Journal of Zoo and Wildlife Medicine 47(1): 196–205, 2016

COMPARISON OF TOTAL LEUKOCYTE QUANTIFICATION METHODS IN FREE-LIVING GALAPAGOS TORTOISES (*CHELONOIDIS* SPP.)

Julie D. Sheldon, B.S., Nicole I. Stacy, D.V.M., Dr. Med. Vet., Dipl. A.C.V.P., Stephen Blake, Ph.D., Fredy Cabrera, and Sharon L. Deem, D.V.M., Ph.D., Dipl. A.C.Z.M.

Abstract: Reptile hematologic data provide important health information for conservation efforts of vulnerable wildlife species such as the Galapagos tortoise (*Chelonoidis* spp.). Given the reported discrepancies between manual leukocyte counts for nonmammalian species, two manual leukocyte quantification methods, the Natt and Herrick's (NH) and the Eopette (EO), were compared to white blood cell (WBC) estimates from blood films of 42 free-living, clinically healthy, adult female Galapagos tortoises. To investigate the effects of delay in sample processing, estimated WBC counts and leukocyte differentials were compared for blood films prepared at time of collection under field conditions (T0) to blood films prepared from samples that were stored for 18–23 hr at 4°C in the laboratory (T1). Passing-Bablok regression analysis revealed no constant or proportional error between the NH and WBC estimates (T0 and T1) with slopes of 1.1 and 0.9, respectively. However both constant and proportional errors were present between EO and WBC estimates (T0 and T1) with slopes of 3.1 and 2.7, respectively. Bland Altman plots also showed agreement between the NH and WBC estimates where the points fell within the confidence-interval limit lines and were evenly distributed about the mean. In contrast, the EO and WBC estimate comparisons showed numerous points above the upper limit line, especially at higher concentrations. WBC estimates obtained from T0 and T1 films were in agreement, whereas heterophil and monocyte percentages based on differentials were not. Cell morphology and preservation were superior in T0 blood films because thrombocytes exhibited swelling after storage, becoming difficult to differentiate from lymphocytes. In this study, the highest quality and most reliable hematologic data in Galapagos tortoises were obtained by combining immediate blood film preparation with the NH leukocyte quantification method and a confirmatory WBC estimate from the blood film.

Key words: *Chelonoidis* spp., Eopette, Galapagos tortoise, manual leukocyte counts, Natt and Herrick's, reptiles.

INTRODUCTION

Giant Galapagos tortoises (*Chelonoidis* spp.) are one of two remaining taxa of giant tortoises and are listed as Appendix I of the Convention on International Trade of Endangered Species (CITES).[12] Hunted to near extinction in the 16th–18th centuries, Galapagos tortoises remain threatened today because of poaching, habitat destruction, human encroachment, predation, and invasive species, including various parasites and pathogens.[13–15,17] Galapagos tortoises remain a conservation icon for the Galapagos Islands and are represented in zoological facilities worldwide. Understanding the health of these species is important for conservation of both free-living tortoises as well as those in human care in zoological collections.

Leukocyte quantification is an essential component of the complete blood count (CBC) and a powerful tool used for identification and characterization of hematologic disease processes in reptiles.[20] Because nonmammalian species have nucleated erythrocytes and thrombocytes, conventional automated hematology analyzers are not suitable, and less precise manual methods are necessary.[6,10,11,21] These include white blood cell (WBC) estimates obtained by blood film evaluation and various manual leukocyte-counting methods by hemocytometer;[24] however, discrepancies between these methods have been documented.[1,9] Because all manual methods have a number of potential sources for laboratory error,

From the University of California Davis School of Veterinary Medicine, One Shields Avenue, Davis, California 95616, USA (Sheldon); Department of Large Animal Clinical Sciences, University of Florida College of Veterinary Medicine, 2015 Southwest 16th Avenue, Gainesville, Florida 32608, USA (Stacy); Charles Darwin Foundation, Avenue Charles Darwin, Puerto Ayora, Ecuador (Blake and Cabrera); Max Planck Institute for Ornithology, Vogelwarte Radolfzell, Schlossallee 2, Radolfzell, D-78315, Germany (Blake); WildCare Institute, Saint Louis Zoo, One Government Drive, St. Louis, Missouri 63110, USA (Blake); and Institute for Conservation Medicine, Saint Louis Zoo, One Government Drive, St. Louis, Missouri 63110, USA (Deem). Correspondence should be directed to Ms. Sheldon (juliedsheldon@gmail.com).

a gold standard for leukocyte quantification in nonmammalian vertebrates currently does not exist. Because blood films are routinely prepared as part of the CBC, the WBC estimate obtained from a high-quality blood film with excellent cell preservation, a monolayer of blood cells, and without leukocyte or thrombocyte clumping, provides a readily performable tool for leukocyte quantification that should always be performed.[22] A high-quality blood film can be identified by even cell distribution, absence of cell lysis, and excellent cell preservation with visualization of nuclear and cytoplasmic detail. The comparison of the WBC estimate from a blood film can be helpful to confirm manual leukocyte counts performed using a hemocytometer. This can be useful in identification of potential sources of laboratory error if discrepancies are identified. Reporting results of both methods would be informative, along with potential issues that were present in the specimen, for instance, major cell clumping in the hemocytometer, or cell lysis on the blood film. The method that was used to calculate leukocyte concentrations should also be clearly indicated.

