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Hematological parameters of the Bolson tortoise *Gopherus flavomarginatus* in Mexico

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Abstract. We present findings of our preliminary study to determine biometry and blood chemistry values of healthy wild individuals of the critically endangered Bolson tortoises (*Gopherus flavomarginatus*) in Mexico. Given the absence of previously published data regarding hematology parameters for this species, these results represent an important base for additional research. Hematocrit determination, stains, and cell counts were performed, as well as 18 parameters of blood chemistry. Values of biometry and blood chemistry for *G. flavomarginatus* were similar to reference values those already reported for *G. agassizii*, *G. polyphemus,* and *G. berlandieri*. These similarities reflect the phylogenetic relationships among these species. However, slight differences may point to particular adaptations that each has developed to their own habitat, and so point to questions to be addressed with future research.

Keywords. Chelonia, lymphocyte, desert, Mapimí Biosphere Reserve.

Hematological analysis is a common technique for evaluating the health status of wild animals (Campbell, 2004; Tavares-Dias et al., 2009). Blood biometry and chemistry of terrestrial, semiaquatic and marine tortoises have been described already (Stacy et al. 2011; Campbell, 2015). However, there are still species of ecological importance whose blood information is unavailable. This is the case of *Gopherus flavomarginatus*, a tortoise endemic to Mexico and considered the largest terrestrial tortoise in North America, with a carapace length of up to 40 cm (Morafka et al., 1989). This species is in danger of extinction according to the Official Mexican Standard 059 (SEMARNAT, 2010) and critically endangered according to the IUCN red list (Kiester et al., 2018). Its geographical distribution is restricted to the Mapimí Bolson in the Mexican Chihuahuan Desert, where it currently has protected status within the Mapimí Biosphere Reserve (CONANP, 2006). Tortoises of the genus *Gopherus* are keystone organisms for the ecosystems in which they live because, due to their feeding habits (herbivory), they perform the ecological function of seed dispersal (Carlson et al., 2003), and because the burrows that they excavate are deep and provide shelter for at least 300 species of invertebrates and 60 of vertebrates (Lips, 1991). Due to the importance of protecting this tortoise, we conducted this preliminary study to determine biometry and blood chemistry values of healthy wild individuals. This information will serve as a basis for future hematological studies carried out on this tortoise.

From May 2015 to September 2017, we captured 44 adult individuals of *G. flavomarginatus* (16 males and 28 females) within the Mapimí Biosphere Reserve in Mex-

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ico (26°00' and 26°10'N, and 104°10' and 103°20'W). Blood samples were collected from the subvertebral vein. Three milliliters of blood were collected from each specimen; one milliliter was placed in a Vacutainer® tube with lithium heparin as anticoagulant and the rest in a red Vacutainer® tube. The tubes were stored in a cooler at a temperature of approximately 4 °C. Each tortoise was determined to be healthy following the observation protocols of Jacobson (2014) and USFWS (2016). Tortoises were then released at the site of their capture. Biometric analysis of blood was performed following the protocols suggested by Thrall et al. (2006) and Turgeon (2012). The volume percentage of red cells (Hematocrit, Ht in%) was determined in the blood contained in the green Vacutainer® tube, using the microhematocrit technique. The total number (TR) and percentage (PR) of red cells were obtained for each blood sample. The formula used to obtain hematocrit values was Ht% = (PR/TR) × 100. Hemoglobin concentration was quantified with the Drabkin colorimetric method, using the Spinreact® commercial kit, in a VetTest® spectrophotometer. The reaction product was centrifuged (12,000 g × 5 min) to precipitate the nucleus of the cells and keep them from distorting the color to be measured (Thrall et al., 2006).

