Variations in Blood Biochemical Values in Male Hermann’s Tortoises (Testudo hermanni)

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Abstract
Hermann’s tortoises (Testudo hermanni) are popular pets. Blood biochemistry is an essential tool for the early diagnosis of the diseases. The objective of the study was to investigate the biological variations in selected blood biochemical values in healthy tortoises through repeated samplings in the same animals. We also aimed to evaluate the values in time-related manner of sampling. Blood samples were taken five times (in two-month intervals) from the dorsal coccygeal vein. Twelve clinically healthy 2,5 year-old male tortoises were housed indoors. For each dataset, the mean, the standard deviation, and the 95% confidence interval of the mean were calculated. Mixed effect linear model was fitted with blood parameters as response variables, samples as fix factors and individuals as random factors.

The mean levels and standard deviations are as follows: Ca, 2.3 ± 0.3 mmol/l; ionized Ca, 1.3 ± 0.4 mmol/l; P, 1.1 ± 0.3 mmol/l; uric acid, 121.1 ± 56.3 µmol/l; AST, 76.8 ± 42.8 IU/l; sodium, 127.7 ± 4.0 mmol/l; potassium, 4.5 ± 0.6 mmol/l. Except for AST, sampling time had significant effect on the parameters.

Despite the statistically significant difference between the collected samples, standard deviations were small in all samplings except for the uric acid. Accordingly, the clinical relevance of the sampling seems to be important especially for uric acid. In order to eliminate the possible effects of the time of sampling, repeated measurements are recommended if biochemical parameters are not in the reference intervals.

Keywords
calcium – metabolic bone disease – reptile – nutrition

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Introduction
Tortoises, such as Hermann’s tortoises (Testudo hermanni), are popular pets. Nutritional problems in these reptiles are common, especially metabolic bone disease (MBD; 25, 27). One tool for the early diagnosis of these disorders is blood biochemistries. Several studies have been conducted in order to set up blood reference values for the tortoises. In several studies clinically healthy animals were examined only once thus providing just a glimpse into their physiological status (34, 35). Repeated samplings have been performed only in a limited number of studies (15, 18, 23); however, even in such cases, levels of some biochemistries can be influenced by season, sex and/or species (2, 3, 7, 11, 18, 21,24, 26, 29, 31, 32, 38, 39, 46). The use of serum or plasma may also have impact on the value of the parameters (5, 19). Information about feeding and housing conditions of the tortoises has also often been limited or unknown. As the previous conditions may have effect on some parameters, to collect and consider relevant information prior to interpreting the results is essential (e.g.: feeding proteins of animal origin, hibernation, indoor or outdoor housing, etc.).

According to our previous data collection in a veterinary clinic, pet owners housed their tortoises indoor, without or with limited access to UVB light. Metabolic bone disease (MBD) was the most frequent disorder. Diet of these animals was mainly based on vegetables grown for human consumption. In our study, the conditions created followed the above mentioned practice. The objective of this study was to investigate the biological variations in selected blood biochemical values in healthy Hermann’s tortoises through repeated samplings in the same animals. We also aimed to evaluate the values in time-related manner of sampling.

Material and Methods
Twelve 2.5 year-old male Hermann’s tortoises were housed indoors in two 1.2 x 1.6 m wooden terrariums (6 animals in each), using natural soil as substrate. Animals were marked with a red number on their carapace. A 60W spot bulb provided the local temperature of 30-35 °C. They received light for 12 hours. Tortoises were raised in the department from the age of 1 month. Animals were not hibernated. A wooden house served as a hiding place for each group, and tap water was given ad libitum. The air temperature was 25-28 °C during the daytime and 20-22 °C at night. The relative humidity was between 50-60%. Tortoises were bathed in warm water for 15-20 minutes once a week.

None of the tortoises had ever received protein of animal origin, and the diet was based on garden weeds (80%;
dandelion, chickweed, grass, plantain, cloverleaf) and field vegetables (20%; 2/3 part cucumber, 1/3 part apple and carrot) were offered. A dietary vitamin and mineral supplement (Korvimin ZVT + Reptil, containing 150 g/kg Ca, 50 000 IU/kg vitamin D₃) was given on a daily basis. Tortoises were fed once a day, in the morning.