In addition to manual hematology methods, proper sample collection, handling, and processing techniques also contribute to the quality of hematologic data. To obtain the most accurate results, it is recommended that blood films be prepared and samples be analyzed as soon as possible after collection to avoid storage artifacts, such as lysis, clumping, and degeneration of blood cells.[6,7,22]

The first objective of this study was to compare the Natt and Herrick's (NH) and the Eopette (EO) leukocyte quantification methods to the WBC estimate from blood films by performing all three methods on blood collected from free-living Galapagos tortoises. Because of conflicting reports of agreement between the NH and EO methods, the WBC estimate from the blood film was set as the standard for comparison in an effort to determine which hemocytometer count was more accurate. The second objective was to compare WBC estimates and WBC differentials from blood films prepared immediately upon collection in the field (T0) to those obtained from blood films prepared later under laboratory conditions (T1) to investigate potential effects from delay in processing. It was hypothesized that total leukocyte counts using each manual leukocyte quantification method would be in agreement, and that T0 and T1 WBC estimates and differentials would also be in agreement.

MATERIALS AND METHODS

This study was conducted as part of an ongoing giant tortoise ecology and health project on Santa Cruz Island of the Galapagos, located 1,000 km west of continental Ecuador in the Pacific Ocean. Santa Cruz is one of six islands in the archipelago that Galapagos tortoises currently inhabit, and is home to the two populations of free-living tortoises, located in regions called La Reserva and Cerro Fatal, used in this study (Fig. 1).[3] Blood samples were collected from 44 free-living adult female tortoises that were clinically healthy upon veterinary physical examination and distributed widely across their range on Santa Cruz. All animal-handling procedures followed the guidelines of the Galapagos National Park Service and IACUC protocol 121202 of the State University of New York, College of Environmental Science and Forestry.

To facilitate blood sample collection, tortoises were positioned in dorsal recumbency. Five milliliters of blood were collected from the brachial vein of each tortoise with a heparinized 6-ml syringe and a 20-gauge 1.5-in. needle. If the blood sample was lymph-contaminated during collection, it was discarded and a fresh sample was collected. Two blood films were prepared immediately in the field upon sample collection (T0) on glass slides, air dried, fixed for 5 min in high-quality methanol (Fixative 1, Jorvet™ Dip Quick Stain Kit, Jorgensen Laboratories, Loveland, Colorado 80538, USA), air dried, labeled with patient identification, and stored in a slide box. The remaining blood was immediately transferred to lithium heparin blood collection tubes (Becton, Dickinson and Company, 1 Becton Drive, Franklin Lakes, New Jersey 07417, USA), kept cool during the remainder of the field day, and stored at 4°C overnight. Two blood films were prepared from each blood sample18–23 hr later in an air-conditioned laboratory (T1), fixed for 5 min in high-quality methanol, and air dried. All blood films were stained with the use of the Dip Quick Stain Kit® (Jorgensen Laboratories, Loveland, Colorado 80538, USA) following manufacturer's instructions.

To reduce interanalyst variability, one of the authors (JDS) performed all leukocyte quantification methods and leukocyte differentials. Total leukocyte counts were performed in the laboratory per manufacturer's instructions on each sample using the Natt-Herricks-TIC® 1 : 200 plus (Bioanalytic GmbH, Waldmatten 10-13, D-79224, Umkirch/Freiburg, Germany) staining kit

