxes), Hct_{eq} remained unchanged (d11 and 13 at 37 °C and 42 °C) or decreased (d11 and 13 at 24 °C, d15–18 at all T_a's), with the decrease becoming larger at later developmental stages. Equilibration of blood to P_{CO_2} = 2.9 kPa (grey boxes in Fig. 1), at T_a = 24 °C resulted in an unchanged Hct_{eq} in d11 and 13 embryos and a slight decrease in Hct_{eq} of advanced embryos. Conversely, at T_a = 37 °C (P_{CO_2} = 2.9 kPa), Hct_{eq} increased maximally in d11 embryos and the increase in Hct_{eq} diminished with development until ΔHct approached 0 in d17 and 18 embryos. At T_a = 42 °C (P_{CO_2} = 2.9 kPa), the increase in Hct_{eq} in d11–15 embryos was further enhanced and the Hct_{eq} of d17 and 18 also increased from Hct_v values. The response of Hct at a blood P_{CO_2} = 5.1 kPa (black boxes) followed a similar (and slightly augmented) pattern to a P_{CO_2} = 2.9 kPa (Fig. 1).

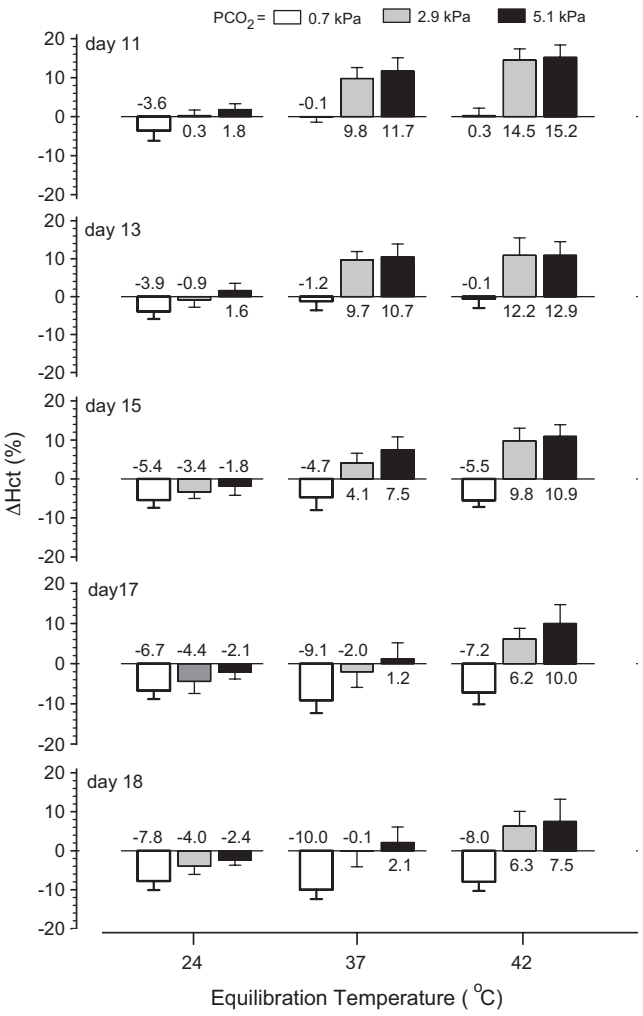

Fig. 1. *In vitro* change in hematocrit from *in vivo* arterial values ($\Delta Hct = 100 \times (Hct_{eq} - Hct_v)/Hct_v$) at different ambient temperatures and P_{CO_2}s in blood of developing chicken embryos. Mean values ± 1 S.E.M. are plotted. 12 embryos for each gas partial pressure at each T_a from each developmental age; i.e., 540 embryos in total.

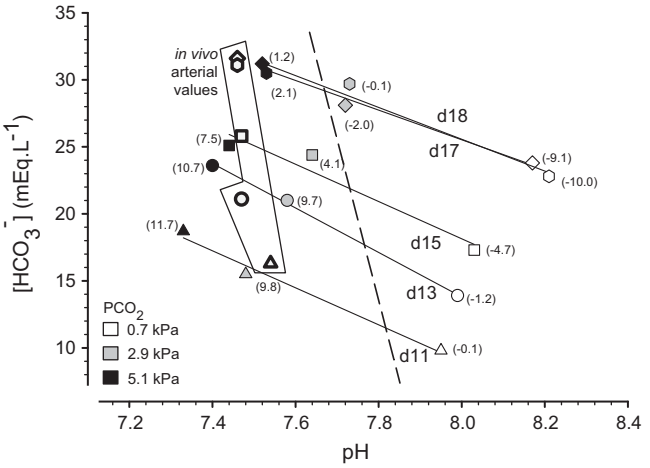

Fig. 2. Davenport diagram representing *in vitro* equilibration of blood (pH_{eq} and $[HCO_3^-]_{eq}$) from day 11 through 18 chicken embryos to $P_{CO_2} = 0.7$, 2.9 and 5.1 kPa at 37 °C. The values in the parentheses next to individual symbols indicate ΔHct (%) shown in Fig. 1. The broken line divides the values into an increase in ΔHct (on left) and a decrease in ΔHct (on right). 12 embryos for each gas partial pressure from each developmental age (i.e., 180 in total). Error bars are too small to visualize for each mean value. The solid lines are the *in vitro* blood buffer lines of embryos at individual ages, with the following equation values: d11: $[HCO_3^-]_{eq} = 120 - 13.8 \times pH_{eq}$; d13: $[HCO_3^-]_{eq} = 147 - 16.4 \times pH_{eq}$; d15: $[HCO_3^-]_{eq} = 129 - 13.9 \times pH_{eq}$; d17: $[HCO_3^-]_{eq} = 114 - 11.1 \times pH_{eq}$; d18: $[HCO_3^-]_{eq} = 121 - 11.9 \times pH_{eq}$. All $r^2 > 0.94$ and $P < 0.05$.

