SHORT COMMUNICATIONS

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Reduce, Replace, Refine: Determining A Posteriori Reference Intervals for Biochemistry in Hermann's Tortoise (*Testudo hermanni*)

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ABSTRACT: Biochemical and hematological analyses are important for the assessment of animal health. However, for most wild species their use is hindered by the scarcity of reliable reference intervals. Indeed, collecting body fluids (e.g., blood, urine) in free-ranging animals is often technically challenging. Further, sampling many individuals would be essential to consider major sources of variations, such as species, populations, sex, age, and seasons. One alternative, according to the reduction, refinement, and replacement framework, is to establish reference intervals a posteriori using literature survey and unpublished data. We produced reference intervals for free-ranging Hermann's tortoises (*Testudo hermanni*), using analyses performed on blood samples collected in previous studies and conservation programs conducted in the field between 2010 and 2016 in southern France (n=195 individuals). Thirteen parameters were analyzed: packed-cell volume, blood concentrations of corticosterone, testosterone, glycemia, cholesterol, triglyceride, urea, uric acid, calcium, sodium, potassium, asparagine aminotransferase (AST), and alanine aminotransferases (ALT). Reference intervals for subgroups defined by sex and season were relevant for corticosterone, triglyceride, and calcium (sex) and cholesterol (season). Comparing our results with those obtained in captive individuals in Germany, except for urea and AST levels the intervals from both free-ranging versus captive tortoises were similar, suggesting that reference intervals established from captive individuals may be suitable for free-ranging populations in this species.

Key words: Baseline values, biochemistry, blood analysis, free-ranging animals, reptiles, wildlife.

Biochemical and hematological analyses are major diagnostic tools in animal medicine, provided that reliable reference intervals (RIs) are available. Individuals in which values fall within the limits of the RI are considered healthy. When a result falls outside of the RI limits, the clinical interpretation depends on the nature of the abnormal parameter, the direction and magnitude of the abnormality, the species, and other individual parameters (Flatland et al. 2020). Various factors may influence RIs: sex, age, season, population, or puncture sites (e.g., vein vs. plexus), for example (Andreani et al. 2014; Bonnet et al. 2016). For this reason, RIs should be species specific and refer to the sampling protocol, analytical methods, and devices used. Decision criteria commonly used to determine whether RIs should be separated into subgroups rely on thresholds and on the percentage of difference between values (Haeckel et al. 2016). This ignores the fact that an increase of a given percent above the limit of reference does not necessarily carry the same clinical significance for all parameters. Although partitioning RIs (e.g., by sex and season) may be relevant (Leineweber et al. 2019), its appropriateness must be assessed and global RIs should be preferred unless subgroups have significantly different RIs (Friedrichs et al. 2012).

The establishment of RIs from samples collected for this purpose (de novo RI) constitutes the best practice (Friedrichs et al. 2012). Practically, this involves sampling a minimum of 20 (preferably >120) healthy individuals. Besides the technical difficulties associated with the capture and sampling of many healthy individuals in the field, ethical and budgetary issues impose additional limits. Several alternatives have been proposed (Geffré et al. 2009), for example, the "validation of a reference interval" that relies on already existing good-quality RIs established in other populations using the same protocol or using distinct, albeit comparable, analytical protocols and analyzers (hence values can be adjusted or confidence intervals relaxed). It is

recommended by Geffré et al (2009) to rely on a sample size ≥20 healthy individuals per population; if at least 90% of the values overlap then the previously established RIs can be used for the population (although they might be too wide compared to the calculated normal values of the targeted population). However, RIs are not available for every species.

Another alternative is the a posteriori determination of RI. Similar preanalytical, analytical and selection factors apply as for the de novo method (e.g., puncture site, protocol, analyzer, sample size). The only distinction is that values are not generated de novo but collected from one or more existing databases (e.g., from surveillance programs, surveys, research projects; Geffré et al. 2009). The statistical method is similar to the de novo method and follows the recommendations of the American Society for Clinical Veterinary Pathology (ASCVP; Friedrichs et al. 2012). Multicentric data can be pooled if similar analytical methods were employed. Finally, given the scarcity of reliable RIs obtained in free-ranging wildlife, it can be judicious to use RIs established in captive animals. However, captivity can have a significant impact on the results (Herbst and Jacobson 2002).

To test whether these alternatives bring relevant information, our objectives were 1) to establish a posteriori RIs using the same statistical method as the de novo method from ASCVP; and 2) to compare RIs established for free-ranging and captive animals. We used Hermann's tortoises (*Testudo hermanni*) as a study case, because data sets were available for a free-ranging population and RIs have been established in captive animals.

