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Source: Journal of Herpetological Medicine and Surgery, 29(3-4) : 113-122

Published By: Association of Reptilian and Amphibian Veterinarians

URL: https://doi.org/10.5818/18-07-159.1

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THE OFFICIAL PUBLICATION OF THE ASSOCIATION OF REPTILIAN AND AMPHIBIAN VETERINARIANS

ORIGINAL ARTICLE

The Journal of Herpetological Medicine and Surgery, Volume 29, No. 3–4, 2019 pp. 113–122
DOI: 10.5818/18-07-159.1

Changes in Plasma Chemistry Parameters in Hermann's Tortoises (*Testudo hermanni*) Influenced by Season and Sex

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Abstract

The metabolism of tortoises is influenced by many factors, which in turn also influences the clinical chemistry parameters in these animals. There are currently limited data available on species, sex, and season-specific reference intervals. The goal of this study was to establish new reference intervals for adult Hermann's tortoises (*Testudo hermanni*), which include variations according to season and sex. Lithium heparinized blood samples from 256 Hermann's tortoises were collected and biochemistry parameters (alkaline phosphatase (ALP), glutamate-dehydrogenase (GLDH), alanine–aminotransferase (ALT), aspartate–aminotransferase (AST), bile acids (BA), creatine kinase (CK), total protein (TP), albumin (Alb), urea, uric acid (UA), inorganic phosphorus (P), total calcium (Ca), sodium (Na), and potassium (K)) were measured from May 2016 to October 2017. The results showed many variations depending on sex and season. Male Hermann's tortoises had higher ALT, BA, CK, and UA concentrations, and females had higher Ca concentrations. The concentrations of BA, UA, GLDH, TP, and Alb decreased during the course of the year, and ALT increased over time in males. The present study demonstrates that it is important to establish specific reference intervals for each species that include seasonal and sex specific variations in order to facilitate correct interpretation of blood results.

Key Words: blood, reference interval, male, female, Testudinidae, enzyme

Introduction

Hermann's tortoises (*Testudo hermanni*) occur in Mediterranean Europe, and wild populations have been historically threatened because of habitat loss caused by intensive agricultural use. The species is listed as near threatened in the Red List of the International Union for Conservation of Nature (IUCN, 2017). A large number of Hermann's tortoises are kept in zoological gardens and by hobbyists, and captive breeding of this species is very successful. Improvement of medical diagnostic options for this species is important both to aid conservation efforts and because of their popularity as pets.

The natural habitats of this species are littoral pinewoods, coastal dunes, Mediterranean scrub, and garrigues (Berardo *et al.*, 2015). Most Hermann's tortoises in Germany are kept in outdoor enclosures with greenhouses, which are equipped with basking spots for the cold days in the spring and fall. Tortoises generally brumate from the beginning of November to the end of March. In the wild, these tortoises mainly feed on legumes (e.g., clover and lupins) and plants from the families Poacea, Rubiaceae, Scrophulariaceae, Cruciferae, Ranunculaceae, Araliaceae, Asreaceae, and Rosaceae (Meek, 2010). In captivity, they are often fed wild herbs, like dandelion, red clover and plantain, lettuce, hay, and rarely other vegetables, fruit, and small invertebrates (Zentek and Dennert, 1997). The nutritional offerings vary strongly depending on the season.

The activity of Hermann's tortoises is variable and influenced by climatic conditions and sex. Physiological blood parameters are also influenced by many different factors, including sex, season, growth, and mating season (Erler, 2003; Holz, 2007; Goldberg *et al*., 2013; Scope *et al*., 2013; Sibeaux *et al*., 2016). In order to interpret these parameters, it is important to establish appropriate reference intervals. Most published studies (Kölle *et al*., 2001; Mathes *et al.,* 2006) have not documented the variations in blood values associated with sex and season. Others have shown that these factors play a major role in the concentrations of several parameters, including total calcium (Ca), uric acid (UA), alanine–aminotransferase (ALT), creatine kinase (CK), and total protein (TP), but have not calculated specific intervals according to sex and season (Lawrence, 1987; Erler, 2003; Holz 2007; Andreani *et al*., 2014). An additional difficulty in establishing reference intervals is obtaining reasonable numbers of representative samples (Scope *et al*., 2013). Decisions on which parameters to include in studies on blood biochemistries in tortoises have also differed depending on the study, and only limited numbers of blood parameters have been evaluated in some cases (Sibeaux *et al*., 2016). The goal of this study was to evaluate the effects of sex and season on biochemical blood parameters and to establish new reference intervals that include these variables. For this purpose, plasma biochemistry parameters of captive adult Hermann's tortoises in central Europe were measured over the course of 1½ yr. Results were evaluated according to sex and season.

Materials and Methods

The Hermann's tortoises sampled for this study were kept in different zoological collections, reptile rescue centers, and, in some cases, by private breeders. Most of the samples were obtained from southern Germany ($n = 260$). Some of the samples were obtained from a rescue center in northern Germany ($n = 3$). Work on this project was carried out under an animal experiment proposal submitted to and approved by the animal welfare committee of the University of Veterinary Medicine Hannover (file number TVA-2017-V-18). Blood samples were collected between May 2016 and November 2017. Samples collected between March and May were considered spring samples, June–August summer samples, and September–November fall samples. No samples were collected in the winter. All of the tortoises were adults and their body weights ranged between 310 g and 4.14 kg (mean 1.15 kg; SD 0.63 kg, normally distributed). For those cases in which the age of the tortoise was known, it ranged from 4 to 77 years (median 20 yr; 10–90% percentile 10–50, not normally distributed).