Blood samples (0.5-1ml) were collected 5 times every two months - beginning in February and ending in October - from the dorsal coccygeal vein with 22G needle into heparin collection tubes. The following parameters were measured from the plasma: calcium, ionized calcium, phosphorus, aspartate aminotransferase (AST), uric acid, sodium and potassium. Due to technical problems, we could not measure AST in the second sample collection. Plasma was separated after blood sampling by centrifugation, and frozen at -20 °C until being analyzed. Ions were measured immediately after transportation. Ionized calcium, potassium and sodium were measured using an electrolyte analyzer (Radiometer ABL 500 blood gas analyzer; Radiometer Medical ApS, Brønshøj, Denmark), the other biochemical parameters were measured with Randox Rx Daytona analyzer (Radox Laboratories Ltd, Crumlin, UK).

For each dataset, the mean, the standard deviation (SD) and the 95% confidence interval of the mean were calculated by using R 2.14.2.software (R Development Core Team, 2009, R Foundation for Statistical Computing, Vienna, Austria). Normality was tested by a quantile-comparison plot. Mixed effect linear modeling was used to analyze the data. Blood parameters served as response variables, samples as fixed factors, and individuals as random factors. The Spearman-rank correlation coefficient was used to assess the correlation between calcium and ionized calcium.

All institutional and national guidelines for the care and use of laboratory animals were followed. This experiment also complies with the relevant legislation. The work has been approved in the ethics review by the Hungarian Committee of Animal Welfare and also the Committee of Animal Welfare at the Faculty of Veterinary Science.

Table 1 shows the ranges and mean values with standard deviations by each sample taking (n=12/sample). As demonstrated, except for AST (P=0.153), sampling time had significant effect on the parameters (P<0.001). Table 2 shows the ranges, confidence intervals of the means (95%) and mean values (including standard deviation) of the examined parameters in 12 animals (n=60/parameter).

It can be seen that both blood calcium and ionized calcium levels are fairly standard with relatively low SD values. A positive correlation was found between calcium and ionized calcium (r=0.66; P<0.001). Phosphorus, sodium, potassium, calcium/ionized calcium to phosphorus ratios and solubility indexes were within the reference ranges from the literature (34, 39).

The concentration of uric acid and the activity of AST varied widely with high SD and ranges. Three animals had uric acid levels higher than 200 µmol/l; the mean concentration was close to 100 µmol/l. Extra low activity of uric acid (4.2 µmol/l) was measured only once.

Discussion and conclusion

The dorsal coccygeal vein - which was used in this study – is commonly used in chelonians. Peripheral blood samples can contain variable amounts of lymph. Two studies (20, 33) described the significant effect of venipuncture site on several biochemical parameters (e.g.: calcium, uric acid, potassium, total protein). Others (8, 42) found differences only in some parameters (e. g.: potassium), or found no significant effect on the biochemical parameters (9). This phenomenon is in connection with the rate of hemodilution. The activity of enzymes and the concentration of total protein, albumin and globulin are generally lower in lymph than in plasma. None of our samples showed visual signs of lymph contamination although this cannot be necessarily detected by visual observation.

The possible effects of the diet, environmental conditions and sex on the measured parameters were minimized in this experiment. One study (30) proved that tortoises under identical conditions (feeding and housing) had lower SD than those kept under different conditions. As indoor housing (according to own data collection) is widely spread by hobby keepers, blood reference values for such animals are important from the clinical point of view.

Despite the statistically significant difference between the collected samples (Table 1), standard deviations were low in all samplings except for AST and uric acid. Accordingly, the clinical relevance of the samplings seems to be important, especially for AST and uric acid. In order to eliminate the possible effects of time on sampling, repeated measurements are recommended when activities of biochemical parameters are not in the reference intervals.

Some studies also demonstrated the seasonal changes in calcium in males (maximum of 3.8 mmol/l, 4.8mmol/l, 3.3 mmol/l or 6.6 mmol/l), in nature or outdoor housing (3,18, 39, 44). According to our study, indoor housing does not affect this parameter. Due to lower blood calcium level (3.0±0.3 vs. 2.3±0.3 mmol/l) the total calcium-phosphorus ratio (mean 3.7) was lower than that determined by Eatwell (16). Lower levels in our study may be the result of haemodilution or indoor housing. However, the average levels were similar to those from another study (12, 22, 33).

Calcium-phosphorus, ionised calcium-phosphorus ratios and solubility indexes can help in the diagnosis of renal diseases. High solubility index (>9) indicates the risk of soft tissue mineralization, and hyperuricaemic reptiles appear to have high index (41). Eatwell (14) found a solubility index of 14.09 and a total calcium-to-phosphorus ratio of 0.55 in a tortoise with egg binding and metastatic mineralisation. No primary renal disease was identified in that case. A side-necked turtle (Phrynops geoffroanus) with significant renal disease and metastatic mineralisation of multiple tissues had solubility indexes of 3.0 and 2.82, and total calcium-to-phosphorus ratios of 2.08 and 2.82 (36).