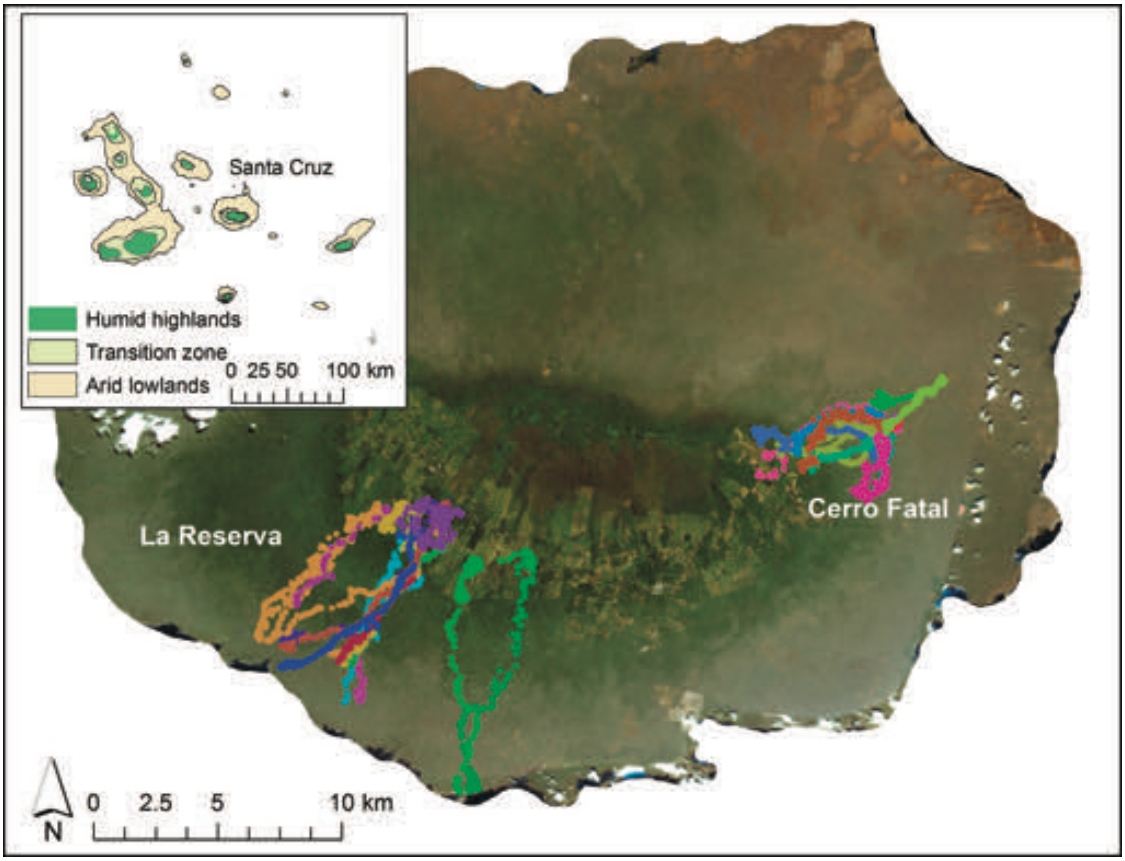

Figure 1. Global positioning system tracks of tagged tortoises from La Reserva and Cerro Fatal Galapagos tortoise populations on Santa Cruz Island. The insert illustrates the location of Santa Cruz and the two tortoise populations within the Galapagos archipelago, and major vegetation types associated with elevation gradients.

charged into a Neubauer hemocytometer, and calculated with the use of the following equation:

WBC/μl = leukocytes counted in nine squares × dilution/counting volume

= leukocytes counted in nine squares × 200/0.9.

Total leukocyte counts were also performed in the laboratory per manufacturer's instructions on each sample with the use of the Eopette™ (Exotic Animal Solutions, Inc., 3516 Sharon Lane, Hueytown, Alabama 35223, USA) staining kit charged into a Neubauer hemocytometer and calculated with the use of the following equation:

WBC/μl = (cells counted in 18 squares × 1.1 × 16 × 100)/(% heterophils + % eosinophils from differential)

WBC estimates were performed in the laboratory on the highest quality blood film from each tortoise and each time point (T0 and T1) with the use of a 40× objective lens. Ten replicate counts were performed in 10 different fields of the monolayer and the results were averaged with the use of the following equation:[22,24]

WBC/μl = (average no. of cells per field) × (objective power)^2

One-hundred-cell leukocyte differentials were performed on the highest-quality blood film from each tortoise and each time point (T0 and T1) with the use of a 100× objective lens under oil immersion.

The distribution of each hematologic data set was evaluated with the use of the Shapiro-Wilk test. The mean ± standard deviation was reported for normally distributed data; whereas the median, 10%, and 90% quartiles were reported for nonnormally distributed data. These statistical analyses were performed with GraphPad Prism® 2015 v. 6.0f (GraphPad, La Jolla, California, USA). Passing-Bablok regression curves were used to determine statistical agreement or disagreement. This test assumes that the data set contains measurement errors, nonnormal distri-

Table 1. Hematologic values from 42 free-living, clinically healthy, adult female Galapagos tortoises (*Chelonoidis* spp.) with the use of three methods and two sample processing times.

Parameter ($\times 10^3$ cells/μl)	White blood cell estimate mean ± SD,[a] or median (range or 10–90% quartile)	NH[b] mean ± SD, or median (range or 10–90% quartile)	EO[c] Mean ± SD, or median (range or 10–90% quartile)
Total leukocytes			
T0[d]	8.85 ± 3.29[e] (2.56–17.1)	6.68 ± 3.32[e] (1.11–14.8)	10.4 (3.03–24.1)
T1[f]	9.23 ± 3.77[e] (1.60–18.2)		10.5 (3.77–24.5)
Heterophils			
T0	1.18 (0.339–2.44)	0.821 (0.194–2.29)	1.09 (0.463–2.98)
T1	0.659 (0.110–1.66)	0.417 (0.067–1.14)	0.730 (0.218–2.50)
Lymphocytes			
T0	5.00 (2.60–9.90)	3.84 (1.44–8.36)	6.80 (1.64–16.3)
T1	6.39 ± 2.90[f] (0.992–14.0)	4.59 ± 2.47[f] (0.688–10.6)	7.09 (2.62–20.9)
Monocytes			
T0	0.351 (0.064–0.759)	0.193 (0.035–0.590)	0.314 (0.053–0.885)
T1	0.180 (0.0–0.395)	0.122 (0.0–0.303)	0.196 (0.0–0.830)
Eosinophils			
T0	0.896 (0.195–1.67)	0.556 (0.109–1.64)	1.02 (0.190–1.63)
T1	1.14 (0.251–2.58)	0.851 (0.149–2.06)	1.45 (0.511–3.03)
Basophils			
T0	0.629 ± 0.402[f] (0.0–1.50)	0.396 (0.066–1.03)	0.589 (0.116–2.21)
T1	0.558 (0.193–1.14)	0.396 (0.105–0.787)	0.680 (0.244–1.85)

[a] SD: standard deviation.
[b] NH: Natt-Herricks-TIC® 1 : 200 plus (Bioanalytic GmbH, Waldmatten 10-13, D-79224, Umkirch/Freiburg, Germany).
[c] EO: Eopette™ (Exotic Animal Solutions, Inc., 3516 Sharon Lane, Hueytown, Alabama 35223, USA).
[d] T0: blood films prepared at time of collection under field conditions.
[e] Data set was normally distributed and presented as mean ± SD (range). Data without footnote were not normally distributed and presented as median (10–90% quartile).
[f] T1: blood films prepared 18–23 hr later under laboratory conditions.

bution, arbitrary sampling distribution and imprecision. A significant disagreement between two methods was considered present if the 95% confidence interval limit (CL) for the y intercept did not include the value 0, or if the 95% CL for the slope did not include the value 1.[2,18] Bland Altman plots were used to describe the bias between methods.[4] Passing-Bablok and Bland Altman plots were generated using Microsoft® Excel® for Mac 2011 (Microsoft, Redmond, Washington, USA).

