



Development of hematological respiratory variables in late chicken embryos: The relative importance of incubation time and embryo mass

Hiroshi Tazawa, Sarah J. Andrewartha, Warren W. Burggren *

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

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ABSTRACT

Oxygen demand increases during embryonic development, requiring an increase in red blood cells (RBCs) containing hemoglobin (Hb) to transport O₂ between the respiratory organ and systemic tissues. A thorough ontogenetic understanding of the onset and maturation of the complex regulatory processes for RBC concentration ([RBC]), Hb concentration ([Hb]), hematocrit (Hct), mean corpuscular indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration ([MCHb])) is currently lacking. We hypothesize that during the last half of incubation when the respiratory organ (the chorioallantoic membrane) envelops most of the egg contents, mean corpuscular indices will stabilize. Accordingly, Hct, [RBC] and [Hb] must also all change proportionally across development. Further, we hypothesize that the hematological respiratory variables develop and mature as a function of incubation duration, independently of embryonic growth. As predicted, a similar increase in Hct (from 18.7 ± 0.6% on day 10 (d10) to 34.1 ± 0.5% on d19 of incubation), [RBC] (1.13 ± 0.03 × 10⁶/μL to 2.50 ± 0.03 × 10⁶/μL) and [Hb] (6.1 ± 0.2 g% to 11.2 ± 0.1 g%) occurred during d10–19. Both [RBC] and [Hb] demonstrated high linear correlation with Hct, resulting in constant [MCHb] (~33 g% from d10 to d19). The decrease in MCV (from ~165 μ³ on d10 to ~140 μ³ on d13) and MCH (~55 pg to ~45 pg) during d10–13, may be attributed to a changeover from larger primary to smaller secondary and adult-type erythrocytes with MCV and MCH remaining constant (~140 μ³ and ~45 pg respectively) for the rest of the incubation period (d13–19). Hematological respiratory values on a given incubation day were identical between embryos of different masses using either natural mass variation or experimental growth acceleration, indicating that the hematological variables develop as a function of incubation time, irrespective of embryo growth.

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1. Introduction

Although an extensive literature exists on the basic hematology (e.g. hematocrit (Hct), red blood cell concentration ([RBC]) and blood hemoglobin concentration ([Hb])) of developing chicken embryos, these parameters are generally measured as subsidiary information to support other primary goals of individual studies (e.g. Clark, 1951; Yosphe-Purer et al., 1953; Barnes and Jensen, 1959; Jalavisto et al., 1965; Bartels et al., 1966; Romanoff 1967; Erasmus et al., 1970/71; Freeman and Misson, 1970; Tazawa 1971; Tazawa et al., 1971a, 1971b; Baumann et al., 1983; Dzialowski et al., 2002; Black and Burggren, 2004b; Khorrami et al., 2008). The knowledge of embryonic hematological development in birds is thus somewhat fragmented and in need of integrated simultaneous measurement across ontogeny. We still know relatively little of the potentially complex ontogenetic interplay between Hct, [RBC], [Hb], mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration

([MCHb]) and mean corpuscular volume (MCV)—all against a backdrop of the normal large egg water loss and its effects on plasma and other body fluid volumes. These variables are likely to change in complex ways as the embryo optimizes blood oxygen transport during dramatic growth in body mass, redistribution of metabolically active tissue, changes in body fluid volumes and complex changes in tissue metabolic intensity, let alone the maturation of the hemato-poietic processes themselves. Moreover, alterations in some of these hematological variables to meet the changing needs of the developing embryo may have far-reaching effects beyond mere alteration of blood O₂ transport capabilities. A key consideration here is how the maturing vessels of the intra- and extra-embryonic (chorioallantoic) vascular beds change in radius early in development. If radius becomes fixed early on (Wangensteen et al., 1970/71; Tazawa and Ono, 1974), then significant increases in MCV during the last half of embryonic development could increase blood viscosity and apparent vascular resistance perhaps to the point of diminishing blood flow to the embryo's vascular beds. Thus, for example, a 'simple' increase in Hct (i.e. increase in [RBC] with constant MCV) to increase blood O₂ carrying capacity would necessarily also increase blood viscosity. Similarly, any change in MCV associated with blood osmotic changes

* Corresponding author. Tel.: +1 940 565 2497.

E-mail address: Warren.Burggren@unt.edu (W.W. Burggren).

would also potentially affect blood viscosity and flow. Consequently, because increases in MCV could have potentially profound and negative implications for overall blood transport through blood viscosity alteration, we hypothesize that MCV should remain relatively constant during the last half of incubation. This would leave a suite of changes involving increases in [RBC] and [Hb] (along with the former's impact of potentially changing Hct) and the increase in blood pressure that occurs across development (e.g. Tazawa, 2004) as the most likely mode of increasing the overall blood O₂ transport capabilities.

In developing chicken embryos, oxygen is acquired primarily by diffusion for the first several days of ontogeny, despite the onset of convective blood flow around day 3 (Burggren et al., 2000, 2004; Burggren, 2004). As development further progresses, however, convective oxygen transport by the blood must sharply increase to support the increasing tissue demands of the embryo. Such an increase can occur through an increased blood flow, an increased blood oxygen capacity, or a combination of both mechanisms (Dejours, 1966). In fact, blood oxygen capacity increases markedly with embryonic development during the last half of incubation, for example from ~8 vol.% on day 10 (d10) to ~12.8 vol.% on d18 of incubation in chicken embryos (Bartels et al., 1966; Girard, 1971; Tazawa, 1971; Tazawa and Mochizuki, 1977). As development continues and embryonic mass increases, then not surprisingly the overall oxygen consumption continues to increase as the embryos advance towards hatching (Romanoff, 1967). Mass-specific oxygen consumption falls (e.g. Black and Burggren, 2004a) however, so another key unanswered question in avian developmental hematology is whether values of Hct, [RBC] and [Hb] track more closely with the developmental time (reflecting maturity of hematopoietic systems) or with body mass (reflecting absolute oxygen requirements of the embryo). Hct is independent of body mass on any given incubation day (Xu and Mortola, 1988), indicating that Hct (and potentially other hematological variables) develops independently of body mass. We additionally hypothesize, therefore, that the developmental values of Hct, [RBC] and [Hb] are a function of incubation duration, not embryonic body mass.

2. Materials and methods

2.1. Egg incubation

Fertile eggs of the domestic fowl (*Gallus gallus domesticus*, layer strain) were obtained from Texas A & M University (College Station, Texas, USA). Eggs were weighed (± 0.01 g), then incubated at a temperature of 37.5 ± 0.1 °C and relative humidity of ~50% (Incubator: 1502, G.Q.F. Manuf. Co., USA). Individual eggs were removed from the incubator on one of d10 through d19, assigned to the "control group" and their hematological variables measured as described below.