In one study, the level of ionised calcium – despite high total calcium (5.8 mmol/l) - was not higher than 1.9 mmol/l (23). This parameter is less influenced by season and sex (10, 15, 18, 23, 40). Confidence intervals in our experiment were similar to those measured by Eatwell (15, 16). Minimums were lower than in the other studies but
of drinking water, high protein diets and dry environment (especially in winter) can lead to gout. Uric acid levels lower than 250 µmol/l are considered normal, however, in the immediate post-hibernation period, values of up to 350 µmol/l can also be accepted as normal (41). For this parameter, the reference values show wide (e.g.: 105-467.5 µmol/l; 89.2-344.9 µmol/l; 50-539 µmol/l) ranges (3, 22, 34). 4.2 µmol/l as measured in our study seemed to be very low and rarely described in the literature, although similar data (5.9 µmol/l) were found by Weinzierl (44). Three of our animals had values higher than 200 µmol/l, and in two of these high potassium levels (5.8 and 6 mmol/l) were measured at the same time. As mentioned before, such values may indicate dehydration.

One study (1) demonstrated markedly higher (13.1 vs. 4.7 mmol/l) potassium levels in Burmese brown tortoises (Manouria emys) when heparinised blood was stored at 25°C for two hours compared to fresh heparinised samples. The same differences were not demonstrated in Aldabra tortoises (Geochelone gigantea). In cases of potassium and sodium, the storage of the samples at 4°C significantly improved their stability (1), and after 24 hours there were no appreciable changes in the biochemical parameters (17). Seasonal variation occurs in potassium and sodium (18, 23, 45) also. Tortoises with potassium levels greater than 5.5 mmol/l appear to be dehydrated (41), but hyperkalaemia can be found in haemolysed blood samples as well. Hypokalaemic reptiles (1.2mmol/l) might have enteropathy or dietary deficiency such as anorexia, enteritis or muscle weakness (45). Other studies (3, 13, 18, 23) also report higher values in clinically healthy animals (up to 8.7 mmol/l, 7.1mmol/l or 13.9 mmol/l).

Sodium levels were similar to those reported in the other studies (3, 34, 35). Sodium levels may be elevated in conditions followed by extensive water loss or reduced water intake (e.g. inability to drink because of stomatitis or non-availability of water; 6, 45).

Enzymes are not tissue-specific and may vary within species, which makes their interpretation difficult. Although AST can be measured at moderate levels in many different

**Table 1.** Ranges and mean values with standard deviations (SD) of blood plasma samples in five samplings (n=12/sampling)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P</th>
<th>s1</th>
<th>s2</th>
<th>s3</th>
<th>s4</th>
<th>s5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mmol/l)</td>
<td>&lt;0.001</td>
<td>Range: 2.1-2.5</td>
<td>2.2-2.8</td>
<td>2.2-2.5</td>
<td>1.6-2.4</td>
<td>2.3-2.8</td>
</tr>
<tr>
<td>Ionised calcium (mmol/l)</td>
<td>&lt;0.001</td>
<td>Range: 1.0-1.5</td>
<td>1.4-1.9</td>
<td>1.5-1.8</td>
<td>0.5-1.3</td>
<td>1.2-1.6</td>
</tr>
<tr>
<td>Phosphate (mmol/l)</td>
<td>&lt;0.001</td>
<td>Range: 0.9-1.5</td>
<td>0.5-0.8</td>
<td>0.9-1.4</td>
<td>0.5-0.9</td>
<td>1.0-2.0</td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>&lt;0.001</td>
<td>Range: 90.4-266.0</td>
<td>27.9-200.6</td>
<td>4.2-151.2</td>
<td>71.7-256.8</td>
<td>42.2-156.7</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>0.154</td>
<td>Range: 50.0-133.0</td>
<td>35.0-141.0</td>
<td>35.0-140.0</td>
<td>51.0-280.0</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Ranges, the 95% confidence intervals of the means and mean values with standard deviations (SD) of the blood plasma parameters (n=60/parameter)