All animals were examined before blood collection and were considered healthy. The clinical examination included evaluation of the general health, nutritional, and care status, as well as observation of the nares, eyes, oral cavity, skin, extremities, tail, cloaca, and the form and strength of the shell. Additionally, a fecal sample was collected and screened for endoparasites using standard microscopic techniques, including both a native smear and a flotation.

The blood samples were collected from the dorsal coccygeal vein or the subcarapacial sinus. Blood was transferred into lithium heparinized tubes and, for the biochemical parameters, the tubes were cooled (8°C; 46.4°F) and stored in an upright position. Samples were centrifuged at 5,500 *g* for 10 min with a Heraeus Multifuge 3 L-R (Heraeus Holding GmbH, Hanau, Germany) no later than 4 h after collection. Only samples with no visible lymph dilution were included in the study. The plasma was sent to the laboratory overnight and analyzed 1 day after collection. For the differential blood count and the packed cell volume (PCV), lithium heparinized whole blood was also collected from each animal. Blood smears were prepared and air dried directly after blood collection. The PCV was measured by microhematocrit capillaries, which were centrifuged for 5 min at 12,000 *g* in a Haemofuge (Thermo Fisher Scientific Inc., Breda, The Netherlands). Hemoglobin and thrombocytes were measured with Sysmex XT-2000i (Sysmex Deutschland GmbH, Norderstedt, Germany). The smears were stained with Diff-Quick® (Labor+Technik Eberhard Lehmann GmbH, Berlin, Germany) and evaluated microscopically. Leukocytes were calculated at 400× magnification in 10 fields (Sheldon *et al*., 2016). The number of thrombocytes were also evaluated microscopically. For the differential blood count, 100 cells were evaluated at 1,000× magnification. The results of the hematological examinations were used to determine the health of the animals included in the study. Tortoises with leukocyte counts $>15.3 \times 10^3$ cells/µl (Bielli *et al*., 2015) were excluded from the statistical analysis. Samples with a PCV under 10% were also excluded from the statistical analysis. Biochemistry parameters were measured with the use of the cobas® 8000 module c701 analyzer series (Roche Diagnostics, Mannheim, Germany). The following analytes were measured: alkaline phosphatase (ALP), glutamate–dehydrogenase (GLDH), ALT, aspartate–aminotransferase (AST), bile acids (BA), CK, TP, albumin (Alb), urea, UA, inorganic phosphorus (P), Ca, sodium (Na), and potassium (K). TP concentration was measured by the biuret method and Alb by the bromocresol green (BCG) dye-binding method.

The statistical analyses were carried out with statistical analysis software (SAS; SAS Institute Inc., Cary, NC, USA) and the reference intervals were calculated according to the recommendations of the American Society of Veterinary Clinical Pathologists (ASVCP) (Friedrichs *et al*., 2012). Histograms were used to determine initially whether each parameter was normally distributed. This evaluation was confirmed by determining whether or not 95% of the values were within 2 standard deviations (SDs) of the mean (Friedrichs *et al*., 2012), as well as reviewing Shapiro-Wilk tests and Q-Q plots. The values that were not normally distributed ($P < 0.05$) are marked accordingly in Tables 1 and 2, and the reference intervals for these parameters were calculated with the use of the nonpara-

metric method (10th–90th percentiles). However, these parameters were normalized with the use of a logarithmic transformation so that parametric statistics could be performed. An ANOVA mixed model with a Bonferroni test as a *post hoc* test was used to determine if sex and season and the interaction of sex and season (independent variables) had an effect on the biochemistry parameters (dependent variables), with $P < 0.05$ considered the cutoff for significance.

Results

Blood was collected from a total of 193 adult clinically healthy Hermann's tortoises. Fifty-four tortoises were sampled repeatedly, but in different seasons, and 139 tortoises were sampled only once. A total of 263 samples were collected; 7 were excluded from the statistical analysis based on the exclusion criteria noted previously. The remaining 256 samples were included in the analysis, 148 from males (Table 1, Fig. 1) and 108 from females (Table 2, Fig. 2).