A major thrust of this study was to examine the effect of embryo size on hematology. Increased incubation temperature produces avian embryos of a larger mass for any given day of development (Romanoff, 1960, Zhang and Whittow 1992, Maltby et al., 2004, Hammond et al., 2007) Embryos designated as the "growth-accelerated group" were separated from the control group on d7 of incubation, placed into a second incubator and maintained at 38.5 ± 0.1 °C (i.e. 1 °C > control). Growth-accelerated embryos were removed from the incubator on d13, d15 or d17 and their hematological variables were measured.

2.2. Blood sampling

The allantoic vein or artery was located via candling and marked on the overlying eggshell 1 day before the target developmental day. Previous studies showed no difference in Hct between arterial and venous blood (e.g. Tazawa et al., 1971a). On the target day, the marked region of the eggshell (~0.6–0.8 cm in diameter) was removed and the

underlying allantoic blood vessel was gently lifted by forceps through the small hole in the eggshell. The blood vessel was punctured for sampling by a 25 gauge needle on a 1 mL sampling syringe that had been flushed with heparinized saline (ca. 100 mg in 100 mL saline). Approximately 200 μ L of blood was collected from individual d10 embryos, although occasionally blood from two individual d10 embryos was pooled to provide adequate volume for all hemocytometric measurements. Blood samples of ~200–250 μ L and at least 250 μ L were collected from individual embryos on d11 and d12–19 of incubation, respectively.

Sampled eggs were then placed into a cold and anoxic environment for 2 h and embryos were removed from their eggshell, following confirmation of cardiac arrest. Yolk and extra-embryonic membranes were removed, and the embryo's body mass was measured (± 0.01 g) with an electronic balance.

2.3. Hematology measurements

Blood from a total of 223 control embryos and 65 growth-accelerated embryos was measured. Duplicate preparations of 60 μ L of blood were transferred into sealed hematocrit tubes and centrifuged for 4 min at 10,000 rpm and the mean % Hct was determined ($\pm 0.1\%$, READACRIT Centrifuge, Becton Dickinson). On 169 blood samples, a glass hemocytometer was used to determine [RBC] using 4 μ L of each blood sample following standard dilution and measurement techniques as outlined by the manuscript provided for the Corpuscle Counting Chamber (Levy–Hausser Corpuscle Counting Chamber).

[Hb] in g% was determined on ~20 μ L of blood from each sample using a Coulter counter hematology analyzer (Beckman Coulter ACT10) (although the Coulter counter was not used for all possible hematology variables, e.g. Hct, [RBC] see below and Appendix). Calibration using a control sample of hemoglobin ([Hb] = 6.2 g%, Streck Co.) demonstrated an accuracy of ± 0.5 g%. Similarly, preliminary comparisons of [Hb] of chicken embryo blood determined by a Radiometer OSM2 hemoximeter (as used in previous studies e.g. Dzialowski et al., 2002; Black and Burggren, 2004b) yielded no significant difference in [Hb] between the two methods. Thus, the [Hb] value measured by the Coulter counter was assumed to be accurate for this study.

The initial intent was to use a Coulter counter for all hematology measurements because the analyzer provides a high throughput data collection on a relatively small blood sample (~20 μ L). Coulter counter (and other automated) hematology analyzers, however, typically use internal mathematical constants based on adult human blood. To verify the hematological values determined on avian embryonic blood using the Coulter counter, Hct and [RBC] were also determined on a subset of the blood samples by traditional manual methods using a hematocrit centrifuge and a hemocytometer, respectively, as mentioned above, and the data were subsequently compared (Appendix). Careful validation comparing values obtained with 'traditional' methodologies (centrifugation and hemocytometry) and the Coulter counter demonstrated an overestimation of Hct and underestimation of [RBC] by the Coulter analyzer resulting in an overestimation of MCV and MCH by approximately 10% (see Appendix). [MCHb] was underestimated by the Coulter analyzer, and thus, in the present report; the mean corpuscle indices were derived using expressions (1), (2) and (3):

$$\text{MCV} (\mu\text{m}^3) = 10 \times \text{Hct} / [\text{RBC}] \quad (1)$$

$$\text{MCH} (\text{pg}) = 10 \times [\text{Hb}] / [\text{RBC}] \quad (2)$$

$$\text{and } [\text{MCHb}] (\text{g}\%) = 100 \times [\text{Hb}] / \text{Hct} \quad (3)$$

where Hct is the value obtained via centrifugation (%), [Hb] is the value determined from the Coulter counter (g%) and [RBC] (10^6 cells/ μ L) is calculated from an individual value determined by the Coulter analyzer,

using a previously obtained regression equation relating values simultaneously determined using the Coulter analyzer and hemocytometry (Eq. (A2) in Appendix).

2.4. Statistical analysis

Differences between the control and growth-accelerated groups were assessed using unpaired *t*-tests. Differences in hematological

respiratory variables between incubation days were examined using one-way factorial ANOVA. When ANOVA revealed significant differences between incubation days, Tukey *post hoc* tests detected the differences between group means. The effect of the two different incubation temperatures on embryo mass and the difference of mass at three incubation days were examined by two-way ANOVA. A significance level of $P < 0.05$ was adopted for all tests. Mean values ± 1 s.e.m. are presented.