RESULTS

Results from two animals were excluded from the final statistical analysis due to poor-quality blood samples and blood film preparations, providing a final sample size of 42 blood samples from 42 free-living, clinically healthy, adult female tortoises. Total leukocyte count results from all three quantification methods (WBC estimate, NH, and EO) and leukocyte differentials from both blood film preparation times (T0 and T1) are reported in Table 1. For the NH total leukocyte count in Table 1, only one set of values is reported, because the NH does not rely on a blood film to calculate a result, unlike the WBC estimate and EO. Thus, the NH was only performed once in the laboratory on each of the 42 blood samples and there are no NH results for time T0. The absolute values for each leukocyte type were calculated by multiplying the percent from the blood film differential times the total leukocyte count (Table 1). This was performed for each blood film (T0 and T1) and for each leukocyte quantification method (WBC estimate, NH, and EO).

The results of the Passing-Bablok regression analyses, including the slopes, intercepts, and upper and lower 95% CLs are presented in Table 2. Passing-Bablok regression curves and Bland Altman plots of the T0 WBC estimate vs. the NH and T0 EO are depicted in Figure 2. The total leukocyte counts obtained via the NH method were in agreement with the WBC estimates with regression slopes of 1.1 for T0 and 0.93 for T1 (Fig. 2a and Table 2). In contrast, the EO method showed both proportional and systematic differences when compared to WBC estimates with regression slopes of 3.1 for T0 and 2.7 for T1 (Fig. 2b and Table 2). Bland Altman plots revealed a negative bias between the NH and T0 WBC estimate (–2,172 ± 5,800 cells/μl) and a larger

Table 2. Summary of Passing-Bablok regression analyses for blood samples collected from 42 free-living, clinically healthy, adult female Galapagos tortoises (*Chelonoidis* spp.) with the use of three leukocyte quantification methods. Leukocyte differentials were performed on both sets of blood films for each sample to obtain the percent of each cell type.

Methods compared	Slope	Lower 95% CL[a] of slope	Upper 95% CL of slope	Intercept	Lower 95% CL of intercept	Upper 95% CL of intercept
NH[b] vs. T0[c] WBC est.	1.1	0.76	1.6	−2,695	−6,840	55.2
NH vs. T1[d] WBC est.	0.9	0.67	1.1	−1,066	−2,997	577.9
T0 EO[e] vs. T0 WBC est.[f]	3.1	1.8	6.8	−15,823	−44,277	−5,377
T1 EO vs. T1 WBC est.[f]	2.7	1.7	4.5	−10,826	−26,698	−1,858
T0 WBC est. vs. T1 WBC est.	0.8	0.63	1.0	1,382	−386	2,624
Heterophil % T0 vs. T1[f]	1.9	1.2	3.4	−0.44	−9.3	3.8
Lymphocyte % T0 vs. T1	1.1	0.8	1.6	−15	−47	8.6
Monocyte % T0 vs. T1[f]	2.0	1.5	4.0	−1.0	−4.25	0.5
Eosinophil % T0 vs. T1	0.9	0.67	1.0	−0.4	−2.5	1.3
Basophil % T0 vs. T1	1.3	0.86	2.0	−1.9	−6.5	1.7

[a] CL: confidence interval limit.
[b] NH: Natt-Herricks-TIC® 1 : 200 plus (Bioanalytic GmbH, Waldmatten 10-13, D-79224, Umkirch/Freiburg, Germany).
[c] T0: blood films prepared at time of collection under field conditions.
[d] T1: blood films prepared 18–23 hr later under laboratory conditions.
[e] EO: Eopette™ (Exotic Animal Solutions, Inc., 3516 Sharon Lane, Hueytown, Alabama 35223, USA).
[f] Methods were in disagreement because the 95% CL of the slopes did not include the value 1, and/or the CL of the intercept did not include the value 0.

positive bias between the T0 EO and T0 WBC estimate (2,981 ± 17,766 cells/μl) (Fig. 2c, d). The Passing-Bablok regression curves and Bland Altman plots illustrate the marked difference in agreement between the NH and EO results when compared to the WBC estimates, where the points for the former are evenly distributed about the mean and are within the confidence interval limit lines, whereas the points for the latter show greater dispersal about the mean (Fig. 2c, d).

According to the Passing-Bablok analysis, T0 WBC estimates were in agreement with T1 WBC estimates with a regression slope of 0.80 (Fig. 3a). The Bland Altman plot revealed a small positive bias (378 ± 4,958 cells/μl) with most points falling within the confidence-interval limit lines and even distribution about the mean (Fig. 3b). Although the total leukocyte counts were in agreement, heterophil and monocyte differential percentages, with regression slopes of 1.9 and 2.0 respectively, were in disagreement between T0 and T1 blood films. Lymphocyte, eosinophil, and basophil differential percentages were in agreement and had regression slopes of 1.1, 0.85, and 1.3, respectively (Table 2).