In vitro equilibration of the blood to altered P_{CO_2} consequently affected blood pH_{eq} and $[HCO_3^-]_{eq}$ in a highly stage-specific manner largely due to differences in blood $[HCO_3^-]$ between the age groups (Fig. 2). Equilibration at 37 °C to normoxic gas with a $P_{CO_2} = 0.7$ kPa resulted in an increase in pH_{eq} from 7.95 pH units (d11) to 8.21 units (d18). There was also an increase in $[HCO_3^-]_{eq}$ from 10 up to 25 mEq L^{-1} across the same developmental range. Concurrently, ΔHct decreased from −0.1% in d11 to −10.0% in d18 embryos. Equilibration of the blood to $P_{CO_2} = 2.9$ kPa at 37 °C resulted in pH_{eq} increasing from 7.48 in d11 to 7.73 in d18 embryos, with $[HCO_3^-]_{eq}$ increasing from 16 mEq L^{-1} to 30 mEq L^{-1} across the same age span (Fig. 2). Concurrently, ΔHct decreased from 9.8% in d11 to −0.1% in d18 embryos (Figs. 1 and 2). Equilibration of the blood to $P_{CO_2} = 5.1$ kPa at 37 °C resulted in a pH_{eq} increase from 7.33 in d11 embryos to 7.53 in d18 embryos, accompanied by $[HCO_3^-]_{eq}$ increases from 19 mEq L^{-1} to 31 mEq L^{-1} for the same age span (Fig. 2). Concurrently, a large increase in Hct_{eq} was observed in d11 and 13 embryos but no significant change in Hct_{eq} in d17 and 18 embryos (Figs. 1 and 2). In summary, large increases in Hct_{eq} occurred as pH_{eq} decreased in response to increased P_{CO_2} (= 2.9 and 5.1 kPa) in young embryos (d11–15) in which $[HCO_3^-]$ was low (Fig. 2). Conversely, Hct_{eq} was lower at higher pH_{eq} (in lower P_{CO_2} i.e., air) in advanced embryos that had a higher $[HCO_3^-]$.

3.2.2. Inter-relationships between changes in pH, Hct and $[HCO_3^-]$ in vitro

In blood of d11 and 13 embryos (where Hct_{eq} increased in response to high P_{CO_2}), pH_{eq} decreased and $[HCO_3^-]_{eq}$ increased relative to arterial values (Fig. 2), creating a negative ΔpH and a positive $\Delta[HCO_3^-]$ (Fig. 3A). At the other end of development, d17 and 18 embryo blood, which largely decreased Hct_{eq} in response to equilibration with normoxic gas with $P_{CO_2} = 0.7$ kPa, had the largest positive ΔpH with the largest negative $\Delta[HCO_3^-]$ (Fig. 3A). The changes in ΔpH and $\Delta[HCO_3^-]$ of d15 embryos were between these two extremes and there was a negative linear relationship between ΔpH and $\Delta[HCO_3^-]$ (Fig. 3A). Similarly, the largest increase in ΔHct and the largest negative ΔpH were observed in d11 and 13 embryo blood exposed to $P_{CO_2} = 5.1$ kPa (Fig. 3B),

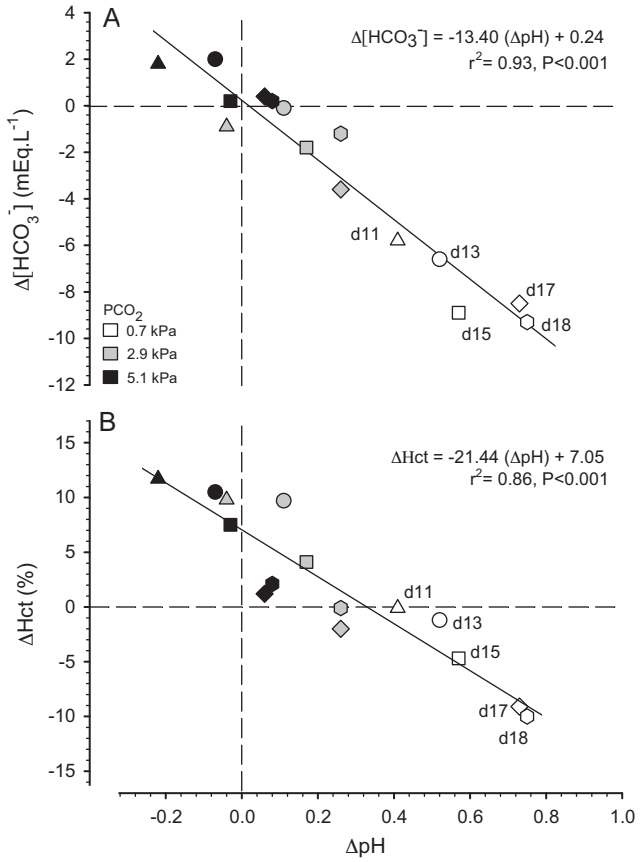

Fig. 3. The *in vitro* relationship between ΔpH ($pH_{eq} - pH_v$) and (A) $\Delta[HCO_3^-]$ and (B) ΔHct at 37 °C from day 11 through 18 embryos exposed to $P_{CO_2} = 0.7$, 2.9 and 5.1 kPa. 12 embryos for each P_{CO_2} from each developmental age; i.e., 180 in total. Error bars are too small to visualize for each mean value. The dashed lines parallel to the axes represent the origin.

while the largest decrease in ΔHct and largest positive ΔpH occurred in d17 and 18 embryo blood exposed to normoxia (Fig. 3B). Again, the changes in ΔHct and ΔpH of d15 embryos were between these two extremes and there was a negative linear relationship between ΔpH and ΔHct (Fig. 3B). Because $\Delta[HCO_3^-]$ was negatively related to ΔpH, ΔHct was linearly related to $\Delta[HCO_3^-]$; i.e., $\Delta Hct = 6.24 + 1.60 \cdot [HCO_3^-]$ ($r = 0.8756$, $t = 6.536$, $P < 0.001$, $N = 15$).

