

Trace Element Levels in the Serum of Hermann's Tortoises (*Testudo hermanni*)

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Abstract: Up to now, blood reference values for trace elements (zinc, copper, iodine, iron, selenium and manganese) in the serum of *Testudo spp.* have not been established. Most studies concerning trace element levels in blood of reptile's regard environmental pollution esp. concerning selenium. In the present study levels of iron, copper, iodine, zinc, selenium and manganese were evaluated in 130 blood samples of clinically healthy Hermann's Tortoises (*Testudo hermanni*), originating from pet tortoises in Germany (n= 64), extensively kept captive-hatched tortoises from Turkey (n=20), and from free-ranging specimens of two locations in France (n=46). The French habitats differ in the nature of the soils, one being low in lime and another high in lime. The levels of trace elements in the blood were compared between the different groups and to the levels of trace elements in the respective feed rations resp. food plants. The aim was to check the correlation between origin, husbandry, feeding and sex on the levels of trace elements in the blood. Blood levels of trace elements, regardless of origin, gender, or diet, generally showed a wide range. Also there existed a poor correlation to contents in diet concerning iron, selenium, and manganese, but a statistically significant correlation to zinc, copper and iodine. Preliminary reference values of zinc, iodine and copper can be a useful tool to diagnose nutrient deficiencies, poisoning and deficiency symptoms in *Testudo hermanni*.

Keywords: European Tortoises, Copper, Zinc, Iodine, Iron, Selenium

1. Introduction

Pet tortoises of the genus *Testudo* have high emotional and financial value for their owners. Because of this, owners expect extensive diagnostics of these pets. According to Dennert [5], the proportion of tortoises presented in veterinary practices due to nutritional diseases or corresponding symptoms represents 24% of all patients. Trace element levels in the serum are considered helpful for checking imbalances in diets, for example in dairy cows [35]. To our knowledge, reference values of trace element levels in serum of clinical healthy *T. hermanni* have not yet been established.

Trace element levels were investigated in blood samples of recently diseased reptiles, including tortoises [27]. They have also been evaluated in Loggerhead Sea Turtles (*Caretta caretta*) as indicators of environmental pollution [25] and in whole body analysis of chelonians [20].

2. Material and Methods

Blood samples of 130 clinical healthy *T. hermanni* from four different habitats were examined (70 male and 60 female specimens).

Sixty-four samples originated from pet tortoises in Germany, 20 samples were from extensively kept captive-

hatched tortoises from Turkey. Samples of pet captive tortoises from Germany were received from a reptile shelter in Munich. The history of these animals is mostly unknown.

Forty-six samples were collected from free-ranging *T. hermanni* from two locations in France, with 37 from Habitat "France 1" (soil poor in calcium) and 9 from Habitat "France 2" (lime rich soil).

Remnants of blood samples were collected within the scope of health checks (Germany and Turkey) and genetic tests (France), respectively. These blood samples were taken from the dorsal tail vein (*Vena coccygealis dorsalis*).

The body weight of the tortoises showed a range from 113 to 2,396 grams.

The average weight was 750 grams. For each animal a protocol was created including a detailed data and history (if known).

The food rations of German and French habitats were analyzed by Weender Analysis. Plants fed to the tortoises from the reptile rescue center, from one private owner and from two locations in France, were analyzed for the contents of trace elements zinc, copper, iron, iodine, selenium and manganese. All analyses were performed by LUFA Kiel, Doktor-Hell-Straße 6, 24107 Kiel, Germany.

The contents of trace elements in the diets were compared to

the blood levels in serum to figure out food - blood correlations.

The study was approved by the Ethics Commission of Veterinary Faculty of LMU, Munich, Germany (AZ 5902112015).

After centrifugation (2.000 U/min for 10 minutes) of the blood samples, serum was deep frozen (-18° Celsius). Trace elements analyses were performed by "Laboklin", Bad Kissingen, Germany.

Contents of copper, iron, zinc were evaluated by photometrical methods. Levels of selenium and manganese were measured by atomic absorption spectroscopy.

Statistics were performed, regarding sex and origin, i.e. pets versus free-ranging or different habitats. Statistics were performed by IBM SPSS Statistics. The Mann-Whitney Test was used for comparison of "gender" and "habitat" groups. The Shapiro-Wilk Normality Test, the Kruska-Wallis Test and the Dwass-Steel-Crichtlow-Fisher Test were used for checking seasonal differences. Statistically significant differences were defined with $p < 0.05$. Possible significant correlations were checked by the Spearman Correlation Coefficient.