Blood films prepared at the time of blood collection (T0) exhibited superior blood cell preservation and more distinctive cell characteristics compared to blood films prepared 18–23 hr later in the laboratory (T1). In T1 films, thrombocytes appeared more morphologically similar to lymphocytes than in T0 films (Figs. 4, 5). Thrombocytes from T0 blood films possessed classic morphological features. They were oval or spindle shaped, with elliptical nuclei, wispy cytoplasm (Fig. 4A, B), and appeared in small clumps. Lymphocytes had distinct round nuclei with scant colorless or pale basophilic cytoplasm (Fig. 4C). However, thrombocytes in T1 blood films were round with scant cytoplasm, appearing more similar to lymphocytes (Fig. 5).

DISCUSSION

Significant discrepancies were observed between the NH and EO leukocyte quantification methods when applied to a population of free-living, clinically healthy, adult female Galapagos tortoises. In this study, the NH method showed better agreement with the WBC estimate than did the EO method. The WBC estimate is easily performed on high-quality blood films. Although the WBC estimate method may lack accuracy and precision depending on the quality of the stained blood film, it is an excellent means of quality control for confirming and comparing results from manual cell counting methods.[22]

Other reptile hematology studies have also documented discrepancies between manual leukocyte quantification methods.[1,9] Total leukocyte counts of foraging and stranded loggerhead sea turtles (*Caretta caretta*) were performed with the use of both the WBC estimate and the Eosinophil

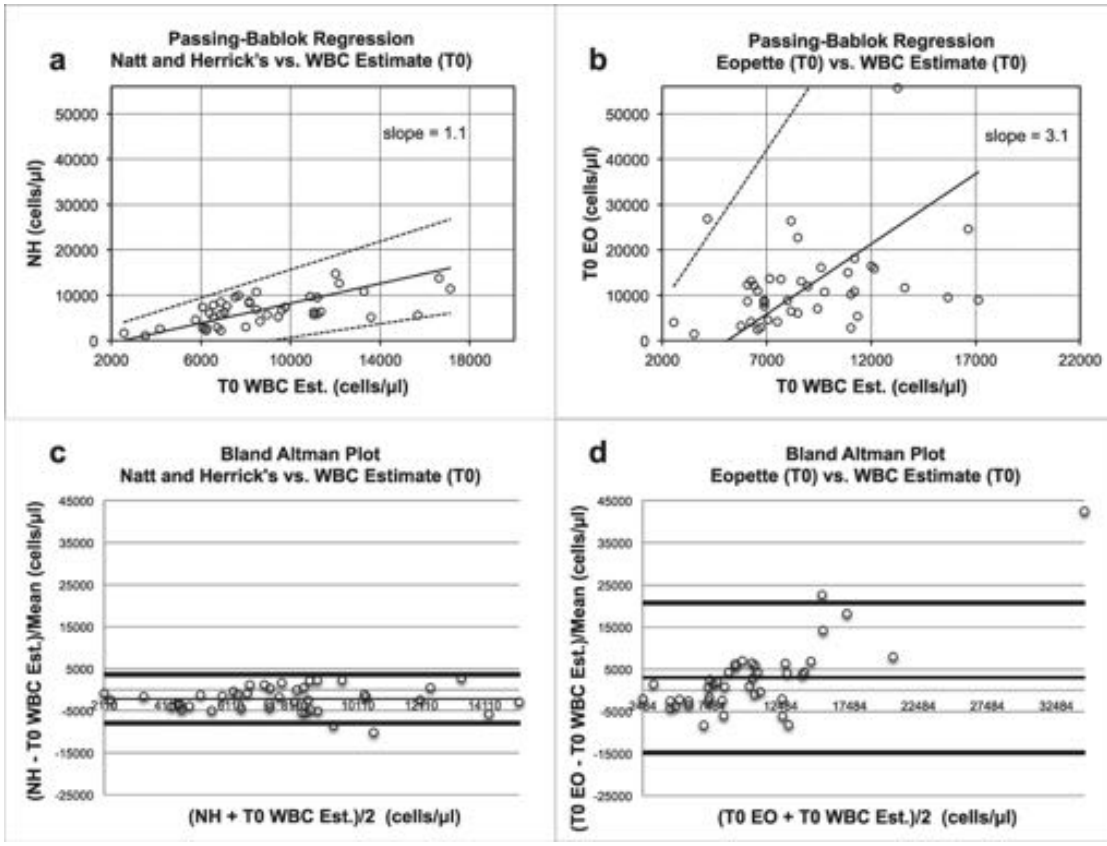

Figure 2. (**a**, **b**) Passing-Bablok regression curves comparing the white blood cell (WBC) estimate (T0) (x axis) to the Natt and Herrick's (NH) (y axis of graph a) and Eopette (EO) (y axis of graph b). Solid line, mean regression slope; dashed lines, 95% confidence intervals. (**c**, **d**) Bland Altman plots comparing the biases between the NH (c) and EO (d) with regards to the WBC estimates. Thin line, mean difference between methods; thick lines, mean ± 2 SD.