Erythrocyte and leukocyte counts were carried out using a Thoma pipette with red bead and a Neubauer hemocytometer with Natt and Herrick's stain (Thrall et al., 2006). The erythrocyte count was carried out under 10× magnification, on both sides of the Neubauer chamber, in the four corner squares and the center square within the large center square of the chamber. The following formula was applied (Kemal, 2014): erythrocytes ($\times 10^6$ ul) = mean erythrocytes × 10000. The leukocyte count was carried out under 40x magnification in the nine large squares on both sides of the Neubauer chamber. The following formula was applied: leukocytes ($\times 10^3$ ul) = mean leukocytes × 50 (Kemal, 2014). The erythrocyte indices were calculated according to the formulas described by Ball (2014) and Kemal (2014): mean corpuscular volume, MCV (fL) = Ht% × 10/erythrocytes ($\times 10^6$ ul); mean corpuscular hemoglobin, MCH (pg) = (Hg × 10)/erythrocytes ($\times 10^6$ ul); mean corpuscular hemoglobin concentration, MCHC (g/dl) = (Hg × 100)/Ht%. Two smears were prepared from each sample on glass slides using Wright's dye (Analytyka®). One hundred leukocytes were counted in the body of the smear of each of the two slides. The average of both slides was obtained, and the results were expressed as the proportion of each cell type (relative differential count). To obtain the absolute differential count, the total value of leukocytes ($\times 10^3$ cel/ul) was multiplied by the average percentage of each type of leukocyte and the result was divided by 100 (Thrall et al., 2006).

The H:L ratio was obtained by dividing the percentage of heterophiles by the percentage of lymphocytes in each sample (Davis et al., 2008). Blood from the red Vacutainer® tube was centrifuged at 12,000 g for five minutes; the serum was separated from the red cells and placed in a sterile plastic tube. No hemolysis was observed in any sample. The following parameters were analyzed: glucose, uric acid, urea, blood urea nitrogen (BUN), creatinine, total proteins, albumin, globulins, cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), chlorine, sodium, calcium, phosphorus and osmolality (mOsm/kg=1.86 (Na [mmol/L]) + glucose [mg/dL]/18 + BUN [mg/dL]/2.8 + 9). Human serum Spintrol H Spinreact® was used as control. These analyzes were performed in a VetTest® spectrophotometer with Spinreact® reagents. Descriptive statistics (mean, standard error, median, standard deviation, minimum, and maximum) were obtained in PAST 3.14 (Hammer et al., 2001).

Blood biometry results for *G. flavomarginatus* are shown in Table 1 and for blood chemistry in Table 2. A comparison among biometry and chemistry variables of *G. flavomarginatus* and other *Gopherus* species are shown in Tables 3 and 4, respectively. Blood biometric and chemistry values obtained for *G. flavomarginatus* were similar to those reported for *G. agassizii* (Christopher et al., 1999; Dickinson et al., 2000), *G. polyphemus* (Taylor and Jacobson, 1982), and *G. berlandieri* (Teare, 2013a) (Tables 3 and 4). Christopher et al. (1999) reported that the most abundant leukocytes in *G. agassizii* are heterophils, followed by lymphocytes, basophils, and finally eosinophils and monocytes. In our findings for *G. flavomarginatus*, the order of abundance of the leukocyte cells was similar to the one mentioned by Duguy (1970), with lymphocytes as the most abundant leukocytes (males = 53.43%, 4.79×10^3 cel/ul; females = 44.03%, 4.20×10^3 cel/ul). This agrees with the findings of Diaz-Figueroa (2005) for *G. polyphemus*, and other authors who indicate that lymphocytes are predominant in the peripheral blood of most reptile species (Davis et al.2008; Stacy et al., 2011).

Heterophils were the second most abundant leukocyte cells in *G. flavomarginatus*. In general, the abundance of this type of leukocyte in reptiles ranges from 30 to 45% (Duguy, 1970; Frye, 1991); however, in tortoises it can reach up to 50% (Alleman et al., 1992; Christopher et al. 1999). Basophils were the third most abundant leukocytes in the blood of the Bolson tortoise. Alleman et al., (1992), and Duguy, (1970) estimated that the percentage of basophils in healthy terrestrial and aquatic tortoises can be higher than 40%. In *G. flavomargi-*

Table 1. Descriptive statistics of blood biometric variables from male/female *Gopherus flavomarginatus*. SE = standard error, SD = standard deviation, Min = minimum, Max = maximum.