The majority of the biochemical parameters measured in the Hermann's tortoises (Tables 1 and 2) fluctuated depending on both the season and the sex of the animal. In males, ALP activity increased from the spring (mean = 87.2 U/L) to the summer (mean = 122.6 U/L) and then decreased in the fall (mean = 103.0 U/L). In females, ALP activity was highest in the spring (mean = 112.6 U/L) and decreased continuously until the fall (mean = 106.2 U/L). The variations in the ALP activity were significant ($P = 0.0458$; $F = 3.12$, depending on an interaction of sex and season, but not significant for sex ($P = 0.3913$; $F = 0.74$) or season ($P = 0.8556$; $F = 0.16$) individually. GLDH activity decreased in males from spring (mean = 3.9 U/L) to fall (mean = 2.8 U/L); in females it increased from spring (mean = 3.5 U/L) to summer (mean = 4.0 U/L) and then decreased in fall (mean = 1.8 U/L). The variations were significant between seasons ($P = 0.0014$; $F = 6.75$), but not significant for sex ($P = 0.0520$; $F = 3.81$) or an interaction of sex and season ($P = 0.1291$; $F = 2.06$). Overall, ALT activity was higher in males, with the highest activity in the fall (male, mean = 5.1 U/L; female, mean = 3.4 U/L). The variations in ALT were highly significant ($P < 0.0001$; $F = 30.52$) between the sexes and for an interaction of sex and season ($P = 0.0306$; $F = 3.54$), but not significant between the seasons ($P = 0.0796$; $F = 2.56$). AST activity was lowest in males (mean = 49.2 U/L) and highest in females (mean = 37.1 U/L) in the summer. The variations for AST were highly significant between the sexes ($P < 0.0001$; $F = 35.31$) and the seasons ($P < 0.0001$; $F = 9.59$), but not significant for an interaction of sex and season ($P = 0.0575$; $F = 2.89$). BA decreased in both sexes from spring (male, mean = 4.3 μmol/L; female, mean = 2.6 μmol/L) to summer (male, mean = 3.8 μmol/L; female, mean = 2.0 μmol/L). The variations in BA between the sexes were highly significant ($P < 0.0001$; $F = 28.13$), and significant between the seasons ($P = 0.0354$; $F = 3.39$), but not significant for the interaction of sex and season ($P = 0.8243$; $F = 0.19$). CK activity was high in males and decreased from spring (mean = 163.8 U/L) to fall (mean = 120.0 U/L). Females had lower CK activity levels, which were lowest (mean = 29.9 U/L) in the summer. The variations in the CK activity were highly significant ($P < 0.0001$; $F = 38.54$) between the sexes, but not significant between the seasons ($P = 0.0914$; $F = 2.42$) or for the interaction of sex and season ($P = 0.8880$; $F = 0.12$). TP and Alb were the highest in both sexes in the spring (TP: male, mean = 36.8 g/L, female, mean = 39.9 g/L; Alb: male, mean = 16.6 g/L, female, mean = 17.3 g/L) and decreased continually to the fall in males (TP: mean = 29.0 g/L; Alb, mean = 10.1 g/L). Females had the lowest concentration of TP in the summer. The seasonal variations were significant for TP ($P = 0.0055$; $F = 5.32$), but variations between the sexes ($P = 0.1131$; $F = 2.53$) and in the interactions of sex and season ($P = 0.5900$; $F = 0.53$) were not significant. The variations in Alb were significant between the seasons ($P = 0.0013$; $F = 6.80$) and the interaction of sex and season ($P = 0.0170$; $F = 4.15$), but not between the sexes ($P = 0.2676$; $F = 1.23$). Urea and UA values were high in the spring (urea: male, mean = 9.3 mmol/L; female, mean = 7.0 mmol/L; UA: male, mean = 308.9 μmol/L; female, mean = 240.8 μmol/L) and the UA decreased in males during the year (mean = 200.8 μmol/L), but urea decrased to summer (mean = 3.5 mmol/L) and slightly increased again to fall (mean = 4.2 mmol/L) In females, these values decreased in the summer (urea, mean = 4.5 mmol/L; UA, mean = 144.5 μmol/L) but increased in the fall (urea, mean = 4.8 mmol/L; UA, mean = 184.1 μmol/L). The seasonal variations in urea were significant ($P = 0.0408$; $F = 3.24$), whereas the variations between the sexes ($P = 0.5114$; $F = 0.43$) and the interaction of sex and season ($P = 0.9433$; $F = 0.06$) were not. The seasonal variations in the UA values were significant ($P = 0.0458$; $F = 3.12$), but the variations between the sexes ($P = 0.2951$; $F = 1.10$) and the interaction of sex and season ($P = 0.3889$; F = 0.95) were not. P increased in males and decreased in females from spring (male, mean = 1.2 mmol/L; females, mean = 1.6 mmol/L) to summer (male, mean = 1.3 mmol/L; females, mean = 1.5 mmol/L). The seasonal variation in P was highly significant ($P < 0.0001$; $F = 12.02$) and the variation by sex was significant ($P = 0.0085$; $F = 7.04$), but the interaction of sex and season was not ($P = 0.3438$; $F = 1.07$). Females had higher Ca concentrations than males throughout the study period, and the concentrations of Ca in the females decreased from spring (mean = 4.4 mmol/L) to summer (mean = 3.8 mmol/L). The variations in Ca between the different sexes were highly significant ($P < 0.0001$; $F = 165.84$), but the variations between the seasons ($P = 0.0740$; $F = 2.63$) and the interaction of sex and season ($P = 0.2371$; $F = 1.45$) were not significant. Na levels were higher in males (mean = 141.8 mmol/L) in spring. The differences noted in Na were significant between the sexes ($P = 0.0500$, $F = 3.88$), the seasons ($P < 0.0001$, $F = 23.52$), and the interaction of sex and season ($P = 0.0461$; $F = 3.12$). K

Table 1. Reference intervals for plasma chemistry parameters in male Hermann's tortoises depending on the season.