3.2.3. In vitro response of pH and Hct to altered T_a and P_{CO_2}

Changes in T_a affected pH_{eq}, particularly the pH_{eq} of blood exposed to air (Fig. 4). Equilibrating blood to lowered T_a (24 °C) in normoxic gas increased pH_{eq} and a consequent increase in ΔpH. Changes in Hct_{eq} were small however, resulting in small changes in ΔHct in spite of large changes in ΔpH. Conversely, small changes in pH_{eq} occurred when blood was equilibrated to increased T_a (42 °C) and relatively large changes in ΔHct occurred against the restricted changes in ΔpH. The changes that occurred at 37 °C were of a similar magnitude to those observed at 42 °C.

3.2.4. In vitro response of pH and Osm to altered T_a and P_{CO_2}

The mean *in vivo* Osm of blood collected from the allantoic artery (Osm_v) was 276 ± 1 mmol kg^{-1} ($N = 46$), 277 ± 1 mmol kg^{-1} (42) and 278 ± 1 mmol kg^{-1} (60) on d13, 15 and 17 of incubation, respectively. At a T_a of 37 °C, for instance, *in vitro* Osm was increased by equilibration with normoxia and $P_{CO_2} = 0.7$, 2.9 and 5.1 kPa gas mixtures to mean values of 292 ± 1 ($N = 12$), 339 ± 4 ($N = 12$) and 342 ± 3 mmol kg^{-1} ($N = 18$), respectively (Fig. 5). Further, Osm_{eq} demonstrated a negative linear relationship with pH_{eq}, which was

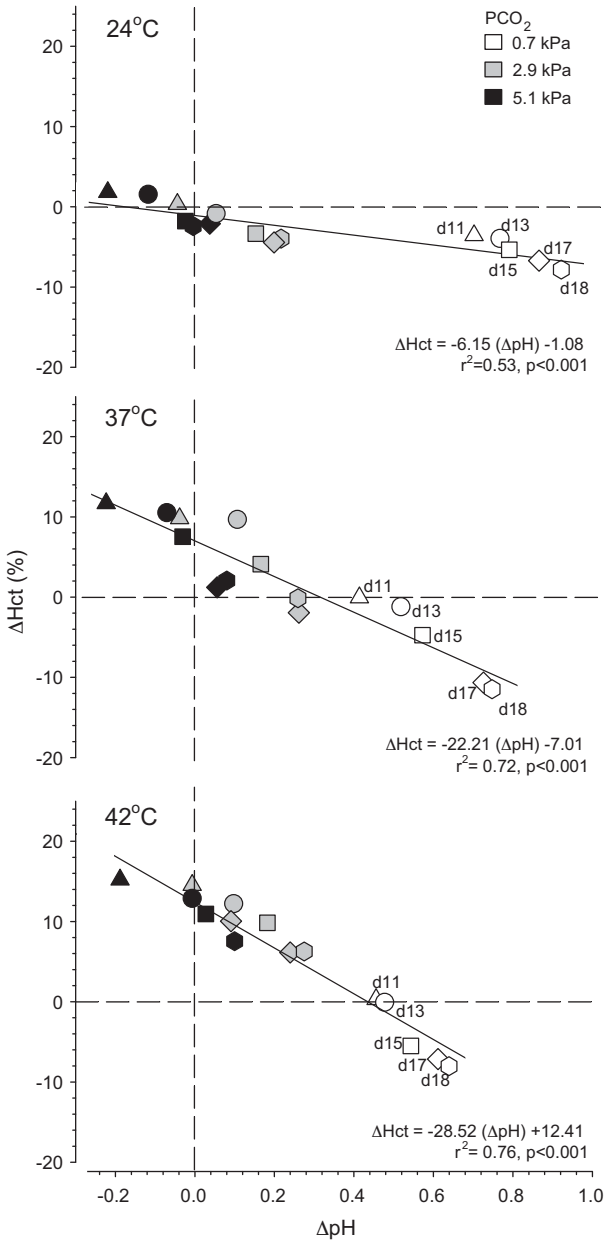

Fig. 4. *In vitro* relationship between ΔpH ($pH_{eq} - pH_v$) and ΔHct from day 11 through 18 embryos exposed to $P_{CO_2} = 0.7$, 2.9 and 5.1 kPa at 24 °C, 37 °C and 42 °C. 12 Embryos for each gas partial pressure at each developmental age (i.e., 180 embryos at each T_a). Error bars are too small to visualize for each mean value.

likely age specific although this is still to be confirmed due to limited sample sizes per age group in the present study. Increased P_{CO_2} caused an increase in ΔOsm (= $Osm_{eq} - Osm_v$) (Fig. 5) and resulted in the ΔHct changing from a decrease (with normoxic equilibration) to an increase (with increased CO_2 equilibration) (Fig. 6). Accordingly, ΔHct increased as ΔOsm increased.