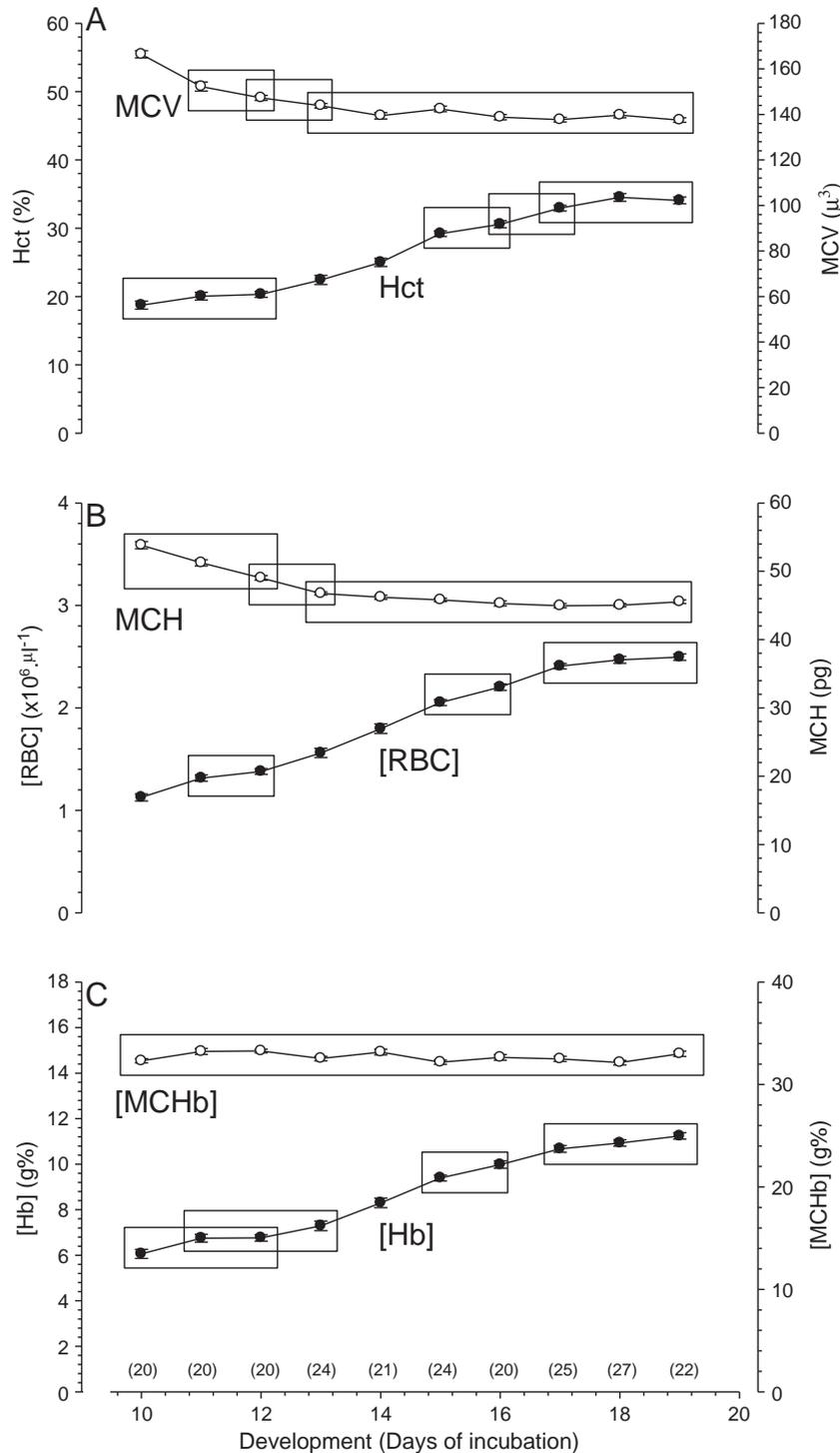


Fig. 1. Developmental changes in whole blood hematology as a function of development in control embryos. (A) Hematocrit (Hct) and mean corpuscular volume (MCV), (B) red blood cell concentration ([RBC]) and mean corpuscular hemoglobin (MCH) and (C) blood hemoglobin concentration ([Hb]) and mean corpuscular hemoglobin concentration ([MCHb]) as a function of development (days). N values are reported in parentheses on panel C and error bars are ± 1 s.e.m. Boxes enclose means that are not statistically different.

3. Results

3.1. Developmental changes in hematology

Control hematology measurements were made on a total of 223 embryos, with at least 20 embryos measured on each developmental day from d10 to d19 (Fig. 1). Essentially, these data fell into two categories. The first category of variables – Hct, [RBC] and [Hb] – reflecting the general properties of blood hematology, increased significantly in a slightly sigmoid fashion during the second half of development (Fig. 1). Mean values of Hct increased by approximately 80% from $18.7 \pm 0.6\%$ on d10 to $34.1 \pm 0.5\%$ on d19. [RBC] increased 120% from $1.13 \pm 0.03 \times 10^6/\mu\text{L}$ to $2.50 \pm 0.03 \times 10^6/\mu\text{L}$ from d10 to d19. [Hb] increased 80% from $6.1 \pm 0.2 \text{ g\%}$ to $11.2 \pm 0.1 \text{ g\%}$ over this same period. The second category reflected hematological values relating specifically to the red blood cell (MCV) and how Hb is packaged within it (MCH, [MCHb]). These values were actually constant or initially decreased slightly over the second half of embryonic development. MCV decreased by approximately 15% from $\sim 165 \mu\text{m}^3$ on d10 to $\sim 140 \mu\text{m}^3$ on d19, although the significant decrease occurred during the first 3 days (i.e. d10–13). MCV remained unchanged at a mean value of $140 \mu\text{m}^3$ during the remaining d13 to d19 of incubation (Fig. 1). Similarly, a significant decrease in MCH of approximately 22% ($\sim 55 \text{ pg}$ on d10 to $\sim 45 \text{ pg}$ on d13) occurred during the first 3 days and MCH remained unchanged at mean value of 45 pg from d13 to d19. The relationship between the changes of MCV and MCH (during d10–19) was significant; i.e. $\text{MCH} = 7.21 + 0.28 \times \text{MCV}$ ($N = 223$, $r = 0.858$, $t = 24.825$, and $p < 0.001$). Thus, [MCHb] remained constant during the last half of incubation at $\sim 33 \text{ g\%}$ (Fig. 1).

The Hct of a blood sample correlates to both [RBC] and [Hb], as shown by the highly significant linear regressions in Fig. 2.

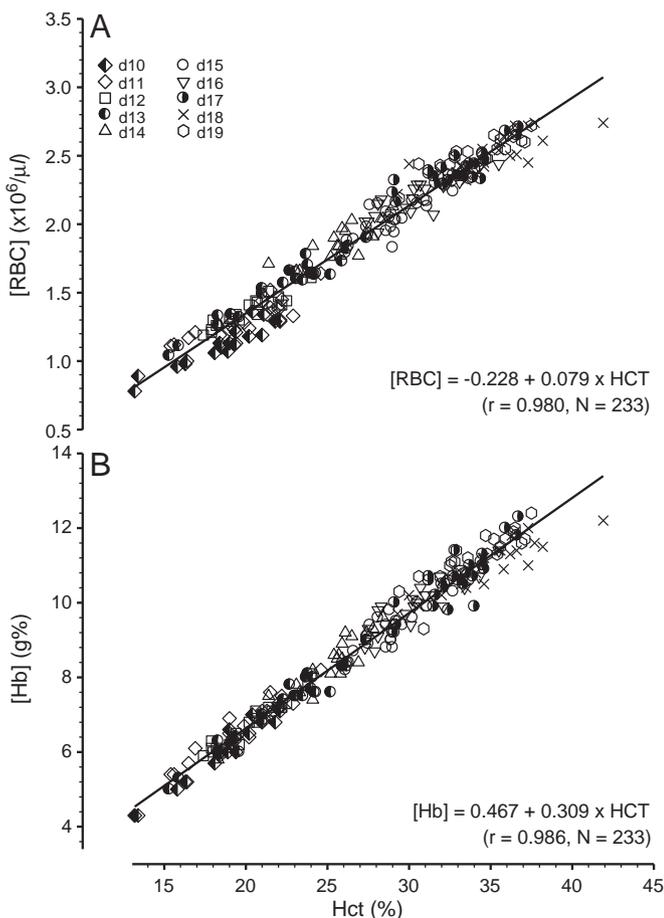


Fig. 2. (A) [RBC] and (B) [Hb] as a function of Hct in control embryos from day 10 to 19.