		Spring					Summer							Fall						
Parameter	Unit	Mean	SD	Median	Min	Max	Mean	SD	Median	Min	Max	10% percentile	90% percentile	Mean	SD	Median	Min	Max	10% percentile	90% percentile
ALP	U/L	87.2[a]	34.5	89.0	36.0	143.0	122.6[b]	69.2	114.5	30.0	354.0	52.0	207.0	103.0[b]	40.1	96.0	28.0	270.0	57.0	156.0
GLDH	U/L	3.9[b]	3.6	2.6	0.7	14.7	2.5[b]	1.9	1.8	0.6	10.1	0.9	5.4	2.8[b]	3.7	1.5	0.3	22.2	0.6	4.8
ALT	U/L	4.5[a]	1.6	4.3	2.2	8.3	5.0[b]	1.7	4.6	0.7	9.6	3.2	7.2	5.1[b]	2.2	4.5	1.0	13.9	3.0	8.0
AST	U/L	61.4[a]	30.7	55.5	15.1	130.6	49.2[b]	21.9	45.5	5.2	104.2	26.5	86.7	48.9[b]	30.4	38.1	10.9	158.7	20.0	86.2
BA	µmol/L	4.3[b]	2.7	4.0	0.8	11.9	3.8[b]	2.1	3.7	0.8	9.6	1.1	6.9	3.0[b]	2.0	2.9	0.0	9.8	0.6	5.4
BA	µg/ml	1.8	1.1	1.6	0.3	4.9	1.6	0.9	1.5	0.3	3.9	0.5	2.8	1.2	0.8	1.2	0	4.0	0.3	2.2
CK	U/L	163.8[b]	218.4	58.0	17.0	691.0	112.8[b]	174.1	41.5	8.0	824.0	12.0	365.0	120.0[b]	145.0	69.0	4.0	719.0	14.0	303.0
Urea	mmol/L	9.3[b]	10.4	4.4	0.5	30.4	3.5[b]	3.9	2.2	0.3	18.0	0.6	9.1	4.2[b]	4.2	2.7	0.3	17.7	0.6	10.8
Urea	mg/dl	26.1	29.1	12.3	1.4	85.2	8.7	10.9	6.2	0.8	50.4	1.7	25.5	11.8	11.8	7.6	0.8	49.6	1.7	30.3
UA	µmol/L	308.9[b]	228.1	210.0	3.0	770.0	220.2[b]	164.8	220.0	4.0	753.0	6.0	397.0	200.8[b]	103.5	195.5	5.0	606.0	77.0	329.0
UA	mg/dl	5.2	3.8	3.5	0.1	12.9	3.7	2.8	3.7	0.1	12.7	0.1	6.7	3.4	1.7	3.3	0.1	10.2	1.3	5.5
Phosphorus	mmol/L	1.2[b]	0.7	0.9	0.5	2.4	1.3[b]	0.7	1.2	0.3	3.8	0.7	2.6	1.0[b]	0.5	0.8	0.5	3.8	0.6	1.5
Phosphorus	mg/dl	3.7	2.2	2.8	1.6	7.4	4.0	2.2	3.7	0.9	11.8	2.2	8.1	3.1	1.6	2.5	1.6	11.8	1.9	4.6
Calcium (total)	mmol/L	2.7[a]	0.4	2.7	2.0	3.6	2.6[b]	0.3	2.6	2.0	3.8	2.3	3.0	2.6[b]	0.4	2.6	1.8	4.9	2.3	3.0
Calcium (total)	mg/dl	10.8	1.6	10.8	8.0	14.4	10.4	1.2	10.4	8.0	15.2	9.2	12.0	10.4	1.6	10.4	7.2	19.6	9.2	12.0
Potassium	mmol/L (mEq/L)	6.5[b]	2.2	5.7	4.0	12.8	6.8[b]	1.9	6.6	4.1	11.9	4.6	9.8	5.2[b]	1.1	5.1	3.7	12.0	4.1	6.1
Sodium	mmol/L (mEq/L)	141.8[a]	6.6	144.0	132.0	153.0	132.6[a]	5.3	132.0	119.0	145.0	126.0	139.0	134.0[b]	5.7	134.0	113.0	151.0	127.0	139.0
TP	g/L	36.8[a]	12.7	38.7	9.4	54.1	32.5[a]	9.4	33.2	14.9	53.2	19.0	45.4	29.0[a]	8.6	28.0	10.3	53.5	18.2	39.4
TP	g/dl	3.7	1.3	3.9	0.9	5.4	3.3	0.9	3.3	1.5	5.3	1.9	4.5	2.9	0.9	2.8	1.0	5.4	1.8	3.9
Albumin	g/L	16.6[a]	6.7	18.4	3.4	26.7	14.2[a]	6.4	14.6	0.8	26.3	5.1	21.1	10.1[b]	5.0	9.2	0.8	26.6	4.9	16.0
Albumin	g/dl	1.7	0.7	1.8	0.3	2.7	1.4	0.6	1.4	0.1	2.6	0.5	2.1	1.0	0.5	0.9	0.1	2.7	0.5	1.6
n		17					48							83						

ALP, alkaline phosphatase; GLDH, glutamate-dehydrogenase; ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; BA, bile acids; CK, creatine kinase; UA, uric acid; TP, total protein [TP].
[a] Normally distributed.
[b] Not normally distributed.