The magnitude of changes in Osm in response to altered P_{CO_2} was correlated with T_a. At T_a of 24 °C, Osm_{eq} was 280 ± 2 mmol kg^{-1} ($N = 16$), 280 ± 2 mmol kg^{-1} ($N = 15$) and 281 ± 2 mmol kg^{-1} ($N = 15$) after equilibration with normoxic gas and $P_{CO_2} = 2.9$ and 5.1 kPa, respectively, resulting in little change in Osm from arterial values (small ΔOsm). Meanwhile, at T_a of 42 °C, large ΔOsm resulted in an increase in Osm_{eq} to 299 ± 2 mmol kg^{-1} ($N = 18$), 379 ± 5 mmol kg^{-1} ($N = 20$) and 391 ± 5 mmol kg^{-1} ($N = 22$) at the three P_{CO_2} levels. Consequently, higher T_a's resulted in large changes in ΔOsm occurring with large changes in ΔHct, whereas

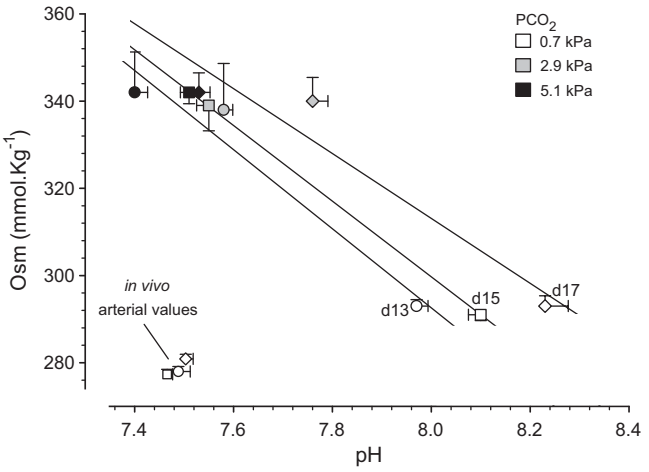

Fig. 5. Relationship between pH and osmolality in d13 ($N = 12$), d15 ($N = 12$) and d17 ($N = 18$) chicken embryo blood equilibrated to $P_{CO_2} = 0.7$, 2.9 and 5.1 kPa at 37 °C. Arterial values represent mean values measured immediately after drawing the samples from the artery. Error bars are ± 1 S.E.M. Solid lines indicate regressions for each developmental day: d13: $Osm_{eq} = 984 - 86 \times pH_{eq}$ ($r^2 = 0.6079$, $P < 0.01$); d15: $Osm_{eq} = 1006 - 88 \times pH_{eq}$ ($r^2 = 0.9234$, $P < 0.001$); and d17: $Osm_{eq} = 880 - 71 \times pH_{eq}$ ($r^2 = 0.7498$, $P < 0.001$).

both ΔOsm and ΔHct were fairly restricted at lower temperature (Fig. 6).

3.3. *In vivo responses of Hct to altered T_a and P_{CO_2}*

3.3.1. *In vivo response of Hct to altered T_a*

Developmental age of the embryo was an important determinant of how Hct responded to altered T_a *in vivo* (Fig. 7). On d11 of incubation, Hct was not statistically different between the four T_a exposure groups, although the mean value of group 3 (exposed to 42 °C) tended to be higher than the other groups. By d13, this trend had become significant and the Hct of embryos exposed to 42 °C was higher than the other groups. Elevated Hct produced by exposure to 42 °C remains even after d15 embryos were returned to a 37.5 °C environment. However, by d17 and 18 Hct in groups 3 and 4 had returned to values not significantly different from control embryos. Instead, Hct of d17 and 18 embryos exposed to 24 °C was significantly decreased compared with control embryos.

3.3.2. *In vivo response of pH and Osm to altered P_{CO_2} and T_a*

The pH_v and Osm_v together with Hct were also measured for a separate group of embryos exposed to T_a of 24 °C, 37.5 °C and 42 °C on d13, 15 and 17 of incubation (Table 2). Overall pH_v was highly T_a dependent with a $\Delta pH/\Delta T$ of −0.023, −0.017 and −0.020 in d13, 15 and 17 embryos, respectively (Table 2). Hct was increased by exposure to T_a of 42 °C in d13 and 15 embryos and decreased at T_a of 24 °C in d17 embryos (i.e., the same changes shown for different embryos in Fig. 7). No changes in Osm_v occurred, and across the T_a range mean Osm_v averaged 275 ± 1 mmol kg^{-1} ($N = 48$), 277 ± 1 mmol kg^{-1} (48) and 278 ± 1 mmol kg^{-1} (48) in d13, 15 and 17 embryos respectively and 276 ± 1 mmol kg^{-1} ($N = 144$) for all embryos.

The effect of > 4 h exposure of incubated eggs to increased ambient P_{CO_2} (= 5.1 kPa) on blood gas variables, Osm_v and Hct_v were determined for d13, 15 17 embryos at T_a of 37.5 °C (Table 3). Pv_{CO_2} and $[HCO_3^-]_v$ increased from control values and consequently pH_v decreased. However, Osm_v and Hct_v were not different from control values at any age (Table 3). Additionally, exposing d17 embryos to an increase in T_a (= 42 °C and $P_{CO_2} = 5.1$ kPa) did not elicit a change in Osm_v and Hct_v values (Table 3).