This relationship was conserved across the developmental range (d10–19).

3.2. Body mass influences on hematology

Control embryos increased their mass approximately 15-fold from $1.73 \pm 0.04 \text{ g}$ ($N = 20$) on d10 of incubation to $25.00 \pm 0.32 \text{ g}$ ($N = 22$) on d19 (Fig. 3), a rate of growth that was anticipated based on previous studies (e.g. Romanoff, 1967). Considerable variation in mass between embryos occurred on any given day of development (Fig. 3A). For instance, on d17 of incubation, control embryo mass ranged from 14.85 g to 20.85 g , with a mean of $17.82 \pm 0.32 \text{ g}$ ($N = 25$). We exploited natural variations in body mass to explore body mass–hematology relationships. Embryos were divided into two populations: the *light group* comprised the 12 embryos with lowest body mass ($16.49 \pm 0.25 \text{ g}$), while the 12 embryos with the heaviest body mass ($19.12 \pm 0.28 \text{ g}$) were designated as the *heavy group* (Fig. 3A). There was no difference in Hct between the *light* ($32.5 \pm 1.9\%$) and *heavy* ($33.3 \pm 2.3\%$) d17 embryos (Fig. 3C). Similarly, no difference in [RBC] between *light* and *heavy* d17 embryos ($2.40 \pm 0.13 \times 10^6$ and $2.41 \pm 0.15 \times 10^6$ cells/ μL , respectively) or [Hb] (10.6 ± 0.7 and $10.7 \pm 0.9 \text{ g\%}$) was observed (Fig. 3E and G). A similar pattern was demonstrated for embryos ranging in age d11–19 with no significant difference in Hct, [RBC] or [Hb] between the *light* and *heavy* groups (Fig. 3C, E, and G). Accordingly, although the difference of embryo mass was highly significant ($P < 0.001$) between populations, there were no significant differences at any day of development between the mean values of Hct, [RBC] or [Hb] of two body mass groups (Fig. 3A, C, E, and G).

The second approach to exploiting embryonic body mass variation involved significantly increasing body mass above mean control values by elevating incubation temperature by 1°C (i.e. 38.5°C) from d7 of incubation. There was no difference in initial egg mass of the *control* (fresh egg mass; $58.27 \pm 0.20 \text{ g}$, $N = 223$) and *growth-accelerated* (fresh egg mass; $58.74 \pm 0.40 \text{ g}$, $N = 65$) embryos ($t = -1.01$, $p = 0.27$). After incubation at elevated temperature, the resulting *growth accelerated* embryos were approximately 10% heavier on d13 ($4.98 \pm 0.09 \text{ g}$ in *control* vs $5.49 \pm 0.14 \text{ g}$ in *growth accelerated* embryos). On d15 and d17, they were $\sim 9\%$ ($10.05 \pm 0.22 \text{ g}$ vs $10.92 \pm 0.20 \text{ g}$) and $\sim 6\%$ ($17.82 \pm 0.32 \text{ g}$ vs $18.95 \pm 0.26 \text{ g}$) heavier than *control* embryos. All embryo mass differences were statistically significant ($P < 0.001$, Fig. 3B). Again, no difference in Hct was observed between the *control* ($33.8 \pm 0.6\%$) and *growth accelerated* ($32.9 \pm 0.4\%$) d17 embryos (Fig. 3D). Similarly, [RBC] ($2.41 \pm 0.04 \times 10^6$ vs $2.41 \pm 0.03 \times 10^6$ cells/ μL) and [Hb] (10.7 ± 0.1 vs $10.7 \pm 0.2 \text{ g\%}$) were also unchanged in *growth accelerated* embryos on d17 (Fig. 3F and H). A similar pattern was demonstrated for d13 and d15 embryos with no significant difference between in Hct, [RBC] or [Hb] between the *control* and *growth accelerated* embryos (Fig. 3D, F, H).

[Hb] increased rapidly as a function of embryonic body mass during early period of measurement, but leveled off in the largest embryos ($\sim 30 \text{ g}$) at a value of $\sim 11 \text{ g\%}$ which approached chicken adult [Hb] of $9\text{--}14 \text{ g\%}$ (e.g., Nightingale and Fedde, 1972; Baumann and Baumann, 1977) (Fig. 4A). However, when mass-specific hemoglobin concentration ([Hb]/g body mass) was considered, there was a sharp decline in the amount of hemoglobin per body mass (Fig. 4B).

4. Discussion

4.1. Changes in hematological variables during development

Development of the embryo is accompanied by relatively profound changes in hematological variables. While such changes in individual variables (Hct, [RBC] and [Hb]) have been well documented (Clark, 1951; Yosphe-Purer et al., 1953; Johnston, 1955; Barns and Jensen, 1959; Macpherson and Deamer, 1964; Jalavisto et al., 1965; Bartels et al., 1966; Romanoff 1967; Ackerman, 1970; Freeman and Misson,