Table 2. Reference intervals for plasma chemistry parameters in female Hermann's tortoises depending on the season

		Spring							Summer							Fall						
Parameter	Unit	Mean	SD	Median	Min	Max	10% percentile	90% percentile	Mean	SD	Median	Min	Max	10% percentile	90% percentile	Mean	SD	Median	Min	Max	10% percentile	90% percentile
ALP	U/L	112.6[a]	63.4	99.0	31.0	273.0	41.0	180.0	89.9[b]	46.2	82.0	20.0	217.0	38.0	154.0	106.2[b]	70.5	90.0	20.0	353.0	43.0	175.0
GLDH	U/L	3.5[b]	7.0	1.6	0.5	35.1	0.9	4.6	4.0[b]	6.8	1.9	0.4	41.2	0.7	8.7	1.8[b]	2.5	0.9	0.3	13.8	0.5	4.4
ALT	U/L	3.4[a]	0.7	3.5	2.1	5.1	2.4	4.1	3.1[b]	1.5	3.5	0.1	6.5	0.2	4.7	3.4[b]	1.4	2.9	1.6	7.6	2.2	5.5
AST	U/L	38.2[a]	13.3	38.1	14.5	68.6	23.9	50.3	37.1[b]	19.6	32.3	4.9	80.4	17.9	72.9	24.8[b]	17.1	17.6	9.6	76.7	10.7	55.4
BA	µmol/L	2.6[b]	1.9	2.3	0.2	8.7	0.5	4.7	2.0[b]	1.4	1.7	0.05	6.7	0.5	4.1	2.0[b]	1.8	1.8	0.02	7.9	0.3	5.1
BA	µg/ml	1.1	0.8	0.9	0.1	3.6	0.2	1.9	0.8	0.6	0.7	0.02	2.7	0.2	1.7	0.8	0.7	0.7	0.01	3.2	0.1	2.1
CK	U/L	73.2[b]	166.6	24.0	5.0	822.0	7.9	135.0	29.9[b]	44.1	14.4	3.0	202.7	4.0	52.0	59.7[b]	104.5	12.0	2.0	467.0	3.0	211.0
Urea	mmol/L	7.0[b]	7.4	3.0	0.5	22.4	0.6	16.5	4.5[b]	9.1	1.8	0.4	45.1	0.6	6.5	4.8[b]	13.8	2.3	0.4	89.0	0.8	5.0
Urea	mg/dl	19.6	20.7	8.4	1.4	62.8	1.7	46.2	12.6	25.5	5.0	1.1	126.3	1.7	18.2	13.5	38.7	6.4	1.1	249.3	2.2	14.0
UA	µmol/L	240.8[b]	169.8	167.5	46.0	584.0	63.0	448.0	144.5[b]	90.7	114.0	22.0	476.0	57.0	244.0	184.1[b]	195.6	113.5	11.0	784.0	29.5	478.5
UA	mg/dl	4.1	2.9	2.8	0.8	9.8	1.1	7.5	2.4	1.5	1.9	0.4	8.0	1.0	4.1	3.1	3.3	1.9	0.2	13.2	0.5	8.0
Phosphorus	mmol/L	1.6[b]	0.9	1.3	0.6	3.3	0.8	3.0	1.5[b]	0.7	1.3	0.4	3.4	0.9	2.0	1.1[b]	0.8	0.8	0.5	4.7	0.6	2.1
Phosphorus	mg/dl	5.0	2.8	4.0	1.9	10.2	2.5	9.3	4.6	2.2	4.0	1.2	10.5	2.8	6.2	3.4	2.5	2.5	1.6	14.6	1.9	6.5
Calcium (total)	mmol/L	4.4[b]	1.4	4.0	2.8	6.9	3.1	6.4	3.8[a]	0.9	3.8	2.0	6.0	2.8	5.1	3.8[b]	1.1	3.4	1.9	6.8	2.7	5.6
Calcium (total)	mg/dl	17.6	5.6	16.0	11.2	27.6	12.4	25.6	15.2	3.6	15.2	8.0	24.0	11.2	20.4	15.2	4.4	13.6	7.6	27.2	10.8	22.4
Potassium	mmol/L (mEq/L)	6.1[a]	1.1	6.4	3.6	7.8	4.5	7.2	5.9[b]	2.4	5.2	2.8	12.9	3.4	9.8	5.4[a]	1.1	5.3	3.4	8.9	4.2	6.6
Sodium	mmol/L (mEq/L)	137.1[a]	4.7	137.0	127.0	148.0	131.0	142.0	131.8[a]	5.3	130.0	123.0	144.0	127.0	140.0	134.6[a]	5.2	136.0	121.0	149.0	128.0	140.0
TP	g/L	39.9[a]	10.3	39.2	23.1	60.6	27.6	54.0	33.4[a]	10.2	35.5	6.4	50.9	20.9	45.6	33.2[a]	12.8	32.1	9.0	59.5	18.4	54.0
TP	g/dl	4.0	1.0	3.9	2.3	6.1	2.8	5.4	3.3	1.0	3.5	0.6	5.1	2.1	4.6	3.3	1.3	3.2	0.9	6.0	1.8	5.4
Albumin	g/L	17.3[a]	5.3	17.6	7.8	25.5	9.4	24.7	12.3[a]	5.6	12.9	1.0	21.8	3.5	18.9	14.1[a]	6.4	13.4	1.6	27.7	7.0	25.6
Albumin	g/dl	1.7	0.5	1.8	0.8	2.6	0.9	2.5	1.2	0.6	1.3	0.1	2.2	0.4	1.9	1.4	0.6	1.3	0.2	2.8	0.7	2.6
n		24							43							41						

ALP, alkaline phosphatase; GLDH, glutamate-dehydrogenase; ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; BA, bile acids; CK, creatine kinase; UA, uric acid; TP, total protein [TP].
[a] Normally distributed.
[b] Not normally distributed.