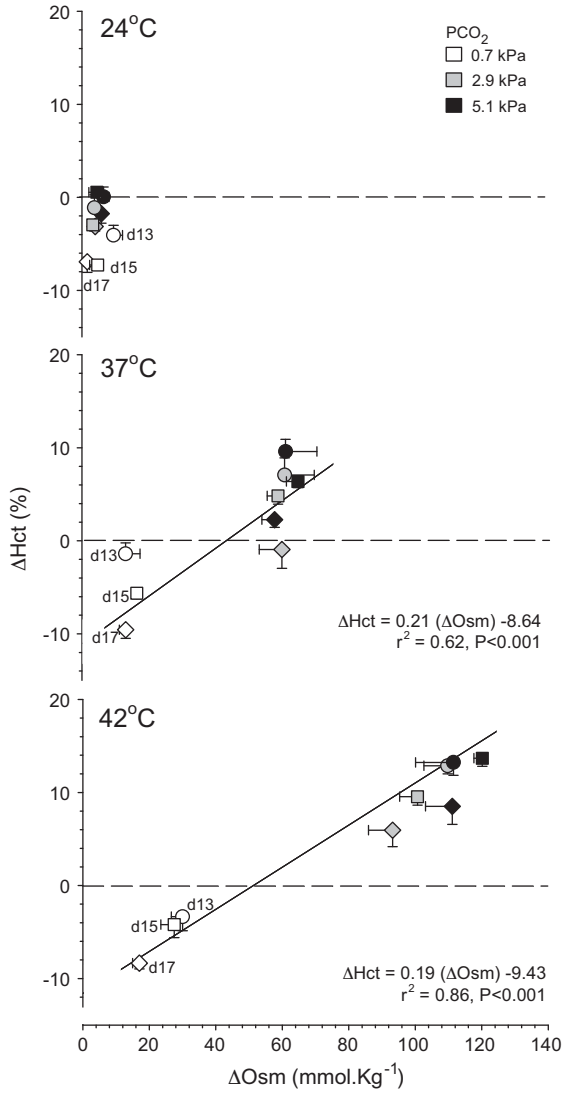

Fig. 6. The relationship between ΔOsm and ΔHct during *in vitro* equilibration of blood (tonometry) from d13 (N = 46), 15 (42), and 17 (60) embryos at 24 °C, 37 °C and 42 °C. Error bars are ± 1 S.E.M. Dashed lines indicate the origin.

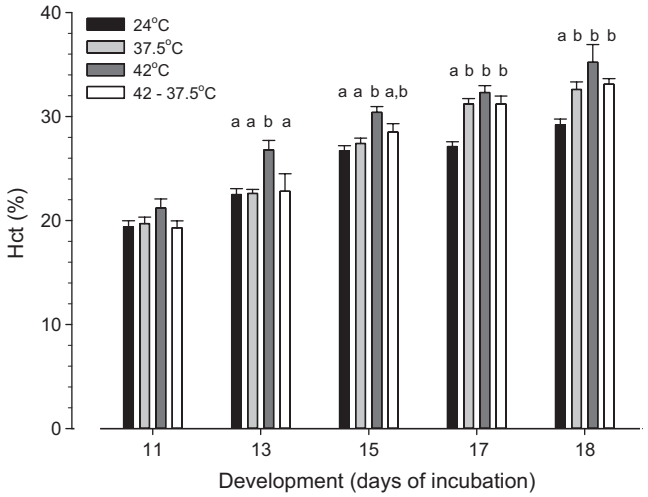

Fig. 7. Hct after *in vivo* exposure to >4 h ambient temperature in d11 through 18 chicken embryos. 42–37.5 °C refers to embryos exposed to 42 °C for 4 h then 37.5 °C for 4 h. Error bars are +1 S.E.M. Different lowercase letter pairs represent significantly different values at a developmental age. N = 64, 99, 104, 132 and 54 for d11, 13, 15, 17 and 18, respectively (total N = 453).

Table 2
Embryo body mass, hematocrit (Hct_v), and osmolality (Osm_v) of allantoic arterial blood of chicken embryos acutely exposed to different ambient temperatures (T_a) in supplementary *in vivo* experiment determining blood pH and Osm.

T_a	24 °C	37.5 °C	42 °C
Day 13			
Body mass (g)	6.54 ± 0.17[a]	6.86 ± 0.19[a,b]	7.34 ± 0.14[b]
Hct_v (%)	23.5 ± 0.7[a]	23.9 ± 0.6[a]	26.8 ± 0.5[b]
pH_v	7.81 ± 0.02[a]	7.49 ± 0.01[b]	7.41 ± 0.02[c]
Osm_v (mmol kg^{-1})	274 ± 1	276 ± 1	276 ± 2
Day 15			
Body mass (g)	12.28 ± 0.30[a]	12.70 ± 0.22[a,b]	13.43 ± 0.23[b]
Hct_v (%)	25.8 ± 0.6[a]	27.5 ± 0.6[a]	30.0 ± 0.7[b]
pH_v	7.71 ± 0.03[a]	7.46 ± 0.02[b]	7.41 ± 0.02[b]
Osm_v (mmol kg^{-1})	275 ± 2	276 ± 3	279 ± 2
Day 17			
Body mass (g)	17.96 ± 0.44[a]	18.50 ± 0.41[a,b]	19.76 ± 0.47[b]
Hct_v (%)	26.3 ± 0.7[a]	32.1 ± 1.1[b]	30.0 ± 0.8[b]
pH_v	7.75 ± 0.02[a]	7.49 ± 0.02[b]	7.40 ± 0.02[c]
Osm_v (mmol kg^{-1})	277 ± 2	276 ± 2	280 ± 2

Values are means ± 1 S.E.M. N = 16 for each group and thus N = 144 in total. Different superscripts for mean values on the same developmental day indicate a significant difference between the individual temperature group means. No significant differences in Osm were observed between any groups.

4. Discussion

We hypothesized that pH alterations *in vitro* would have a larger effect on Hct than *in vivo* because of the mitigating effects of regulatory processes involved in Hct homeostasis during environmental perturbations. Using temperature change and hypercapnia as independent experimental variables, we have determined that, indeed, Hct regulation is a process that appears and matures as the avian embryo develops, as will now be discussed.

Table 3
Blood gas variables (Pv_{CO_2}, pH_v and $[HCO_3^-]_v$), osmolality (Osm_v) and hematocrit (Hct_v) of embryos in control group and test groups examined for *in vivo* responses of Hct and Osm to increased ambient CO_2 (P_{CO_2} = 5.1 kPa) on days 13, 15 and 17 of incubation.