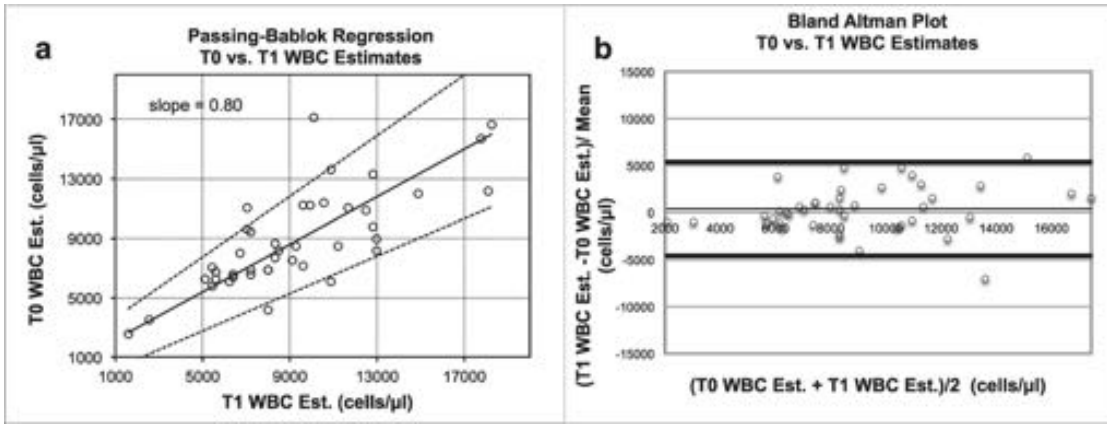

Figure 3. (**a**) Passing-Bablok regression curve comparing the white blood cell (WBC) estimates obtained from blood films prepared in the laboratory 18–23 hr after sample collection (T1) (x axis) to the WBC estimates obtained from blood films prepared immediately in the field (T0) (y axis). Solid line, mean regression slope; dashed lines, 95% confidence intervals. (**b**) Bland Altman plot comparing the bias between T0 and T1 WBC estimates. Thin line, mean difference between methods; thick lines, mean ± 2 SD.

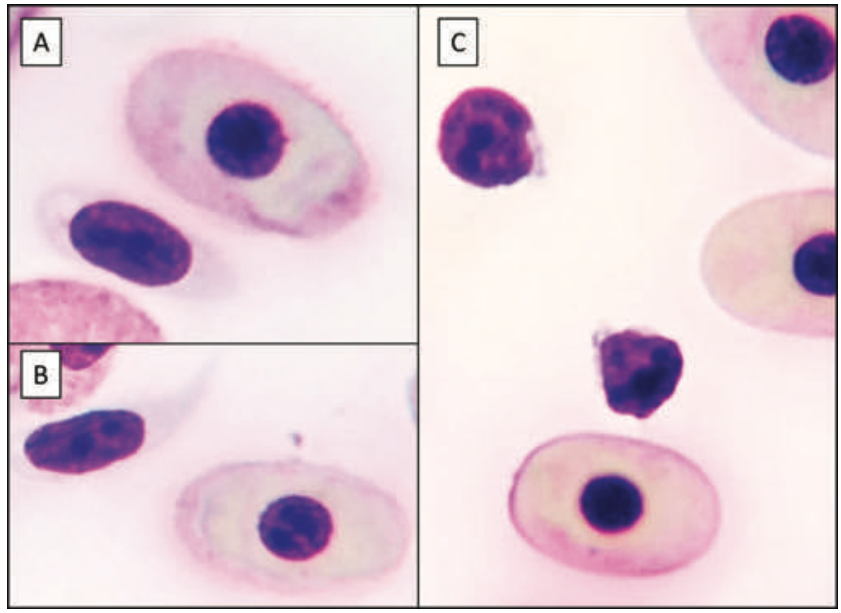

Figure 4. Two thrombocytes (**A**, **B**) and two lymphocytes (**C**) from blood films prepared immediately in the field (T0) depicting expected cell morphology for thrombocytes (elliptical shape, elongated nucleus with clumped chromatin, and moderate amount of clear cytoplasm) and lymphocytes (round shape, high nuclear to cytoplasmic ratio, round nucleus with clumped chromatin, scant amount of pale basophilic or clear cytoplasm). Dip Quick® stain. ×100 objective.

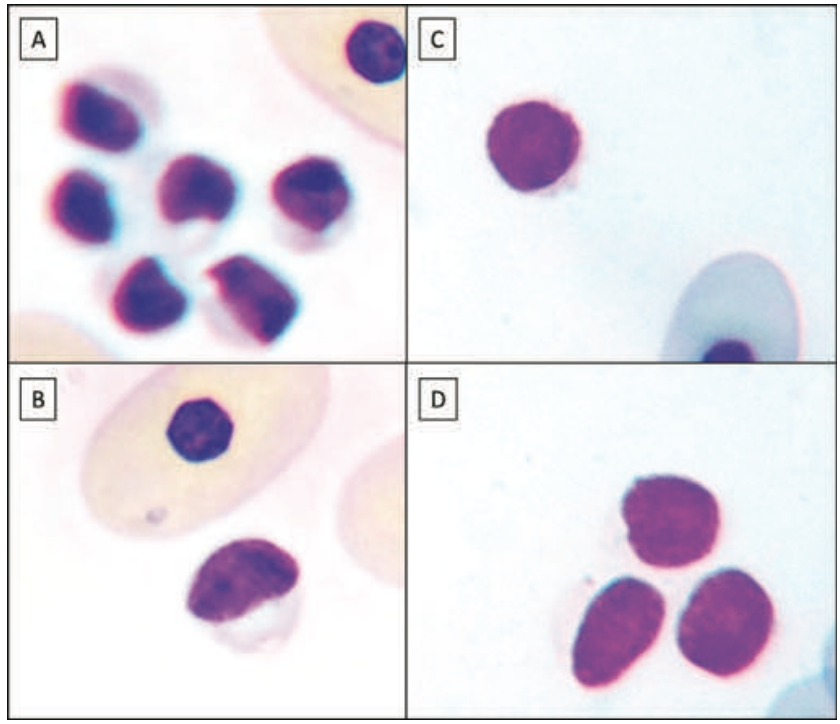

Figure 5. Thrombocytes (**A**, **B**, **D**) and lymphocyte (**C**) from blood films prepared 18–23 hr later in the laboratory (T1) depicting similar morphologic characteristics, likely due to cell swelling during storage. Dip Quick® stain. ×100 objective.