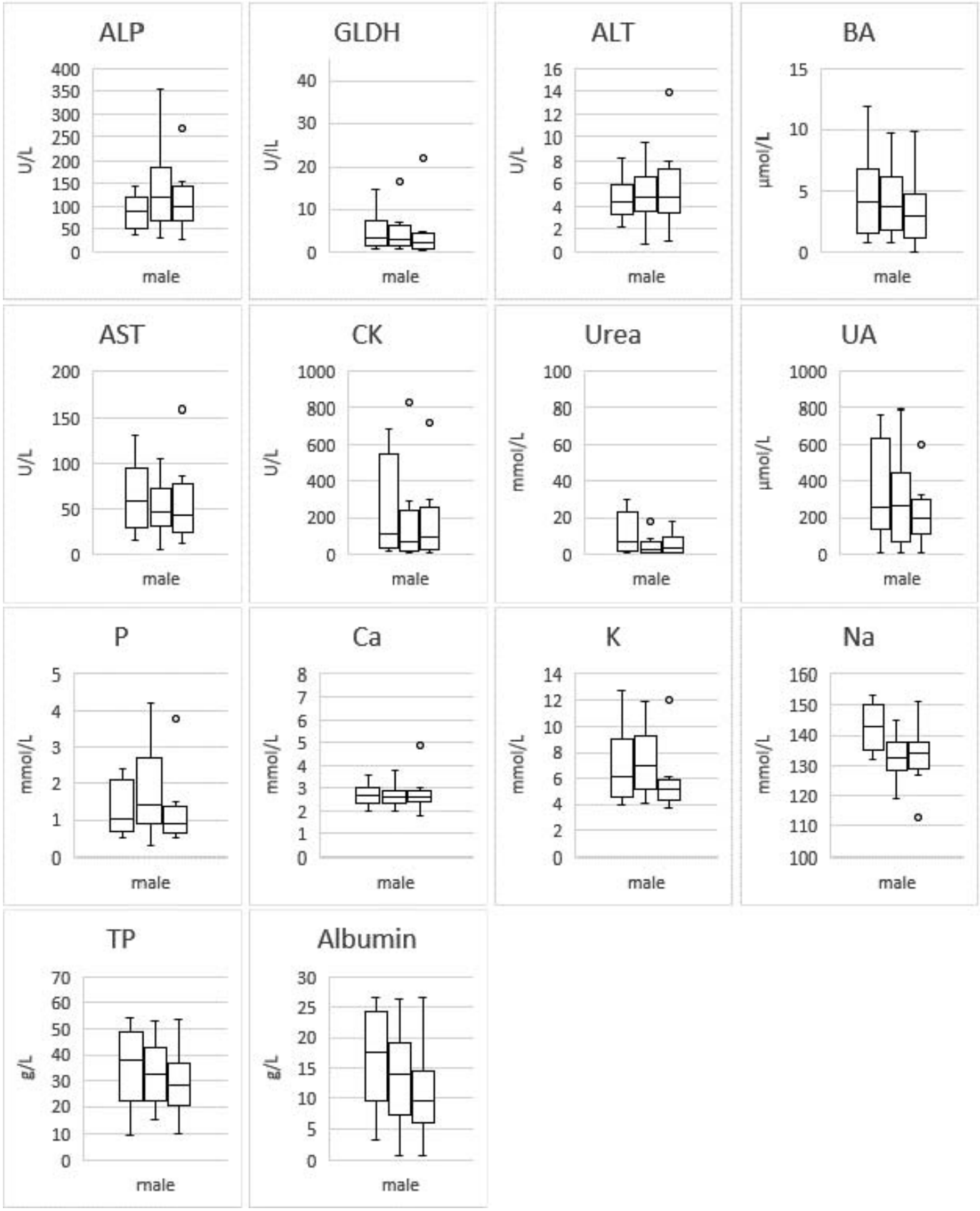

Figure 1. Plasma chemistry parameters for male Hermann's tortoises by season. Box plots with maximum, 90% percentile, median, mean, 10% percentile, and minimum. First box = spring; second box = summer; third box = fall.