Days	T (°C)		N	Pv_{CO_2} (kPa)	pH_v	$[HCO_3^-]_v$ (mEq L^{-1})	Osm_v (mmol kg^{-1})	Hct_v (%)
13	37	Control	12	4.1 ± 0.1[a]	7.50 ± 0.01[a]	27.0 ± 3.1[a]	276 ± 1	23.8 ± 0.6
	37	P_{CO_2} = 5.1 kPa	11	7.2 ± 0.2[b]	7.41 ± 0.02[b]	33.2 ± 1.2[b]	279 ± 2	24.4 ± 0.4
15	37	Control	12	5.1 ± 0.3[a]	7.48 ± 0.02[a]	27.2 ± 1.4[a]	277 ± 3	27.1 ± 0.4
	37	P_{CO_2} = 5.1 kPa	12	8.3 ± 0.2[b]	7.41 ± 0.01[b]	38.5 ± 0.8[b]	272 ± 1	26.9 ± 0.9
17	37	Control	16	5.3 ± 0.2[a]	7.50 ± 0.02[a]	31.0 ± 1.4[a]	277 ± 2	32.2 ± 1.0
	37	P_{CO_2} = 5.1 kPa	8	9.2 ± 0.5[b]	7.35 ± 0.02[b]	37.4 ± 1.9[b]	275 ± 1	31.0 ± 1.9
	42	P_{CO_2} = 5.1 kPa	12	–	7.36 ± 0.02[b]	–	280 ± 1	29.9 ± 0.8

Values are means ± 1 S.E.M. N = 83 in total. Different superscripts for mean values on the same developmental day indicate a significant difference between the individual group means. No difference in Osm and Hct between control (P_{CO_2} = 0.7 kPa) and P_{CO_2} = 5.1 kPa was observed.

4.1. In vitro effects of altered P_{CO_2} and T_a on Hct

4.1.1. Basic blood gas variables and Hct in developing embryos–comparisons with previous literature

The basic variables described in Table 1 align well with the relatively few published data sets that have resulted from measurements of blood gas variables over a wide developmental range. Allantoic arterial blood has properties similar to mixed venous blood (Table 1), with at least 65% from venous origin being shunted into the arterial blood system through the interatrial foramina and ductus arteriosus (Olszowka et al., 1988; Tazawa, 1978; Tazawa and Johansen, 1987; Tazawa and Takenaka, 1985; White, 1974). Both Pv_{CO_2} and $[HCO_3^-]_v$ increased and reached a plateau as embryos developed. Importantly, these increases were statistically correlated with daily increases in Hct_v (Table 1). This pattern is similar to the previously described sigmoid increase in red blood cell concentration ([RBC]) and hemoglobin concentration ([Hb]) during development (e.g., Tazawa et al., 2011). The establishment of blood gas values can also be correlated with developing metabolic rate, which similarly increases in a sigmoid fashion, reaching a plateau before hatching (see Mortola, 2009 for review). Daily values of Pv_{CO_2} and pH_v on d11–18 of incubation were similar to previously reported values for chicken embryos (Tazawa et al., 1971a; Boutilier et al., 1977). However both variables were lower than reported by Freeman and Misson (1970) while pH_v was higher than previously reported by Tazawa et al. (1971a). The source of this variation, whether natural or measurement-based, remains to be identified.

The Hct of developing chicken embryos has been measured in many physiological studies (e.g., Barnes and Jensen, 1959; Bartels et al., 1966; Black and Burggren, 2004; Dzialowski et al., 2002; Erasmus et al., 1970/1971; Freeman and Misson, 1970; Jalavisto et al., 1965; Johnston, 1955; Khorrami et al., 2008; Macpherson and Deamer, 1964; Romanoff, 1967; Tazawa, 1971; Tazawa et al., 1971a, 1971b, 2011; Yosphe-Purer et al., 1953), although rarely as a primary target of interest. Hct values scatter widely amongst individuals on a specific incubation day, with a coefficient of variation (COV; calculated from frequency distributions) ranging from 9% (present study) to 11–14% (Khorrami et al., 2008). Nonetheless, Hct responded to altered environments in this study in a highly predictable fashion in all individuals (see below). Understanding how Hct is regulated in the face of short term environmental perturbations, and whether this regulatory system develops during ontogeny, is of great pertinence. Elevated Hct, through either increased MCV or [RBC], results in increased blood viscosity (and vice versa), potentially constraining blood flow transport at the capillary level and thus affecting tissue respiratory gas exchange. Monitoring Hct may provide insight into the processes of hemopoiesis and RBC sequestration (Khorrami et al., 2008). Further, the high correlation between [Hb] and Hct with constant mean corpuscular hemoglobin concentration in developing chicken embryos allows the calculation of [Hb] (which is more difficult to measure) from Hct (relatively simple to determine) (Tazawa et al., 1971a, 2011).

4.1.2. In vitro response of Hct to altered P_{CO_2} and T_a

There is a complex interaction between Hct and variables in acid–base analysis, and ontogeny (i.e., developmental age) further complicates its interpretation. In this study, *in vitro* changes in blood pH (through equilibration with altered P_{CO_2}) were accompanied by hydration or dehydration of RBC's depending upon embryonic age, resulting in changes in Hct that also were specific to developmental stage (Figs. 1 and 2). Increasing blood pH (by equilibrating with air; $P_{CO_2} = 0.7$ kPa) at T_a of 37 °C, did not change Hct in d11 and 13 embryo blood, and over development resulted in a decrease in ΔHct which reached a maximum of ~10% in d17 and 18 embryo blood (Figs. 1 and 2). This decrease in Hct was due to a decrease in MCV (since there was no change in [RBC] *in vitro*) as the RBC's in the older embryos became relatively dehydrated. Likely mechanistic candidates as driving forces for water movement (and changes in MCV) are plasma Osm and pH which may potentially alter the properties of ion channels in the RBC's. Although there appears to be some effect of developmental age on absolute values (albeit small sample size unfortunately constrains the statistical detection of differences), there was no difference in the magnitude of increase in Osm_{eq} in response to increased P_{CO_2} in d13, 15 and 17 embryos. This created a positive correlation between Osm and Hct (Fig. 6). Thus, Osm appears to be a potential driving force for changes in MCV, although this does not explain the differences between the ages as the initial Osm's were similar (Fig. 5).