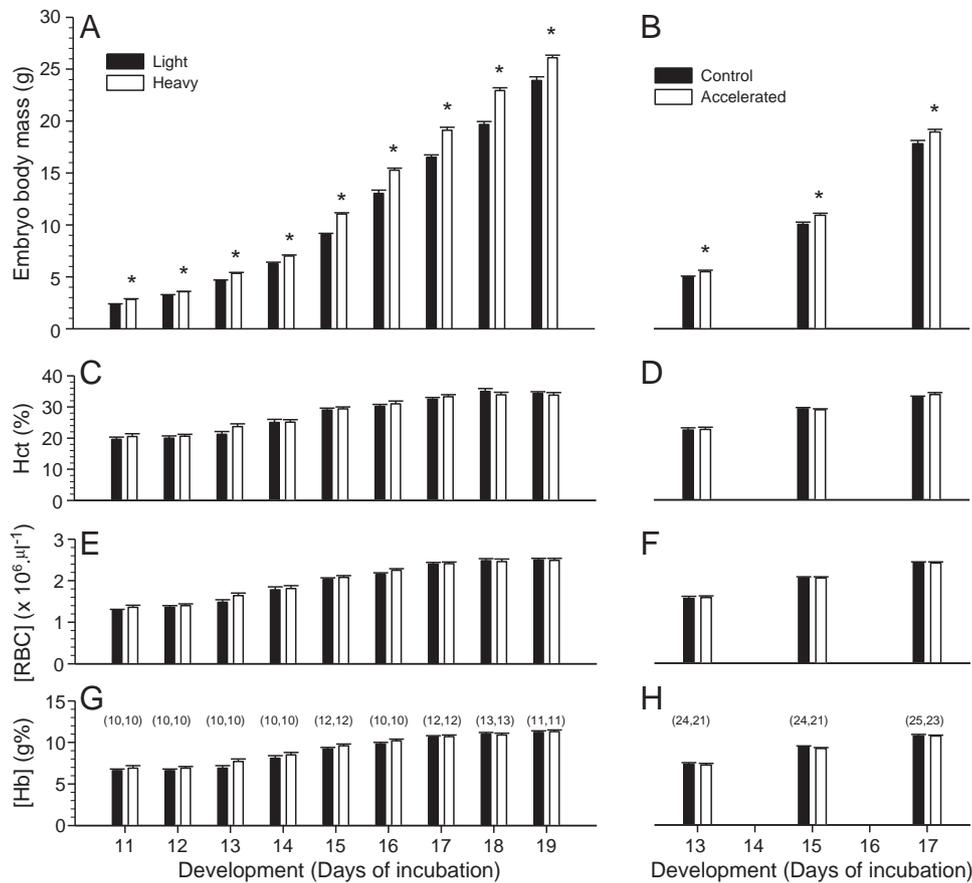


Fig. 3. Embryonic body mass as a function of development in (A) control embryos separated by body mass into lightest (*light*-black bars) and heaviest (*heavy*-clear bars) and in (B) all control embryos (*control*-black bars) and growth accelerated (*accelerated*-clear bars) embryos incubated at 38.5 °C rather than 37.5 °C. The corresponding whole blood hematology values for hematocrit (Hct) (C and D), red blood cell concentration [RBC] (E and F) and blood hemoglobin concentration [Hb] (G and H) are presented for the same embryos. * indicates a significant difference from either the *light* or *control* population. N values are reported in parentheses on panels G and H and error bars are ± 1 s.e.m.

1970; Temple and Metcalfe, 1970; Erasmus et al., 1970/71; Tazawa, 1971; Tazawa et al., 1971a, 1971b; Lemež, 1972; Davis et al., 1988; Dzialowski et al., 2002; Black and Burggren, 2004b; Khorrami et al., 2008), the inter-relationships between hematological variables that reveal how the morphological and biochemical characteristics of the RBC change during development are less clear. This lack of focus on hematological inter-relationships most likely reflects the fact that such correlation studies must depend on extensive data sets that may be laborious to produce.

The relatively large progressive increase in Hct over the last half of development (nearly 1.8-fold) documented in the present study (Fig. 1A) is generally consistent with previously published studies (Yosphe-Purer et al., 1953; Johnston, 1955; Barnes and Jensen, 1959; Macpherson and Deamer, 1964; Jalavisto et al., 1965; Bartels et al., 1966; Freeman and Misson, 1970; Erasmus et al., 1970/71; Tazawa 1971; Tazawa et al., 1971a,b; Davis et al., 1988; Khorrami et al., 2008); however there is a considerable variation in reported Hct values on any given day of development. For instance, reported mean Hct values range from ~16% (Jalavisto et al., 1965) to 24% (Johnston, 1955) on d10 and from ~28% (Erasmus et al., 1970/71) to 37% (Tazawa et al., 1971a, 1971b) on d18. Additionally, the daily increases of Hct in some reports (e.g. Macpherson and Deamer, 1964) are rather irregular over the whole course of development, unlike the relatively smooth, progressive and slightly sigmoid pattern of increase documented in the present study. Similarly, the developmental patterns of [RBC] and [Hb] as well as the mean corpuscular indices collected from previous reports also vary among studies and contain wide ranges of variability (Clark, 1951; Barnes and Jensen, 1959; Macpherson and Deamer, 1964; Jalavisto et al., 1965; Ackerman, 1970; Freeman and Misson,

1970; Temple and Metcalfe, 1970; Lemež, 1972; Dzialowski et al., 2002; Black and Burggren, 2004b). The developmental patterns of [RBC] and [Hb] in the present determination are similar to the patterns of change for Hct with both variables showing progressive increases over development and relatively small variation in the mean values (Fig. 1B and C). In fact, both [RBC] and [Hb] increase linearly with the increase in Hct and the linear relationships of the former two variables with Hct are highly significant (Fig. 2). The clear definition of these relationships with a relatively small variation emphasizes the need for approaches, such as the approach of the present study, that focus directly on hematological variables. Further, this study highlights that increased sample sizes and careful measurement throughout embryonic development are necessary to elucidate patterns and correlations with other developing parameters (see below).

Hct of chicken embryo blood emerges as a highly accurate predictor of both the concentration of RBCs in whole blood (Fig. 2A) and the concentration of Hb in the red cell (Fig. 2B), since the regressions of both of these parameters had correlation coefficients of 0.98 or greater across the range of development examined d10–19 (Fig. 2). The high correlation of Hct and [Hb] is exploited in the clinical measurement of Hct in human blood (see e.g. Myers and Browne, 2007 for review). Determination of Hct through centrifugation, or validation of automated hematology analyzers (as outlined in the Appendix), will ensure that Hct is not overestimated with consequent flow-on effects on any predictions of further hematology variables.

The mean corpuscular indices derived from values of Hct, [RBC] and [Hb] in the present measurement also change relatively smoothly with embryonic development (Fig. 1) when compared with the more saltatory increases of previous reports. Our measurement of [Hb]