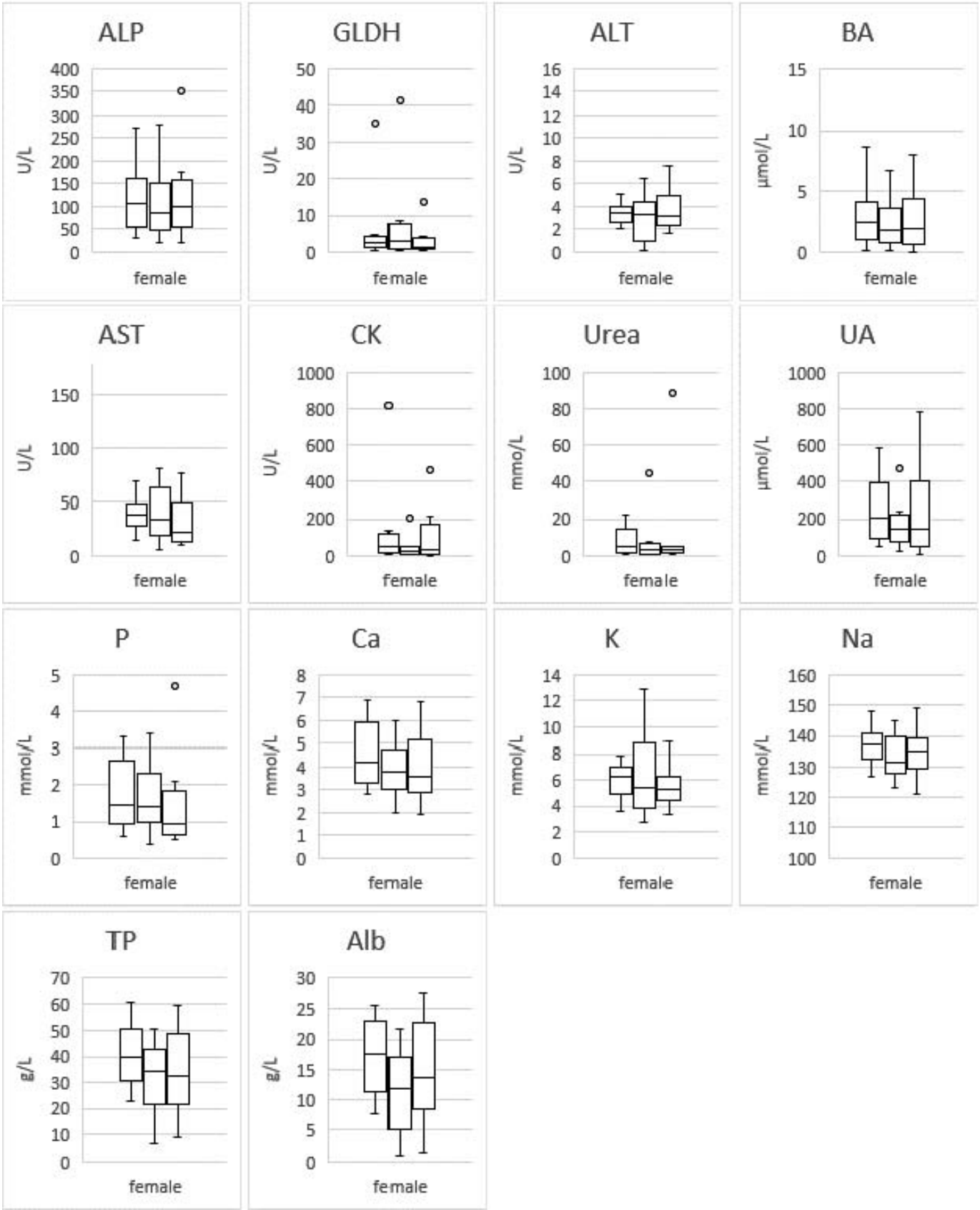

Figure 2. Plasma chemistry parameters for female Hermann's tortoises by season. Box plots with maximum, 90% percentile, median, mean, 10% percentile, and minimum. First box = spring; second box = summer; third box = fall.

increased in male tortoises in summer (mean = 6.8 mmol/L). The K concentrations were also significantly different between the seasons ($P < 0.0001$; $F = 10.68$), and interaction of sex and season ($P = 0.0096$; $F = 4.73$), but not between the sexes ($P = 0.0802$; $F = 3.09$).

Discussion

Populations of Hermann's tortoise are considered near threatened in the wild. At the same time, they are the most commonly kept tortoise species in Europe. A better understanding of their physiology and improvement in diagnostic tools for this species are therefore of both veterinary and conservation interest. In general, it is particularly difficult to establish blood reference intervals in ectothermic vertebrates because these often vary more strongly because of seasonal, environmental, and individual factors than those of endothermic animals.

The calculation of reference intervals generally requires a relatively large number of samples from a defined group of individuals. The ASVCP has established guidelines for reference intervals (Friedrichs *et al*., 2012). These guidelines require a minimum of 20 samples from a defined group in order to determine the 90% percentile for the calculation of reference intervals (Friedrichs *et al*., 2012). In this study, between 17 and 83 individuals were sampled from each sex and in each season. The 17 male tortoises sampled in spring were slightly below the ASVCP cut off, and for this reason the 10 and 90% percentiles were not calculated for this group of animals (Table 1).

The tortoises included in this study were kept in several different parts of Germany under various conditions. Although husbandry was considered good in all cases, conditions for the tortoises in the northern latitudes were generally slightly cooler, with an earlier brumation in the fall and later emergence in the spring. This effect was balanced by grouping of the data into calendar seasons for the statistical analysis and slightly later sample times for spring and slightly earlier sample times for fall in the animals from northern Germany. All of the tortoises included in this study were fed a similar diet consisting of a combination of wild herbs, including dandelion, red clover, and plantain; various lettuces; hay; in rare cases other vegetables and fruit; and cuttlebone. This similar dietary background makes comparison of the individual samples easier, reducing the influence of nutrition on the parameters measured (Liesegang *et al*., 2007; Anderson *et al*., 2011), and also helps highlight the differences in parameters based on sex and season.

Previous studies have shown that the venipuncture site can influence blood parameters, mostly due to the varying risk of lymphatic contamination (Gottdenker and Jacobson, 1995; López-Olvera *et al.,* 2003; Bonnet *et al.,* 2016). In order to reduce these effects, samples that were visibly contaminated with lymph during venipuncture were excluded from the analyses. For all other samples, the PCV was used as an indicator of possible lymph contamination. Samples with a PCV <10% were excluded from the statistical analysis.