Variable	Mean	SE	Median	SD	Min	Max
Hematocit (%)	25.35/23.48	1.41/0.77	25.30/23.61	5.64/4.09	16.47/17.89	35.62/33.33
Hemoglobin (g/dl)	6.53/6.59	0.40/0.26	6.56/7.15	1.62/1.41	4.05/3.89	9.67/8.95
Erythrocytes ($\times 10^6$ cel/ul)	0.54/0.57	0.06/0.03	0.47/0.58	0.25/0.19	0.22/0.32	0.97/0.98
MCV (fl)	539.02/448.98	54.81/27.17	458.56/401.53	219.26/143.78	264.56/196.17	921.72/717.91
MCH (pg)	144.12/122.92	18.96/5.79	124.11/122.53	75.86/30.67	64.66/45.95	292.86/197.78
MCHC (g/dl)	26.37/28.49	1.64/1.23	25.62/28.84	6.56/6.52	17.69/16.20	39.36/44.66
Leukocytes ($\times 10^3$ cel/ul)	8.88/9.50	0.82/0.70	8.55/8.55	3.28/3.73	5.00/2.44	17.67/21.33
Heterophils (%)	25.43/29.32	1.99/1.68	26.00/29.00	7.99/8.89	14.00/13.00	39.00/45.00
Eosinophils (%)	2.75/3.17	0.57/0.51	2.00/2.50	2.29/2.74	0.00/0.00	7.00/11.00
Basophils (%)	17.68/21.42	2.11/1.67	15.50/18.50	8.47/8.86	6.00/8.00	29.00/46.00
Lymphocytes (%)	53.43/44.03	2.99/2.06	54.00/42.50	11.99/10.92	34.00/27.00	73.00/70.00
Monocytes (%)	0.68/2.03	0.17/0.40	1.00/1.00	0.70/2.16	0.00/0.00	2.00/9.00
H:L ratio	0.52/0.73	0.06/0.06	0.45/0.71	0.27/0.36	0.22/0.24	1.11/1.59
Heterophils ($\times 10^3$ cel/ul)	2.17/2.81	0.19/0.29	2.09/2.56	0.77/1.55	0.93/0.66	3.45/7.25
Eosinophils ($\times 10^3$ cel/ul)	0.21/0.34	0.04/0.08	0.18/0.21	0.17/0.45	0.00/0.00	0.66/2.35
Basophils ($\times 10^3$ cel/ul)	1.61/1.95	0.28/0.16	1.45/1.80	1.14/0.85	0.30/0.39	5.12/4.16
Lymphocytes ($\times 10^3$ cel/ul)	4.79/4.20	0.57/0.36	3.96/4.04	2.31/1.95	2.57/0.78	10.32/8.96
Monocytes ($\times 10^3$ cel/ul)	0.07/0.18	0.02/0.03	0.06/0.09	0.09/0.20	0.00/0.00	0.35/0.85

Table 2. Descriptive statistics of blood chemistry variables from male/female *Gopherus flavomarginatus*. SE = standard error, SD = standard deviation, Min = minimum, Max = maximum.

Variable	Mean	SE	Median	SD	Min	Max
Glucose (mg/dl)	79.19/58.34	7.97/8.28	68.33/40.47	31.90/43.82	43.32/13.34	134.67/165.30
Uric acid (mg/dl)	5.63/5.58	1.07/0.71	5.66/5.96	4.28/3.76	0.27/0.24	12.45/13.48
Urea (mg/dl)	25.60/31.22	0.82/0.76	25.39/31.75	3.30/7.86	20.64/14.67	32.86/51.34
BUN	11.96/14.37	0.38/0.82	11.86/14.22	1.54/4.35	9.64/6.86	15.36/23.99
Creatinine (mg/dl)	0.48/0.43	0.06/0.05	0.47/0.40	0.24/0.26	0.01/0.01	0.95/0.89
Total protein (g/dl)	4.12/3.95	0.53/0.24	3.76/3.78	2.13/1.27	1.29/2.00	8.96/7.13
Albumin (g/dl)	0.87/0.68	0.18/0.10	0.56/0.47	0.74/0.56	0.09/0.04	2.50/2.55
Globulins (g/dl)	3.24/3.27	0.42/0.24	3.23/3.09	1.70/1.30	0.99/1.37	6.46/6.50
Cholesterol (mg/dl)	280.55/214.94	45.28/23.50	269.50/167.43	181.13/124.36	41.46/78.32	692.27/508.20
Triglycerides (mg/dl)	207.85/177.57	13.79/16.18	213.80/164.06	55.18/85.62	114.08/42.35	283.63/402.30
ALT (UI/I)	7.74/8.15	1.06/1.06	7.53/6.48	4.25/5.65	2.04/1.74	15.93/22.43
AST (UI/I)	41.79/45.23	6.30/3.53	32.27/47.92	25.21/18.69	14.95/15.63	105.60/80.42
AP (UI/I)	60.30/66.53	8.08/4.58	56.91/70.05	32.32/24.24	14.11/24.86	102.60/99.32
Chlorine (mmol/l)	119.90/121.64	4.24/2.98	118.90/122.31	16.97/15.81	95.68/94.53	139.62/149.14
Sodium (mmol/l)	137.53/138.24	3.20/2.47	136.24/136.42	12.80/13.11	112.36/117.90	160.90/177.60
Calcium (mg/dl)	11.61/13.44	0.65/0.41	12.64/13.29	2.63/2.19	7.46/10.27	15.67/18.30
Phosphorus (mg/dl)	3.95/4.08	0.56/0.36	3.59/4.29	2.24/1.93	1.02/1.31	7.18/7.16
Osmolality (mOsm/kg)	270.50/273.87	5.92/4.73	268.60/272.56	23.70/23.85	225.43/240.81	316.39/345.66