The second likely mechanism is due to the direct effect of plasma pH. In RBCs that are either anucleated (human) or nucleated (fish; presumably similar to the nucleated chicken embryo), Cl^-, HCO_3^- and H^+ become passively distributed across the RBC membrane in Donnan-like equilibrium (Hemming et al., 1986; see Jensen, 2004 for review). In this scenario, increased plasma pH resulted in an increase in intracellular RBC pH, as HCO_3^- combined with H^+ forming CO_2 and water, which diffused down their respective partial pressure and concentration gradients and out of the RBC's. The loss of water from the RBC's then caused a decrease in cell volume, resulting in decreased Hct. Intracellular $[Cl^-]$ also decreased (in exchange for HCO_3^-) due to the chloride–bicarbonate exchange (Jensen, 2004; Nikinmaa, 1992). Conversely, decreasing blood pH (i.e., equilibration with either $P_{CO_2} = 2.9$ or 5.1 kPa) hydrated the RBCs. This in turn led to increased intracellular $[H^+]$ buffered by hemoglobin and organic phosphates in the RBC of d11–15 embryos. Although treating the erythrocyte as a Donnan system to understand transient ion and water movement into and out of RBC's is generally accepted in the comparative respiratory literature (e.g., Berenbrink et al., 2000; Reeves, 1976; see Hoffmann et al., 2009; Nikinmaa, 1992 for reviews), cell volume regulation is also under the influence of many other factors including the potassium-chloride co-transport, taurine transport and sodium-dependent beta-amino acid transport systems. These systems are modulated by a variety of mechanisms including neuronal, hormonal and autocrine stimulation and via changes in osmolality (Osm), P_{O_2}, P_{CO_2} and pH (see Cossins and Gibson, 1997; Lambert et al., 2008; Nikinmaa, 1992 for review). The relative contribution of these many mediators to MCV regulation during environmental perturbations is yet to be elucidated and beyond the scope of the present study.

Developmentally advanced embryos showed little increase in ΔHct (Figs. 1 and 2). When interpreting these results it is important to bear in mind that a P_{CO_2} of 2.9 kPa is normocapnic for d11 embryo blood, whereas a P_{CO_2} of 5.1 kPa is normocapnic for d15 embryo blood and still slightly hypocapnic for more advanced embryo blood (Table 1). These differences in baseline blood gases between the age groups may explain the different sensitivities to P_{CO_2} equilibration in the context of MCV, regulated largely through pH (Donnan equilibrium) mechanisms.

The mean value of $\Delta Hct/\Delta pH$ at 37 °C for developing embryos during the last half of prenatal incubation was −21.4%/(pH unit) (Fig. 3). A large dependency of ΔHct on pH is not surprising considering Hct is the product of MCV and [RBC], and [RBC] is fixed *in vitro*. The high negative correlation of ΔpH with $\Delta[HCO_3^-]$ ($\Delta[HCO_3^-]/\Delta pH = -13.4\ mEq\ L^{-1}\ pH\ unit^{-1}$; Fig. 2), resulted in a positive correlation between ΔHct and $\Delta[HCO_3^-]$ with $\Delta Hct/\Delta[HCO_3^-] = 1.6\%/(mEq\ L^{-1})$. Again, it is not surprising that $[HCO_3^-]$ and Hct are highly interrelated. Changes in $[H^+]$ associated with changes in P_{CO_2} are largely buffered by HCO_3^- and have consequent effects on MCV and hence Hct, particularly *in vitro* where [RBC] cannot change.

Changes in Hct (ΔHct) in response to ΔpH (induced by equilibration of blood to altered P_{CO_2}) were suppressed at lower T_a

(24 °C; ΔHct/ΔpH = −6.2%/pH unit) and augmented at increased T_a (42 °C; ΔHct/ΔpH = −28.5%/pH unit; Fig. 4). A relatively wider ΔpH range could be induced at lower T_a and this occurred with smaller changes in ΔHct. Thus, MCV is more sensitive to pH at higher temperatures. In a different set of embryos, small changes in Hct at 24 °C occurred concurrently with small changes in Osm, with both ΔHct and ΔOsm increasing with increasing T_a (Fig. 6), supporting the implication of osmotic forces in regulation of MCV *in vitro*. Thus, exposure of embryonic blood to higher P_{CO_2} (and consequently lower pH) results in water movement into the RBC's with a consequent plasma osmolality decrease. Potentially, the increased P_{CO_2} results in more CO_2 being hydrated to HCO_3^- by carbonic anhydrase. This reduces the amount of water in the RBC, resulting in water movement into the RBC and an increase in MCV. The tendency for larger Hct at higher T_a's and reduced Hct at lower T_a's *in vitro* is opposite to expected findings if Na^+,K^+ATP-ase was primarily responsible for MCV changes. Na^+,K^+ATP-ase activity is depressed at lower T_a's resulting in less Na^+ pumped out of the RBC in exchange for K^+ and an increase in intracellular Osm and consequent water influx and an increase in MCV (and hence Hct) (e.g., Canestari et al., 1994; see Hoffmann et al., 2009 for review).