Data used for a posteriori determination of the RIs were gathered from long-term monitoring and a one-time survey of free-ranging Hermann's tortoises performed by the Station d'Observation et de Protection des Tortues et de leurs Milieux (SOPTOM; Carnoules, France). The location, time of sampling, and the field and analytical methods used, described elsewhere, have been summarized in the Supplementary Material, as are the assessment methods used to determine sex, health status, and genetic hybridization (Jourdan et al. 2013; Sibeaux et al. 2016; Ballouard et al. 2022; Bech et al. 2022). The parameters measured were packed cell volume (PCV); testosterone, corticosterone, glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities; triglyceride; cholesterol; urea and uric acid; and total calcium, sodium, and potassium. Although sample collection and processing were comparable throughout the whole period and for the different survey data, minor differences in technique occurred for some parameters. Furthermore, some analytical tools and methods used for collection of the data from Sibeaux et al. (2016), such as the Accu-Chek Performa blood glucose meter (Roche Diabetes Care, Mannheim, Germany), have not been validated for reptiles.

Between August 2010 and August 2016, 708 measures from 336 individuals were obtained. Although the information was not available for all data, a few samples were visually considered as hemolyzed or diluted by plasma by SOPTOM; therefore those samples were removed from the data set, as hemolysis might modify biochemical analyses of several parameters and measured values of some parameters might be modified by hemodilution. Data from hybrid, wounded, or unhealthy (abnormal behavior, deformation of carapace, nasal or eye discharge, PCR-positive for herpesvirus or *Mycoplasma* spp.) tortoises were excluded (see Ballouard et al. 2022 for protocols), resulting in a data set of 195 individuals. When several capture events were available for an individual, only one was included; after comparing different methods (mean, first capture, random) and observing similar RIs, we retained values based on the first sampling event, notably because this maximized sample size for each parameter. Finally, at least 120 individuals were tested for 11 parameters (Table 1), whereas 52 and 25 values were available for PCV and testosterone, respectively.

Some tortoises had nonnumeric low values (e.g., some urea values were reported as <0.03 g/L). When the detection of a low value was not clinically relevant (no health abnormalities associated with low values), nonnumeric values were arbitrarily replaced by 0 for computational purposes. For parameters in which a low value was clinically relevant

TABLE 1. Reference intervals (RIs) for 12 biochemical parameters and packed cell volume (PCV) in free-ranging Hermann's tortoises sampled in France. AST=aspartate aminotransferase, ALT=alanine aminotransferase. *n*: sample size, Lower CI, Upper CI: 90% confidence interval for the lower and upper limits of the RI. The RI and CIs were established with nonparametric methods if $n>120$, and otherwise with Horn's robust methods. The RIs were partitioned by sex for corticosterone, triglycerides, and calcium, and by season for cholesterol.

Parameter	Sex	Season	*n*	RI	Lower CI	Upper CI
PCV (%)	Both	All	52	[5.81–37.3]	(2.57–8.88)	(34.81–40.02)
Testosterone ($\times 10^3$ ng/L)	Both	All	25	[0–35.25]	–[a]	(27.73–45.62)
Glucose (mg/L)	Both	All	158	[35.93–1,360.8]	(160–430)	(1,100–1,960)
Urea (g/lL)	Both	All	170	[0–0.17]	–	(0.11–0.23)
Uric acid (mg/L)	Both	All	181	[2.55–50.0]	(0.2–3.8)	(40.0–68.0)
AST (UI/L)	Both	All	173	[39.05–322.65]	(23.0–53.0)	(251.0–389.0)
ALT (UI/L)	Both	All	152	[1–18.0]	(0–2.0)	(15.0–33.0)
Sodium (mmol/L)	Both	All	154	[117.88–157.25]	(105.0–121.0)	(153.0–161.0)
Potassium (mmol/L)	Both	All	154	[3.29–9.41]	(3.0–3.6)	(8.8–9.9)
Corticosterone ($\times 10^3$ ng/L)	Male	All	76	[0–5.94]	–	(5.11–6.82)
	Female	All	84	[0–2.66]	–	(2.13–3.19)
Triglyceride (g/L)	Male	All	73	[0–1.78]	–	(1.33–2.27)
	Female	All	80	[0.11–8.08]	(0–0.74)	(7.44–8.74)
Calcium (mg/L)	Male	All	87	[51.32–154.31]	(38.34–62.77)	(143.07–166.52)
	Female	All	94	[61.74–241.45]	(46.7–75.95)	(226.88–256.07)
Cholesterol (g/L)	Both	Spring	90	[0–2.64]	(0–0.13)	(2.41–2.88)
	Both	Summer	104	[0–5.72]	–	(3.00–8.52)
	Both	Fall	52	[0–2.11]	(0–0.01)	(1.83–2.40)

[a] RI includes 0 as a low value, no CI can be calculated for this value.

(e.g., corticosterone), nonnumeric measures were removed.