natus, the average was 17.68% for males and 21.42% for females, with wide variation between individuals (min = 6%, max = 46%). Eosinophilic leukocytes were the fourth most abundant leukocytes. The seasons and type of diet can influence the amount of eosinophilic leukocytes in some species (Duguy, 1970; Deem et al., 2006). According to Frye (1991), the percentage of eosinophils in healthy reptiles ranges from 7 to 20%; in the case of

Table 3. Comparison among minimum-maximum blood biometry values reported for *Gopherus flavomarginatus* and reference values of other three species of the genus.

Variable	*G. flavomarginatus*[1]	*G.agassizii*[2]	*G. polyphemus*[3,4]	*G. berlandieri*[5]
Hematocit (%)	16.47-35.62	19.50-37.10	15.00-30.00	12.00-44.80
Hemoglobin (g/dl)	3.89-9.67	4.10-9.90	4.20-8.60	-
Erythrocytes ($\times10^6$ cel/ul)	0.22-0.98	0.36-1.08	0.24-0.91	-
MCV (fl)	196.17-921.72	254.00-638.00	200.10-838.60	-
MCH (pg)	45.95-292.86	74.00-186.00	-	-
MCHC (g/dl)	16.20-44.66	20.00-33.00	-	-
Leukocytes ($\times10^3$ cel/ul)	2.44-21.33	1.49-10.92	10.00-22.00	0.00-80.650
Heterophils (%)	13.00-45.00	-	10.00-57.00	-
Eosinophils (%)	0.00-11.00	-	-	-
Basophils (%)	6.00-46.00	-	2.00-11.00	-
Lymphocytes (%)	27.00-73.00	-	32.00-79.00	-
Monocytes (%)	0.00-9.00	-	3.00-13.00	-
H:L ratio males	0.22-1.59	-	-	-
Heterophils ($\times10^3$ cel/ul)	0.66-7.25	0.71-7.15	0.00-6.59	0.00-5.21
Eosinophils ($\times10^3$ cel/ul)	0.00-2.35	0.00-0.95	-	-
Basophils ($\times10^3$ cel/ul)	0.30-5.12	0.06-3.57	0.02-0.92	0.00-1.31
Lymphocytes ($\times10^3$ cel/ul)	0.78-10.32	0.63-2.74	0-4.15	0-3.12
Monocytes ($\times10^3$ cel/ul)	0.00-0.85	0.00-0.32	-	0.00-0.44

[1] Present study. [2] Christopher et al. (1999) in summer season. [3] Taylor and Jacobson (1982). [4] Teare (2013b). [5] Teare (2013a).

Table 4. Comparison among minimum-maximum blood chemistry values reported for *Gopherus flavomarginatus* and reference values of other three species of the genus.