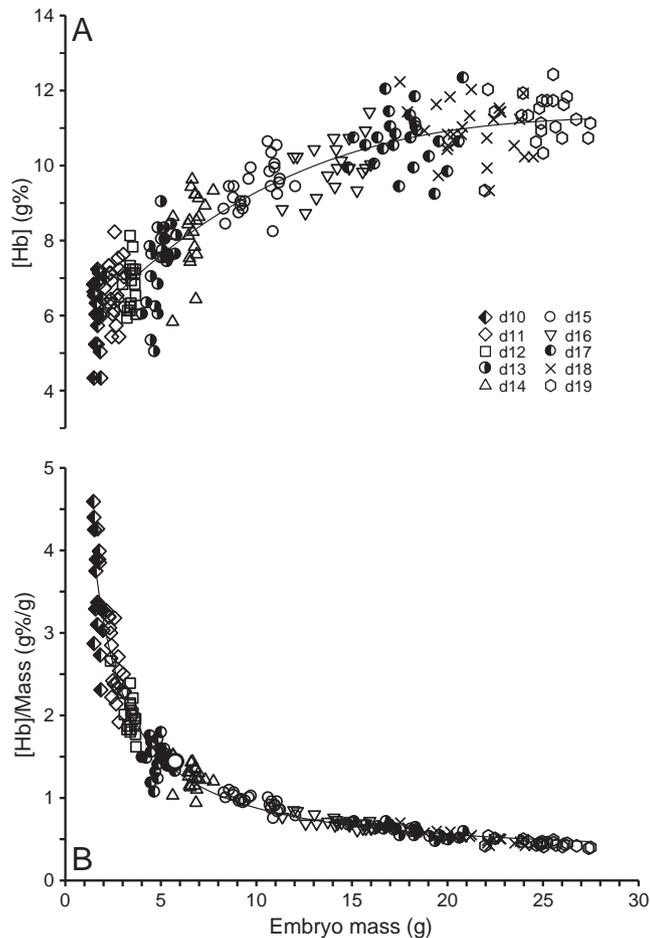


Fig. 4. (A) the relationship of [Hb] and (B) [Hb] per unit mass of embryo (i.e., mass-specific [Hb]) to embryo mass during days 10–19. (N = 223).

increase was toward the highest of reported values, while [RBC] increase was on the lower end. As derivative values, thus, both MCH and [MCHb] are towards the higher end of the reported values across development. Our reported decrease in MCV, especially during d10–13, likely results from changes in the relative proportions of erythrocyte type in bird embryos. During this period of development (d10–13), the larger primary erythrocytes likely still co-exist with the smaller secondary erythrocytes and the maturing adult-type erythrocytes that become prevalent during the last week of prenatal development (Macpherson and Deamer, 1964). The larger primary erythrocytes decrease gradually during the second week of incubation, leaving predominantly mature erythrocytes, which may contribute to the gradual decrease in MCV during d10–13 (Fig. 1). The subsequent constant value of MCV then likely results from the predominance of mature erythrocytes. A future study linking changes in avian embryo hematological variables with changes in proportions of avian red blood cell types is highly warranted.

The radii of the intra- and extra-embryonic (chorioallantoic) vascular beds eventually become fixed in development (Wangensteen et al., 1970/71; Tazawa and Ono, 1974), and thus, an increase in the size of RBC's may potentially impede blood flow. The relative constancy of MCV across the second half of incubation (Fig. 1) will help to ensure that blood flow to the embryo's vascular bed is not restricted. Hct, however, did increase across development through an increase in [RBC] (Fig. 1); potentially blood flow issues may arise late in incubation due to consequential blood viscosity increases. This increase in Hct toward hatching however, may not be large enough to produce an adverse effect on blood viscosity, as Hct reaches 30–44% in adult chickens and presumably the developing vasculature must at

some stage be able to cope with the resulting blood viscosities associated with adult Hct levels (e.g., Chiodi and Terman, 1965; Sturkie, 1967; Baumann and Baumann, 1977). Blood pressure also increases during development (e.g. Tazawa, 2004), further compensating for potential blood flow constraints.

4.2. Relationship between hematology and embryo mass during development

The considerable variation in body mass that naturally occurs during embryonic development allowed us to examine the relationship between body mass and hematological values. Despite significant differences in body mass between the constructed *light* and *heavy* groups, there were no accompanying significant differences in any hematological variables (Fig. 3). Similarly, no hemotological differences between the *control* and *growth accelerated* embryos could be induced using an alternative approach of increased incubation temperature to accelerate embryonic growth to body mass levels even higher than those of the naturally heavier embryos (Fig. 3). Previously, it was also reported that Hct of embryos which were divided into two groups with different egg mass; small (mean mass; 54.1 g, N=60) and large (72.8 g, N=52), resulted in insignificant difference between the two groups (Xu and Mortola, 1988). We conclude, then, that Hct, [RBC] and [Hb] develop as a function of developmental time, not the absolute size of the embryo. A similar chronological development of endothermy (or homeothermy) has been demonstrated in chicks. D22 embryos that remained within the egg (i.e. failed to hatch on d21) responded to egg cooling with similar transient increases in egg temperature, oxygen consumption or heart rate as d22 hatchlings (Tazawa and Rahn, 1987; Tazawa et al., 1988, 2001; Whittow and Tazawa 1991). The external appearance (i.e. embryos or hatchlings) and the stage of maturity (growth and development) of these embryos were not the major determinant for the development of endothermy, which occurred on d22 regardless. This suggests that the chronological development of endothermy is primarily designed and development or growth is secondary.

While Hct, [RBC] and [Hb] increase somewhat linearly as incubation progresses from d10–19 (Fig. 1), the mass-specific changes in these variables are decidedly non-linear (e.g. [Hb], Fig. 4). Interestingly, the pattern of change in mass-specific oxygen uptake of chicken embryos, derived from eight previous studies, closely matches the pattern of change in mass-specific [Hb] across embryonic development (Fig. 5). Noteworthy is that the highest mass-specific levels of [Hb] occur when metabolic intensity is greatest during early development. While it would seem logical to assume that Hb in the blood – and the O₂ it would carry – was directly supporting the very high rates of mass-specific O₂ consumption in early embryos, in fact the role of convective blood oxygen transport is minimal during the first 3–4 days of embryonic development in chicken embryos (Burggren et al., 2000, 2004; Burggren, 2004). Oxygen consumption (and associated growth and development) can continue unabated with a complete elimination of convective O₂ transport. From a comparative point of view, it is interesting to note that high mass-specific [Hb] has also been reported in the highly altricial postnatal opossum (*Didelphis virginiana*, ~130 mg at birth) with a similar decrease occurring in parallel to mass specific oxygen consumption across pouch development (Murphy et al., 1977, see Frappell and MacFarlane, 2006 for review). Gas exchange occurs nearly exclusively via diffusion across the skin in very small postnatal marsupials (e.g. 2 species of dunnart, 10 and 17 mg at birth, see Frappell and MacFarlane 2006, for review), which would likely show a similar mass dependent relationship of [Hb]. Thus, across two taxa, high mass specific [Hb] is seen at a developmental stage where Hb has reduced importance for O₂ transport. It is possible that the high mass-specific levels of [Hb], in chicken embryos (and postnatal marsupials alike) could indirectly aid in O₂ diffusion between, or storage within, different functional