<table>
<thead>
<tr>
<th>Blood parameters</th>
<th>Range</th>
<th>CI mean</th>
<th>Mean and SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mmol/l)</td>
<td>1.6-2.8</td>
<td>2.3-2.4</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>Ionised calcium (mmol/l)</td>
<td>0.5-1.9</td>
<td>1.2-1.4</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>Phosphorus (mmol/l)</td>
<td>0.5-2.0</td>
<td>1.1-1.2</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>Sodium (mmol/l)</td>
<td>122.0-138.0</td>
<td>126.7-128.8</td>
<td>127.7±4.0</td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>3.5-6.0</td>
<td>4.4-4.7</td>
<td>4.5±0.6</td>
</tr>
<tr>
<td>Ca:P</td>
<td>0.9-5.4</td>
<td>-</td>
<td>2.3±0.9</td>
</tr>
<tr>
<td>Ca²⁺+P</td>
<td>0.3-3.6</td>
<td>-</td>
<td>1.4±0.8</td>
</tr>
<tr>
<td>CrP</td>
<td>1.2-4.2</td>
<td>-</td>
<td>2.6±0.7</td>
</tr>
<tr>
<td>Ca²⁺+xP</td>
<td>0.5-2.3</td>
<td>-</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>4.2-266.0</td>
<td>105.8-135.4</td>
<td>121.1±56.3</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>30.0-280.0</td>
<td>63.8-89.3</td>
<td>76.8±42.8</td>
</tr>
</tbody>
</table>

**AST** Aspartate aminotransferase; CI confidence interval, 
Ca:P total calcium to phosphorus ratio,
Ca²⁺+P ionised calcium to phosphorus ratio,
Ca²⁺+xP solubility index, Ca²⁺+xP solubility index

tortoises by bathing and offering ad libitum water access, which had great impact on the blood uric acid level. Lack of drinking water, high protein diets and dry environment (especially in winter) can lead to gout. Uric acid levels lower than 250 µmol/l are considered normal, however, in the immediate post-hibernation period, values of up to 350 µmol/l can also be accepted as normal (41). For this parameter, the reference values show wide (e.g.: 105-467.5 µmol/l; 89.2-344.9 µmol/l; 50-539 µmol/l) ranges (3, 22, 34). 4.2 µmol/l as measured in our study seemed to be very low and rarely described in the literature, although similar data (5.9 µmol/l) were found by Weinzierl (44). Three of our animals had values higher than 200 µmol/l, and in two of these high potassium levels (5.8 and 6 mmol/l) were measured at the same time. As mentioned before, such values may indicate dehydration.

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tissues (43, 38), one study (4) demonstrated higher level of AST in the heart tissue than in the kidney tissue. Values are considered to be normal under 250 IU/l (6). Reference intervals of some biochemical parameters are not well determined for Mediterranean tortoises. Some authors reported 42-134 IU/l (35) or 50-167 IU/l (44), while others described much wider ranges, 48-628 IU/l (3), 0-241 IU/l (18) or 1-138 IU/l (34). In this study, AST values were generally under 100 IU/l (Table 2); the highest (280 IU/l) value was measured once in one tortoise. All other samples from this animal were under 100 IU/l.

In order to have proper blood biochemical references in reptiles, the same housing and feeding systems of the animals are essential as well as information on the past record of the individuals. According to the literature, clinically healthy reptiles may also have pathological blood parameters, while others with clinical signs may have values within the reference range. Using confidence intervals of the mean may be advantageous in variables with wide ranges (e.g.: uric acid, enzymes). Although clinically healthy animals may have higher levels than these, repeated sampling is advised in such situations.

As the indoor housing is common among hobby keepers (according to our own data collection), blood reference values provided in this article can be used by veterinarians in the clinical practice. Seasonal changes in blood biochemical variables are not significant as in the wild animals. In case of indoor housed tortoises the modifying effects (e.g.: season, temperature) on the measured blood parameters can be minimized, which results in the less variable data than mentioned in the literature regarding the outdoor housed or free ranging tortoises. Because the sampling time had significant effect on most of the blood parameters for the proper diagnosis repeated samplings are advised.

### Conflicts of interest

Herewith it is confirmed that neither of the authors has any personal or financial conflicts of interest in the writing of the article.

### References


Varijacije u vrijednostima biohemijskih parametara u krvi mužjaka Hermann kornjača (*Testudo hermanni*)

**Sažetak**

**Uvod i ciljevi**

Nutritivni problemi kod reptila su česti, posebno metaboličke bolesti kostiju. Jedan od načina ranog dijagnosticiranja ovih poremećaja jeste određivanje biohemijskih parametara u krvi. Na aktivnosti nekih biohemijskih parametara mogu utjecati godišnje doba, spol, uvjeti smještaja i ishrana.

Prema našim ranijim prikupljenim podacima, vlasnici kućnih ljubimaca su držali svoje kornjače u zatvorenom prostoru, a ishrana im se uglavnom zasnivala na povrće uzgojenom za ljudsku upotrebu. Uvjeti u kojima je izvedena ova studija su nalikovali gore pomenutim kućnim uvjetima.