Unopette (similar to EO). The resulting WBC concentrations were not in agreement, and results of each method had to be evaluated separately.[9] In addition, large discrepancies were documented between total leukocyte counts of various avian and reptile species when comparing the NH and Eosinophil Unopette methods; the NH was reportedly more precise than the Eosinophil Unopette.[1]

A number of reasons may explain the discrepancies between the NH and EO methods. Laboratory error can occur when using either method, including improper dilution or mixing of stain and the blood sample, incorrect charging of the hemocytometer, and inaccurate cell identification.[20] Another consideration is that the EO equation, designed for eosinophil counts in human blood, may not accurately extrapolate for the unstained lymphocytes and monocytes.[1,7,8] This is important because chelonians reportedly have higher concentrations of lymphocytes than mammals.[19,23]

In addition, NH and EO methods both provide advantages and disadvantages that need to be considered. The NH diluent contains methyl violet dye and is referred to as a direct method because all cell types are visible. The leukocytes appear darker purple than do the erythrocytes and thrombocytes, and the total leukocyte count is determined independent of the blood film differential.[16] However, because all blood cells are stained, morphologically similar thrombocytes and lymphocytes must be differentiated in the hemocytometer chamber, which can be challenging and require substantial training and practice.[7,20,22]

The EO method uses phloxine B dye that only stains the acidophilic cells (heterophils and eosinophils) dark orange, making cell differentiation unnecessary and the method technically easier to perform.[6,8,22] The EO method is also described as an indirect method, because it requires a calculation that includes the percent heterophils and eosinophils from the differential to obtain a total leukocyte count.[5] This requirement adds a possible source of error if the blood film is not of high quality or if cells are misidentified during the leukocyte differential.

In our assessment, the NH was a more reliable manual leukocyte counting method than the EO because it agreed with the WBC estimate. Confirmation of hemocytometer leukocyte counts, as performed in this study, with a WBC estimate obtained from a high-quality blood film, is recommended to help identify any inaccurate results from potential laboratory error associated with manual counting methods.[22] Should a significant discrepancy be found, the manual leukocyte count may be repeated and/or the WBC estimate reported if the hemocytometer count is compromised (e.g., large cell aggregates). In addition, if the cause of discrepant results cannot be identified, interpretation in the context of clinical findings may be helpful to reach a diagnostic conclusion and to plan for further laboratory tests.

This study confirmed previous recommendations for immediate blood film preparation to ensure accurate cellular differentiation.[6,7,22] Although WBC estimates obtained from blood films prepared at the time of collection (T0) were in agreement with those prepared later (T1), the differentials did not agree and the cell morphology altered over time. The thrombocytes were easily differentiated from lymphocytes in T0 blood films, whereas those observed in T1 blood films were rounded, likely due to swelling during storage, and cell differentiation required more time and effort. In addition, there was less cell lysis in T0 blood films. This could explain why the leukocyte differentials were inconsistent between T0 and T1 blood films.

Potential sources of error in this study include possible variation in T0 blood film quality due to environmental challenges (e.g., humidity, rain, temperature), potential laboratory error in loading the hemocytometer chamber for either the EO or NH method, and differentiating thrombocytes and lymphocytes in the hemocytometer during the NH counting method. In order to minimize any potential errors or variations, all blood sample collection, handling, and laboratory procedures during this study were performed in a consistent manner and based on the most accurate laboratory standards available.

Our study compared leukocyte quantification methods commonly used in reptiles with the use of a population of free-living, clinically healthy, adult female Galapagos tortoises, and confirmed the well-documented concerns of accuracy with manual methods. The NH agreed with the WBC estimate and was deemed more accurate in this study, which was consistently performed by a person skilled in cell differentiation after training by a board-certified clinical pathologist (NIS). The EO was not in agreement with the WBC estimate, despite its ease of use. Furthermore, our results align with previous recommendations to compare manual hemocytometer methods with a WBC estimate obtained from a well-prepared

blood film.[20] High-quality blood films provide a wealth of information in addition to the WBC estimate, such as erythrocyte density and morphology, leukocyte differential and morphology, thrombocyte concentration and morphology, and identification of hemoparasites or other infectious agents.[7,20,22,24] It is recommended to prepare and fix blood films immediately upon collection when working in field situations. High-quality results due to immediate processing made up for the logistical and environmental challenges of preparing blood films in the field. This is recommended over processing blood samples later upon return to a climate-controlled laboratory. Practicing consistent sample handling and processing protocols provide the most accurate hematologic data for diagnostic information and establishment of reference intervals.