In this study, Alb was measured using the BCG dye-binding method. Other studies (Müller and Brunnberg, 2010; Macrelli *et al*., 2013) have shown that this method can lead to artificially altered measurements and is not generally recommended for reptiles, but individual studies have not documented these problems (Andreani et al., 2014). This is, however, the most commonly used method, which is why these values were included in this study. Previous studies (Mathes *et al*., 2006; Holz, 2007; Scope *et al*., 2013) have based their calculations for this parameter on the same method. It is, however, important to know what method was used for Alb detection when blood values are being evaluated. A comparison of the results of BCG and capillary zone electrophoresis (CZE) showed higher Alb concentrations in males when measured with BCG, whereas females had lower concentrations (Leineweber *et al*., in press).

The results of this study show multiple cases in which sex and/or season were associated with major changes in the calculated normal intervals for specific parameters. Several of these interdependencies have been previously described. Christopher *et al*. (1999), Erler (2003), and Scope *et al*. (2013) also documented higher Ca concentrations in females. One of the reasons for this is the mobilization of Ca from the bones during vitellogenesis (DeNardo, 2006). In this study, the total Ca concentrations in females were highest at the beginning of the mating season in the spring and decreased in the summer. Females also had higher P concentrations than males in the spring and fall. In the summer, the P concentration increased in males to higher levels than those observed in the females at that time. Scope *et al*. (2013) also described an increase in P in males in the summer, but not above the concentrations measured in females. Seasonal variations were also observed for other electrolytes: K increased in summer and Na decreased from spring to fall, which has also been described previously (Erler, 2003). Possible explanations for these seasonal differences include changes in the amounts of food and water consumed, which also varies with the season, as well as activity levels and the composition of the forage plants over the course of the year (Erler, 2003).

Several of the parameters measured in this study can be used to evaluate the health and function of the liver. AST is located in the cytosol of the hepatocytes, as well as in the skeletal and cardiac muscle cells (Campbell, 2006). An increase in activity of this enzyme in the blood is therefore not specific for liver cell damage and must be interpreted in context with other laboratory parameters, including CK. However, it is important to note that AST has a longer plasma half-life than CK (Grunkemeyer, 2010), so that clinical signs are also important for the correct interpretation of these parameters. Increased ALT activity is nonspecific for hepatocellular diseases in nonavian reptiles (Campbell, 2006). GLDH is localized in the mitochondria of the hepatocytes. Increased activity in the blood of this enzyme is therefore an indication of cell destruction

(Grunkemeyer, 2010; Scope *et al*., 2013). The measurement of BA blood concentrations as an indicator for liver function in reptiles has not been well documented. BA is, however, only synthesized by the hepatocytes and is therefore considered a specific indicator for the liver cell function (Montesinos *et al*., 2002; Grunkemeyer, 2010). Montesinos *et al*. (2002) published reference intervals for BA in Hermann's tortoises, which were higher than those found in the present study, and showed no significant differences between the sexes in contrast to the present study. Higher activities in the liver parameters (AST, ALT, GLDH, BA) in male tortoises were also described by Scope *et al*. (2013) and Erler (2003). However, several enzymes showed slight differences in regard to the season in which the highest activities were measured: ALT increased from the spring to fall in both sexes, GLDH was higher in females than in males in the summer, and ALP activity in females and AST activity in males decreased in the summer in contrast to Scope *et al*. (2013). Why enzyme activity is higher in the blood of males than in females is not known, but there are several possible theories regarding physiological functions, including hormonal regulation, which could explain these differences and which require further study. The increased ALT and GDLH activity in females could, for example, be a result of increased activity of the liver due to vitellogenesis. The high CK activity in males could be a result of physical exertion and dominance behaviors in spring and summer.

Sibeaux *et al*. (2016) described higher UA concentrations in the spring than in the summer in free-living Hermann's tortoises in France. In males, UA and urea had the highest concentrations after hibernation in spring; UA decreased from spring to fall and urea decreased in the summer and increased prehibernation, which was also documented by Scope *et al*. (2013). One reason for this could be the higher protein content in wild herbs and the accumulation of metabolic products. Dehydration posthibernation in the spring could also play a role. Males had higher UA concentrations than females, which was also described by Erler (2003); however, females had an increase in concentrations in the fall. Males and females both had the highest concentrations of TP and Alb in the spring, which corresponds to expected higher protein levels in the food, as well as the highest PCV (data not shown), which could also indicate some dehydration in the spring. In previous studies, the highest TP and Alb concentrations have been found in the summer (Scope *et al*., 2013).

The results of this study demonstrate seasonal changes in numerous important blood chemistry parameters over the course of the year, depending on the sex of adult tortoises. Establishment of specific reference intervals not only for individual reptile species, but also for different sexes and seasons, is critical to allow correct interpretation of blood values and facilitate diagnostics and treatment of Hermann's tortoises. More studies are also needed to evaluate the differences in plasma biochemistry parameters in juvenile tortoises, as these are likely to differ considerably because of differences in metabolic rate due to growth.

Declaration of conflicting interest: Two of the authors (CL and REM) are employed by a private lab (Laboklin) that offers diagnostic services for veterinarians. This employment did not influence study design, interpretation, or publication preparation.

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