Cilj ove studije je bio istražiti biološke varijacije vrijednosti pojedinih biohemijskih parametara zdravih mužjaka Hermann kornjača ponavljanim uzimanjem uzoraka od istih životinja. Cilj je također bio procijeniti vrijednosti parametara u odnosu na vrijeme kada su uzorci prikupljani.

**Materijal i metode**

Dvanasta 2.5-godišnjih mužjaka Hermann kornjača su smješteni u zatvorenom prostoru, u terarijum. Životinje nisu bile u stanju hibernacije. Vođa iz slavine im je davana ad libitum. Temperatura zraka je iznosila 25-28°C (sa lokalnom temperaturom od 30-35°C) tokom dana, i 20-22°C tokom noći. Relativna vlažnost je iznosila 50-60%. Kornjače su provodile u toploj vodi 15-20 min jednom sedmično. Ni jedna od kornjača nikada nije uzimala proteine animalnog porijekla, a ishrana im se zasnivala na vrtnim korovima (80%) i rendanom povrće (20%). Suplementi vitamina i minerala (Korvimin ZVT + Reptil koji sadrži 150 g/kg Ca, 50 000 IU/kg vitamina D3) su davani svaki dan. Kornjače su hranjene jednom dnevno, i to ujutro. Uzorci krvi (0.5-1ml) su prikupljani 5 puta svaka dva mjeseca, počevši u februaru, i završavajući u oktobru, iz dorzalne kokcigealne vene sa iglom od 22G u heparinske epruvete. Za analizu podataka su korišteni mješani efekti linearnih modela. Parametri krvi su korišteni kao izlazne varijable, uzorci kao fiksni faktori, a životinje kao slučajni faktori. Spearmannov koeficijent korelacije je korišten za procjenu korelacije između kalcija i jonskog kalcija.

**Rezultati i interpretacija**

U tabelama 1 i 2 su prikazane razine aktivnosti. Osim kod AST (P=0.153), vrijeme uzorkovanja je imalo signifikantan učinak na parametre (P<0.001). Fosfor, natrij, kalij, omjer kalcij/jonski kalcij i fosfor te indeksi rastvorljivosti su prema literaturi bili unutar referentnih granica.

Utvrđeno je postojanje pozitivne korelacije između kalcija i jonskog kalcija (r=0.66; P<0.001). Kod tri životinje su vrijednosti urične kiseline prelaze 200 µmol/l, a prosječna koncentracija oko 100 µmol/l. Izuzetno niska vrijednost urične kiseline (4.2 µmol/l) je izmjerena samo jednom.

U ovom eksperimentu su minimizirani mogući učinci ishrane, okoliša i spola na izmjerene parametre. Upkrkos statistički signifikantnoj razlici između prikupljenih uzoraka (Tabela 1), standardne devijacije su za sve uzorke bile male osim za AST i uričnu kiselinu. Prema tome, uzorkovanje je bilo klinički relevantno, posebice kod AST i urične kiseline.

Prema rezultatima linearne regresije, postoji relativno jaka veza između jonskog i ukupnog kalcija. Da bi se postavila ispravna dijagnoza, preporučuje se mjerenje i ukupnog i jonskog kalcija. Niži omjer ukupni kalcij-fosfor u našoj studiji može biti posljedica hemodilucije ili života u zatvorenom prostoru. Međutim, pronađene prosječne vrijednosti kalcija su slične onima u drugim studijama.

Najveća koncentracija fosfora (2.0 mmol/l) je izmjerena kod kornjače koja je u isto vrijeme imala najveće koncentracije urične kiseline (211.5 µmol/l) i kalija (5.8 mmol/l). Ova kombinacija ukazuje da je kornjača bila dehidrirana uprkos adekvatnom snabdijevanju vodom. Savjetujemo da se u ovakvim situacijama ponovi uzorkovanje kako bi se dobila ispravna informacija o zdravstvenom stanju.

**Zaključak**

S obzirom da je držanje životinja u zatvorenom prostoru često kod vlasnika koji kornjače drže iz hobija (prema našim podacima), referentne vrijednosti krvnih parametara koje su prikazane u ovom radu se mogu koristiti u veterinarskoj kliničkoj praksi. Sezonske promjene biohemijskih varijabli krvi nisu tako signifikantne kao kod divljih životinja. S obzirom da je vrijeme uzorkovanja imalo signifikantan učinak na većinu krvnih parametara, preporučuje se ponavljanje uzorkovanja kako bi se postavila ispravna dijagnoza.