Acknowledgments: The authors thank the University of California Davis School of Veterinary Medicine Students in Advanced Training and Research (STAR) Program and the University of California Davis School of Veterinary Medicine Global Programs International Externship Travel Fund for financially supporting travel to the Galapagos Islands during the summer of 2014 (for JDS), the Charles Darwin Foundation administration for logistical assistance with travel and housing (for JDS), Freddy Villamar and Walter Ernest for assisting in the field, Bioanalytic GmbH for donating Natt-Herricks-TIC kits, Dr. Phillip Kass of the University of California Davis for providing statistical assistance, and Dr. Walter Boyce for serving as the UC Davis STAR Program faculty mentor. The authors also thank the United States National Science Foundation (Grant 1258062), Max Planck Institute for Ornithology, e-obs GmbH, the National Geographic Society, the Swiss Friends of Galapagos, the Galapagos Conservation Trust, and the Saint Louis Zoo Institute for Conservation Medicine for funding the Galapagos Tortoise Movement Ecology Programme.

LITERATURE CITED

1. Arnold J. White blood cell count discrepancies in Atlantic loggerhead sea turtles: Natt-Herricks vs. eosinophil Unopette. In: Proc Assoc Zoo Vet Tech; 1994. p. 15–22.

2. Bilic-Zulle L. Comparison of methods: Passing and Bablok regression. Biochem Med (Zagreb). 2011; 21(1):49–52.

3. Blake S, Guezou A, Deem SL, Yackulic CB, Cabrera F. The dominance of introduced plant species in the diets of migratory Galapagos tortoises increases with elevation on a human-occupied island. Biotropica. 2015;47(2):246–258.

4. Bland J, Altman D. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986;1:307–310.

5. Campbell T. Clinical pathology of reptiles. In: Mader DR (ed.). Reptile medicine and surgery. Marathon (FL): Elsevier; 2006. p. 453–470.

6. Campbell T. Hematology of birds. In: Thrall MA, Weiser G, Allison R, Campbell T (eds.). Veterinary hematology and clinical chemistry. 2nd ed. Ames (IA): John Wiley & Sons; 2012. p. 238–276.

7. Campbell T. Hematology of reptiles. In: Thrall MA, Weiser G, Allison R, Campbell T (eds.). Veterinary hematology and clinical chemistry. 2nd ed. Ames (IA): John Wiley & Sons; 2012. p. 277–297.

8. Costello R. A unopette for eosinophil counts. Am J Clin Pathol. 1970;54:249–250.

9. Deem SL, Norton TM, Mitchell M, Segars A, Alleman AR, Cray C, Poppenga RH, Dodd M, Karesh WB. Comparison of blood values in foraging, nesting, and stranded loggerhead turtles (*Caretta caretta*) along the coast of Georgia, USA. J Wildl Dis. 2009;45(1):41–56.

10. Dein FJ, Wilson A, Fischer D, Langanberg P. Avian leukocyte counting using the hemocytometer. J Zoo Wildl Med. 1994;25(3):432–437.

11. Harvey JW. Hematology procedures. In: Harvey J (ed.). Veterinary hematology: a diagnostic guide and color atlas. Saint Louis (MO): Elsevier; 2012. p. 11–32.

12. International Union for Conservation of Nature and Natural Resources (IUCN)™ 2014. The IUCN Red List of Threatened Species. http://www.iucnredlist.org/. Accessed 19 May 2015.

13. Jacobson ER. Causes of mortality and diseases in tortoises: a review. J Zoo Wildl Med. 1994;25:2–17.

14. MacFarland CG, Villa J, Toro B. The Galapagos giant tortoises (*Geochelone elephantopus*) part I: status of the surviving populations. Biol Conserv. 1974;6(2): 118–133.

15. Marquez C, Wiedenfeld DA, Landazuri S, Chavez, J. Human-caused and natural mortality of giant tortoises in the Galapagos Islands during 1995–2004. Oryx. 2007;41(3):337–342.

16. Natt MP, Herrick CA. A new blood dilutent for counting the erythrocytes and leucocytes of the chicken. Poult Sci. 1952;31:735–738.

17. Ontaneda S, Trueba G, Sevilla A, Sevilla E. Informe del analisis microbiologico de muestras de tortugas [Report of microbiological analysis of samples of turtles]. Charles Darwin Foundation, Puerto Ayora, Galapagos, Ecuador. 2001.

18. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies

in clinical chemistry, part I. J Clin Chem Clin Biochem. 1983;21(11):709–720.

19. Rousselet E, Stacy NI, LaVictoire K, Higgins BM, Tocidlowski ME, Flanagan JP, Godard-Codding CA. Hematology and plasma biochemistry analytes in five age groups of immature, captive-reared loggerhead sea turtles (*Caretta caretta*). J Zoo Wildl Med. 2013; 44(4):859–874.

20. Stacy NI, Alleman AR, Sayler KA. Diagnostic hematology of reptiles. Clin Lab Med. 2011;31(1):87–108.

21. Stacy NI, Barnhard K, Fry M. Comparative hematology. In: Greer J, Arber D, Glader B, List A, Means Jr. A, Paraskevas F, Rodgers G, Foerster J (eds.). Wintrobe's clinical hematology. 13th ed. Philadelphia (PA): Lippincott Williams & Wilkins; 2013.

22. Strik NI, Alleman AR, Harr KE. Circulating inflammatory cells. In: Jacobson ER (ed.). Infectious diseases and pathology of reptiles: color atlas and text. Boca Raton (FL): CRC Press; 2007. p. 167–218.

23. Sypek J, Borysenko M. Reptiles. In: Rowley AF, Ratcliffe NA (eds.). Vertebrate blood cells. Cambridge (UK): Cambridge University Press; 1988. p. 211–256.

24. Weiss D. Uniform evaluation and semiquantitative reporting of hematologic data in veterinary laboratories. Vet Clin Pathol. 1984;13(2):27–31.

Received for publication 14 July 2015