Outliers, detected with either Cook's or Horn's methods, were removed only if abnormal values suggestive of disease or transcriptional mistakes were present. We applied this method because removing too many outliers might artificially narrow the RI, and increase the erroneous classification of individuals as "suspected of illness" (Friedrichs et al. 2012). Subgroup RIs are relevant when sufficiently different. The difference was considered significant when the RI differed by either 20%, 50%, or 100%, depending on the magnitude of change considered clinically significant in each parameter based on clinical experience of the authors and solicited collaborators. Subgroup analysis was not possible for testosterone and ALT because of low sample size and unbalanced samples (i.e., most of the data had equal values, so that Q1 and Q3 quantiles were equals). Nonparametric (when $n\geq120$) or robust (when $n<120$) methods were used to calculate 95% confidence RIs and the 90% confidence intervals (CI) for the limits (Table 1, package referenceIntervals, v1.3.0 R; Finnegan 2022). Finally, we compared the width of the RIs with the width of the CIs, because, in domestic animals, a CI broader than 20% of the RI would suggest that these RI are unable to represent the normal variation of the parameter reliably (Friedrichs et al. 2012).

To compare RIs from free-ranging versus captive animals, we used RIs based on pet Hermann's tortoises in Germany. These were established as the 10th–90th percentile (80%) intervals, partitioned by season and sex (Leineweber et al. 2019) and are the only available RIs with a sufficient sample size ($n=256$) for captive *T. hermanni*. To obtain comparable values, we calculated 80% partitioned RIs for AST, ALT, urea, uric acid, calcium, potassium, and sodium. The robust method was used in groups with $20<n<120$.

For the 95% confidence RIs based on wild tortoises, we retained seven parameters with no

relevant differences among groups (Table 1): PCV (threshold: 20%), urea (50%), uric acid (50%), sodium (20%), potassium (20%), AST (100%), and glycemia (20%). Males had higher corticosterone RIs (100%) and lower Ca (20%) and triglyceride RIs (100%) than females (Table 1). For cholesterol (100%), higher values were observed during summer (Table 1) than in spring and fall. For all parameters, at least one of the two CIs was broader than 20% of the RI.

The 80% confidence RIs of AST and urea significantly differed between captive and free-ranging populations (see Supplementary Material). The upper limits of AST RIs were two times lower in captive than in wild tortoises, whereas the upper limit of urea RI was higher in captive than in free-ranging tortoises. Regarding the upper limit of AST references, the discrepancy between captive and wild tortoises might be due to differences in laboratory methods or to biological differences. However, the differences remained limited, enabling the detection of sick animals, as most diseases would trigger a strong elevation of AST (Eatwell et al. 2014). Our values for the upper limit of urea intervals were in the range found in captive tortoises kept in outdoor enclosures (Scope et al. 2013), but differed from those documented in tortoises maintained under diverse captivity conditions (Leineweber et al. 2019). This inconsistency might be due to differences in the laboratory methods used, or because contrasted captivity conditions can entail divergent physiological responses, for example, via the inclusion of subhealthy captive individuals and the timing of sampling regarding hibernation (e.g., individuals with metabolic alteration induced by suboptimal feeding). Nonetheless, the lack of substantial difference in most RIs between captive and free-ranging Hermann's tortoises contrasts with previous studies where major differences between captive and free-ranging populations were reported (Herbst and Jacobson 2002; Moore et al. 2020). Therefore, reference values obtained in captive individuals might be suitable for wild *Testudo* sp.

The use of validated analyzers and methods is recommended for any biochemical measure to establish or use RIs. We nonetheless included glycemia values measured with a nonvalidated analyzer because of the wide availability and ease of use of portable glucometers. The precision and accuracy of this analyzer should be validated before further use. Inadequate analyzer calibration, precision, or accuracy could result in repetitive error or in random bias. Repetitive error would prevent the use of RIs for comparison with values obtained with different analyzers, but RIs would still be adequate to compare with data from the same analyzer. Conversely, random bias would result in totally inadequate RIs. If a method or analyzer proves to be inadequate for the considered species, RIs determined using the method or analyzer must not be considered for use regardless of any other adequacy. For instance, the RIs for albumin presented by Leineweber et al. (2019) were obtained with the bromocresol green method, which is not recommended in reptiles (Macrelli et al. 2013).

Tortoises are ectothermic reptiles; the observed broadness of CIs reflects the great variability of their physiological parameters and metabolism in response to environmental fluctuations (Bradshaw 2003; Scope et al. 2013). Therefore, the criteria set for domestic endotherms may be too stringent for free-ranging tortoises, and more generally to ectothermic reptiles. Large CIs nonetheless offer useful indexes; values outside the RIs should be critically appraised considering the magnitude of the change and the clinical context.

Overall, for Hermann's tortoises, the determination of a posteriori RIs appears likely to provide relevant information. This method is an efficient way to replace, refine, and reduce the use of animals while improving our ability to perform informed health assessments. Further research is needed to determine the extent to which this conclusion is relevant for other parameters and species.

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SUPPLEMENTARY MATERIAL

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