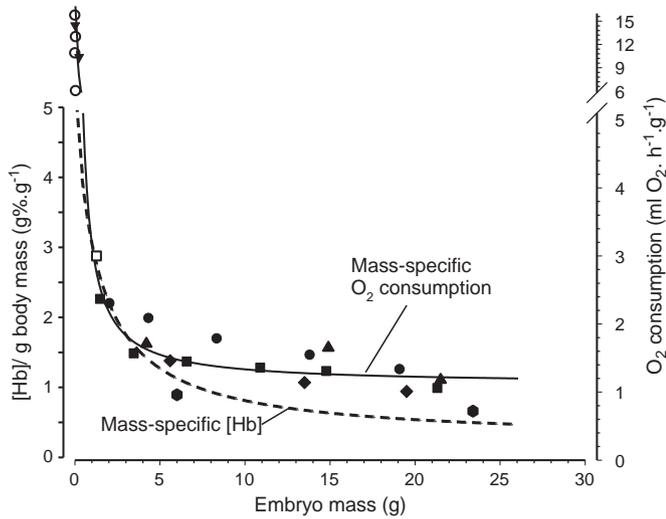


Fig. 5. Comparison of mass-specific embryonic [Hb] and mass-specific O_2 consumption in chicken embryos. The dashed line shows the inverse second order regression for [Hb] with body mass taken from the [Hb] plot in Fig. 4B. The solid line represents the inverse third order regression of previously published mass-specific oxygen consumption against body mass using the data from Altimiras and Phu (2000) (open square), Black and Burggren (2004a) (diamonds), Burggren et al. (2000) (downward triangles), Dzialowski et al. (2002) (hexagons), Romanoff (1941) (open circles), Temple and Metcalfe (1970) (squares), Tazawa (1973) (circles), and the data calculated from masses published by Tazawa and Mochizuki (1977) and the oxygen consumption data from Tazawa et al. (1992) (upward triangles).

compartments of the growing embryo. Additional experiments are warranted to determine the full level of co-dependency of Hb and O_2 consumption in early development.

5. Conclusions

Focusing on respiratory hematological parameters in this study has highlighted their progressive interrelationships during development. MCV decreased during d10–13 of incubation, likely due to changes in the relative proportions of embryonic and adult RBC's, then remained constant. MCH changed in a similar fashion to MCV, resulting in [MCHb] remaining constant throughout late incubation. Hct, however, did increase across development through an increase in [RBC] potentially leaving embryos vulnerable to blood flow issues late in incubation due to consequential blood viscosity increase, although it is likely that the resultant blood viscosities are still affordable to the embryo when compared with adult values. Variations in body mass by 10–20% on a given developmental day had little effect on respiratory hematological variables, which tracked incubation duration and the development of mass-specific metabolic rate more closely. Future studies focusing on how proportions of avian RBC types change with embryonic hematological variables and the relative reliance of O_2 consumption on Hb in early development will further broaden our understanding of the ontogeny of these interconnected parameters.

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Appendix A

Avian red blood cells (embryos or adults) are nucleated and somewhat elliptical in shape (Romanoff, 1960; Lucas and Jamroz, 1961; Macpherson and Deamer, 1964; Tazawa and Ono, 1974). Moreover, avian hemoglobins absorb light at slightly different

wavelengths than mammalian hemoglobins (Romanoff, 1960; Tazawa et al., 1974). These properties make the measurement of avian hematology potentially problematic when using automated, low volume ($\sim 20 \mu\text{L}$) hematological measuring devices, such as Coulter counters with internal constants based on the optical characteristics of the human RBC's which are smaller, biconcave and anucleate. Thus, to ensure such calculations resulted in values appropriate for avian embryonic blood, methodological validation of hematological variables (Hct and [RBC]) using traditional methods was performed on a subset of blood samples as follows.

Hct was determined (in duplicate) for 288 embryos (i.e., 223 the control embryos plus 65 growth-accelerated embryos) from d10 to d19 of incubation using a hematocrit centrifuge (READACRIT Centrifuge, Becton Dickinson) as outlined in the Methods. [RBC] was determined using a glass hemocytometer (Levy-Hausser Corpuscle Counting Chamber) for 81 randomly selected embryos from d10 to d19 (to maximize the [RBC] range) of incubation as outlined in the Methods. All remaining blood was then measured for Hct, [RBC] and [Hb] by the Coulter analyzer and the mean corpuscular indices of MCV, MCH and [MCHb] were calculated using Eqs. (1)–(3) in the methods from the measured values of Hct, [RBC] and [Hb] by the Coulter analyzer and the data from traditional (i.e. centrifugation and hemocytometer) methods (mean where applicable) and Coulter analyzer were compared.

Our careful comparisons of values determined by a Coulter analyzer with those determined by centrifugation and glass hemocytometer methods indicated small but significant measurement differences between the two methods. Each method has its own sources of error, and advantages/disadvantages. The high throughput afforded by the Coulter counter allows processing of large numbers of samples, which increases the accuracy of the predictive models for hematological relationships. At the same time, however, the more basic, “mechanical” process of controlled centrifugation has fewer assumptions and derivative calculations. High precision was demonstrated by the centrifugation technique as no difference in duplicate Hct values was observed among the 169 embryos measured ($t = 0.415$, $P = 0.678$). Hct determined by the Coulter analyzer tended to be 0.5–2.5% (mean difference $1.8 \pm 0.2\%$, $N = 223$; and $P = 0.004$) points higher than by centrifugation, with the differences slightly augmented at the highest Hct values (Fig. A1a). Thus, assuming that the more laborious centrifugation method is also the most accurate, we would suggest that Hct determined by the Coulter analyzer should be calculated by the correlation expression:

$$\text{Hct}_{\text{cal}} = 0.983 + 0.905 \times \text{Hct}_{\text{Coulter}} \quad (\text{A1})$$

[RBC] measured by the Coulter analyzer ($1.73 \pm 0.05 \times 10^6/\mu\text{L}$) was significantly lower than that determined visually by the hemocytometer ($1.89 \pm 0.06 \times 10^6/\mu\text{L}$) ($t = -9.312$, and $P < 0.001$, Fig. A1b) with a difference of $0.16 \pm 0.02 (\times 10^6/\mu\text{L})$. Hemocytometer methods have their own sources of error, particularly associated with pipetting error. However, once more assuming the more simple mechanical methods to be most accurate, we suggest that [RBC] determined by the Coulter analyzer should be calculated by the following expression:

$$[\text{RBC}]_{\text{cal}} = 0.07 + 1.05 \times [\text{RBC}]_{\text{Coulter}} \quad (\text{A2})$$