Variable	*G. flavomarginatus*[1]	*G.agassizii*[2]	*G. polyphemus*[3,4]	*G. berlandieri*[5]
Glucose (mg/dl)	13.34-165.30	65.00-186.00	55.00-128.00	9.00-157.00
Uric acid (mg/dl)	0.24-13.48	1.70-9.20	0.90-8.50	0.00-8.60
Urea (mg/dl)	14.67-51.34	-	1.00-130.00	-
BUN	6.86-23.99	1.00-37.00	2.00-29.00	0.00-13.00
Creatinine (mg/dl)	0.01-0.95	0.20-0.40	0.10-0.40	-
Total protein (g/dl)	1.29-8.96	2.30-5.30	1.30-4.60	1.20-7.70
Albumin (g/dl)	0.04-2.55	0.80-1.90	0.50-2.60	0.50-2.70
Globulins (g/dl)	0.99-6.50	1.30-3.90	0.50-4.60	0.60-5.10
Cholesterol (mg/dl)	41.46-692.27	33.00-381.00	19.00-150.00	
Triglycerides (mg/dl)	42.35-402.30	7.00-603.00	-	-
ALT (UI/I)	1.74-22.43	1.00-5.00	2.00-57.00	-
AST (UI/I)	14.95-105.60	15.00-123.00	57.00-392.00	0.00-265.00
AP (UI/I)	14.11-102.60	25.00-114.00	11.00-71.00	-
Chlorine (mmol/l)	94.53-149.14	101.00-138.00	35.00-128.00	89.00-122.00
Sodium (mmol/l)	112.36-177.60	127.00-176.00	127.00-148.00	123.00-153.00
Calcium (mg/dl)	7.46-18.30	8.60-23.90	10.00-14.00	5.00-17.40
Phosphorus (mg/dl)	1.02-7.18	1.10-6.50	1.00-3.10	0.70-4.90
Osmolality (mOsm/kg)	225.43-345.66	252.00-352.00	-	-

[1]Present study. [2]Christopher *et al.* (1999) in summer season. [3]Taylor and Jacobson (1982). [4]Teare (2013b). [5]Teare (2013a).

G. flavomarginatus, the average percentage found in the present study was 2.75% for males and 3.17% for females, with a total variability of 0-11%. The average percentage of monocytes in the Bolson tortoise was 0.68% for males and 2.03% for females (min = 0, max = 9); these values are within the range of abundance reported for reptiles (Duguy, 1970).

The H:L ratio (heterophils/leukocytes) has been used as a reliable method to evaluate the exposure of vertebrates to chronic stress due to the relationship between the leukocyte profile and the adrenal response (production of cortisol). When the level of cortisol rises, the number of circulating heterophils increases, while the number of lymphocytes decreases (Davis et al., 2011). Davis (2009) indicated that blood biometry studies conducted with terrestrial tortoises in which the possibility of individuals being stressed was not considered (including studies with *G. agassizii*, *G. berlandieri,* and *G. polyphemus*), the H:L ratio was less than 2.0 (mean of 0.65, SD=0.34), which coincides with the values obtained in the present study for *G. flavomarginatus*.

Blood chemistry variables provide important information for establishing levels of hydration (BUN, uric acid, osmolality), nutrition (glucose, total protein, albumin, colesterol, phosphorus), and metabolic activity (alanine aminotransferas, aspartate aminotransferase, and alkaline phosphatase activities) of desert tortoises (Christopher et al., 1999). Christopher et al. (1999) showed that blood chemistry values in *G. agassizii* change due to the effect of physiological (reproductive cycle, hibernation) and environmental factors (precipitation patterns, availability of water and food). However, we observed similar blood values between *G. agassizii and G. flavomarginatus* at the same times of year (summer). In general, the phylogenetic relation among *G. flavomarginatus*, *G. agassizii*, *G. polyphemus*, and *G. berlandieri* (Reynoso and Montellano-Ballesteros, 2004) may be reflecting the similarity between reported hematology values for these species, and the slight differences may point to particular adaptation conditions that each one has developed in their own habitat.

Our small sample size (n = 44) precluded the calculation of formal reference intervals, a process that would require a minimum of 120 individuals (Geffre, et al., 2009). However, given the limited sample size available, and the lack of previously published data regarding hematology parameters in *G. flavomarginatus*, these results provide a useful starting point for researchers.

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