4.2. *In vivo* responses of Hct to altered T_a and P_{CO_2}

Overall, Hct *in vivo* did not respond consistently to changes in temperature (and thus pH) across the developmental ages examined. There was a trend towards increased Hct at a higher T_a, but the T_a that induced differences in Hct was different across development and no changes in Hct were observed in the youngest embryos (d11). The RBC's of d17 and 18 embryos may have been dehydrated at the lower T_a of 24 °C as the Hct (and potentially the MCV) of these advanced embryos was significantly lower compared with control embryos exposed to 37 °C (Fig. 7 and Table 2). This decrease in Hct in d17 and 18 embryos at 24 °C may also have been caused by sequestration of RBC's by the spleen or other organ (though nothing of this capability is known in avian embryos). Increased T_a may potentially hydrate RBC's (due to decreased pH or changes in Osm as *in vitro*) at all ages examined. However, an increase in Hct occurred only in d13 and 15 embryos (Fig. 7). This increase in Hct in d13 and 15 embryos at 42 °C could potentially occur through erythropoiesis. Yet, the length of the time course (4–6 h) appears to make changes in MCV a more likely contender for both the decrease in Hct in older embryos at lowered T_a and the increase in Hct in younger embryos at higher T_a. Interestingly, these changes in Hct were not accompanied by changes in blood Osm (Table 2). A similar lack of correlation between changes in MCV and plasma Osm has been shown in humans in response to exercise training (e.g., Staübli and Roessler, 1986; Van Beaumont and Rochelle, 1974). Seemingly, Osm alone is not the primary driving force for MCV (and consequently Hct) regulation. It is likely that Osm is just one of many instigating forces for MCV regulation which include pH (see above for mechanism) and potentially cell properties under hormonal or nervous influence (see Hoffmann et al., 2009; Jensen, 2004 for reviews). The Na^+-K^+-cotransport system in avian erythrocytes, for example, can be activated either through changes in blood plasma Osm or by β-adrenergic drugs (Kregenow et al., 1976; Palfrey and Greengard, 1981; Uberschar and Bakker-Grunwald, 1983). Further, β-adrenergic stimulation has also been shown to affect MCV in nucleated teleost erythrocytes (see Nikinmaa, 1992 for review) and increased norepinephrine causes cell swelling in carp erythrocytes (Salma and Nikinmaa, 1990). Similar regulation of MCV may occur via β-adrenergic stimulation in the potentially comparable, nucleated avian erythrocytes. Again, similar to *in vitro,* the tendency for larger Hct at higher T_a's and reduced Hct at lower T_a's does not support Na^+,K^+ATP-ase as a primary regulator of MCV in response to T_a change. Depressed Na^+,K^+ATP-ase activity at lower T_a's (e.g., Canestari et al., 1994) should result in higher MCV (and Hct), thus, other mechanisms, such as those mentioned above, may be more important in regulating MCV.

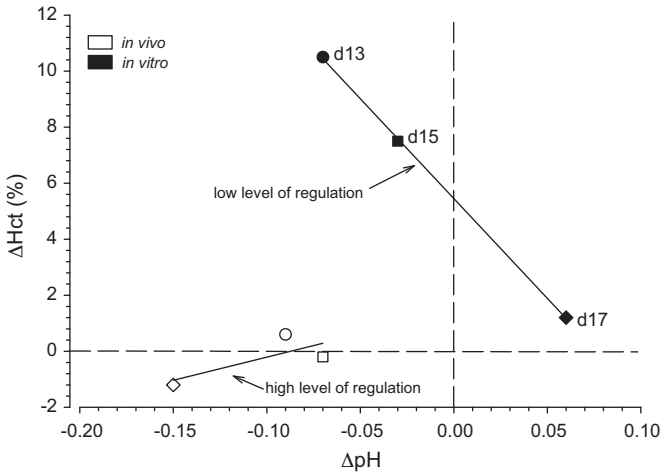

Fig. 8. High level *in vivo* and relatively poor *in vitro* Hct regulation during hypercapnia (P_{CO_2} = 5.1 kPa). *In vivo* data are the mean Hct_v and pH_v values for the hypercapnic embryos minus the mean values for the control embryos as reported in Table 3. *In vitro* data represent the relationship between ΔpH and ΔHct during *in vitro* equilibration of blood (tonometry) to P_{CO_2} = 5.1 kPa as reported in Fig. 3.

Exposing embryos to hypercapnia (P_{CO_2} = 5.1 kPa) resulted, as expected, in significantly altered blood Pv_{CO_2}, pH_v and $[HCO_3^-]_v$. No changes in Osm occurred and, in contrast to *in vitro*, Hct was also maintained constant (Fig. 8 and Table 3). The ΔpH achieved through hypercapnic exposure *in vivo* was smaller than *in vitro.* However the relatively small ΔpH of d15 embryos, for example, produced a large ΔHct whereas *in vivo* no change occurred. Thus, Hct is successfully regulated *in vivo* even in the face of large ΔpH changes. Further, alterations in Hct or Osm could not be induced by the additive effect of elevating T_a to 42 °C in d17 embryos. Potentially, the time-course of the perturbations in this study was not severe enough to disturb the homeostatic mechanisms maintaining Hct at normal *in vivo* levels (Fig. 8). Previous studies induced hypercapnia by decreasing eggshell conductance by coating the egg with an impermeable material (Nakazawa and Tazawa, 1988; Tazawa et al., 1988). Such treatment increased Hct through increased MCV in d16–17 embryos. Hypercapnic exposure in these studies was of longer duration (1d or entire incubation period) than in the present study (4–6 h). Apparently, *in vivo* homeostatic mechanisms regulate MCV in the face of challenges (such as hypercapnia and altered T_a), at least in the short term (h) whereas *in vitro* Hct responds to increased P_{CO_2} within 1.5 h (Fig. 8).

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