In addition to automated determination by the Coulter analyzer, mean corpuscular indices were also calculated using the Hct and [RBC] values determined via centrifugation and hemocytometry respectively, using expressions (1)–(3) as detailed in the methodology. MCV_{cal} and MCH_{cal} were low compared with the values determined by the Coulter analyzer (Fig. A1c and d). $[\text{MCHb}]_{\text{cal}}$ became larger than the value by the Coulter analyzer ($r = 0.475$, $t = 9.064$, and $P < 0.001$) and their relationship can be described by $[\text{MCHb}]_{\text{cal}} = 16.69 + 0.52 \times [\text{MCHb}]_{\text{Coulter}}$. Because the Coulter analyzer overestimated Hct and underestimated [RBC], then MCV and MCH derived from Hct and [RBC] determined by

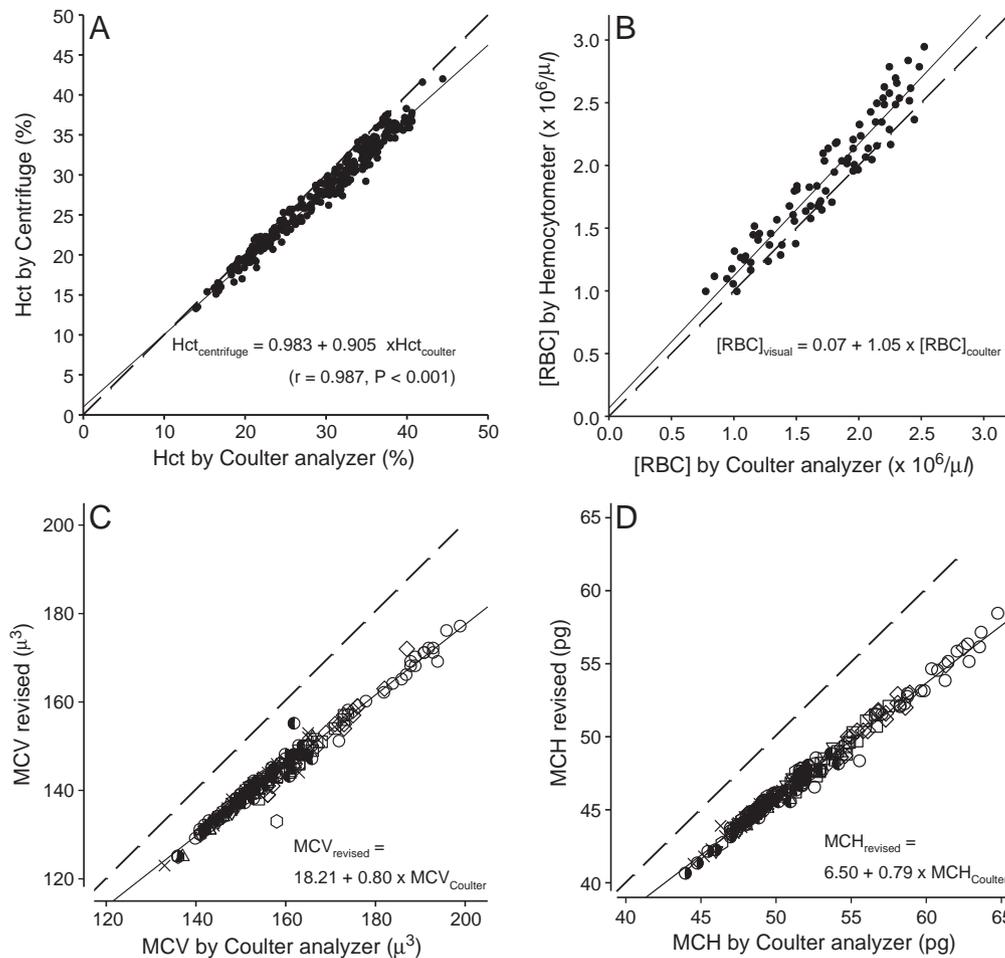


Fig. A1. Validation of hematological measurements in 288 d10 to d19 embryonic chickens. (A) Relationship between Hct values determined by the Coulter analyzer ($\text{Hct}_{\text{coulter}}$, abscissa) and by centrifugation ($\text{Hct}_{\text{centrifuge}}$, ordinate). (B) Relationship between [RBC] determined by the Coulter analyzer ($[\text{RBC}]_{\text{coulter}}$, abscissa) and by visual count using glass hemocytometer ($[\text{RBC}]_{\text{visual}}$, ordinate). (C) Relationship between MCV which was calculated from Hct and [RBC] determined by the Coulter analyzer ($\text{MCV}_{\text{coulter}}$, abscissa) and MCV calculated from Hct determined by centrifugation and [RBC] derived from correlation expression between [RBC] determined by the Coulter analyzer and by the glass hemocytometer using expression (1) in the Materials and methods ($\text{MCV}_{\text{revised}}$, ordinate). (D) Relationship between MCH which was calculated from [Hb] and [RBC] determined by the Coulter analyzer ($\text{MCH}_{\text{coulter}}$, abscissa) and MCH calculated from [Hb] determined the Coulter analyzer and [RBC] derived from correlation expression between [RBC] determined by the Coulter analyzer and by the glass hemocytometer using the correlation expression (2) ($\text{MCH}_{\text{revised}}$, ordinate).

the Coulter analyzer were approximately 10% larger than the value calculated from expressions (1) and (2) using the centrifugation (Hct) and hemocytometer ([RBC]). [MCHb] of the chicken embryo blood was also overestimated by the Coulter analyzer compared with the values calculated by expression (3). Accordingly, to avoid an overestimation of MCV, MCH and [HCHb], the mean corpuscular variables in the present report were derived from calculation using the Hct determined by centrifugation and [RBC] corrected using Eq. (A2) from the value determined by the Coulter analyzer (from centrifugation and hemocytometry) in expressions (1), (2) and (3) in the methodology.

MCV can also be independently validated. The elliptical blood cells of birds are deformed to some extent as they pass through the tissue capillaries, however, when the blood cells flow through the array of capillaries on the amniotic membrane, their elliptic form remains (Tazawa and Ono, 1974). This allows independent estimation of the size of the RBC. If we assume the RBC to be an elliptic disk with long and short axes of 14 μm and 6.5 μm , respectively, and thickness of 2 μm in a d13 embryo (Tazawa and Ono, 1974), the volume of a single RBC can be calculated as 143 μm^3 , which is almost identical to the MCV of d13 embryos determined by using Eq. (1) (Hct values determined by centrifugation and [RBC] calculated from Eq. (A2)) in the present determination.

The potential corrections for hematological parameters outlined above are potentially specific to our Beckman AcT10 Coulter counter.

It is possible – indeed likely – that different corrections would be necessary for a different Beckman counter or certainly for other brands of automated hematological measurement devices. Nonetheless, the procedures for validation of hematological variables outlined in this study can be used in future studies where the high throughput capabilities of a Coulter counter are desirable.

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