3. Results

All trace element levels are provided in Table 1.

Table 1. Levels of Trace elements.

	Zinc (n=130) umol/l	Copper (n=131) umol/l	Iron (n=130) umol/l	Selenium (n= 118) ug/l	Manganese (n=125) ug/l	Iodine (n= 93) ug/l
2,5% -97,5% quantile	5,02 - 57,65	2,65 - 15,3	1,47 - 21,61	1,69 - 51,34	1,01 - 39,4	1,15 - 41,11
median	26,05	7,9	7,95	15,95	6,85	4,5
minimum	3,8	1,6	0,6	1,1	1	1
maximum	62,7	17,9	17,9	72,2	51,8	100,5

All trace element levels in food rations are provided in Table 2.

Table 2. Levels of Trace elements in food rations.

	France 1	France 2	Reptile Rescue Station	Private Owner
Cooper mg/kg dry matter	4,5	5,91	3,31	12
Manganese mg/kg dry matter	34	50,9	25,8	81
Zinc mg/kg dry matter	20,6	22,1	13,8	43
Iron mg/kg dry matter	133	469	385	570
Iodine mg/kg dry matter	0,33	0,46	3,98	50,6
Selenium mg/kg dry matter	< 0,1	<0,05	<0,1	0,11

There was a wide range of the particular trace element levels comparing all animals from different habitats. There were no statistically significant differences regarding gender, habitat for selenium, iron and manganese. There were statistically significant differences seasonally for zinc, iron, selenium and manganese.

3.1. Zinc

In zinc serum levels, statistically significant differences regarding habitat and gender were detected. Zinc showed higher levels in male tortoises ($p=0.049$). There were significant statistical differences when comparing the habitats of "Germany" (average level 22.15 $\mu\text{mol/l}$) and "France 1" (average level 29.0 $\mu\text{mol/l}$).

When comparing the French habitats, there were statistically

significant differences and lower values for Habitat "France 2", which has lime rich soil ($p < 0.001$).

There were also statistically significant differences between the Turkish tortoises (40.7 $\mu\text{mol/l}$.) versus the German and both French groups. Turkish tortoises versus "France 1" tortoises (29.0 $\mu\text{mol/l}$.) showed significant higher levels ($p < 0.001$). Turkish versus "France 2" specimens (18.4 $\mu\text{mol/l}$) had statistic significantly higher levels ($p < 0.001$) and Turkish versus German pet tortoises (22.15 $\mu\text{mol/l}$) showed a statistically significant higher level ($p < 0.001$). Seasonal differences with statistical significance existed in spring level (21.73 $\mu\text{mol CI } 95$) which increased to fall (30.06 $\mu\text{mol CI } 95$) ($p < 0.001$). For zinc, there was a statistically significant correlation between blood and feed values, with a Spearman correlation coefficient of 0.202.

3.2. Copper

Levels of copper yielded habitat differences. Levels were significantly lower in German tortoises (on average 7.7 $\mu\text{mol/l}$) vs “France 1” (10.4 $\mu\text{mol/l}$) tortoises ($p = 0.003$). Comparing German tortoises (7.7 $\mu\text{mol/l}$) and Turkish tortoises (4.35 $\mu\text{mol/l}$), there were statistically significant lower levels in Turkish tortoises ($p=0.001$). Both groups of French tortoises showed significant higher levels than Turkish tortoises. There was a statistical significance between Turkish tortoises (4.35 $\mu\text{mol/l}$) and “France 1” tortoises (10.4 $\mu\text{mol/l}$) ($p < 0.001$) and the significance between Turkey and “France 2” (8.7 $\mu\text{mol/l}$) ($p=0.003$).

For copper there are also statistically significant differences depending on sex ($p= 0.007$). Female tortoises (7.503 $\mu\text{mol/l}$) showed lower levels in blood serum when compared to male tortoises (9.087 $\mu\text{mol/l}$). For copper there was a statistically significant correlation between blood and food values, with a Spearman correlation coefficient of 0.312.

3.3. Selenium

For selenium there were detected statistically significant differences depending on habitat. Comparing habitats “Germany” (on average 18.4 $\mu\text{mol/l}$) and “Turkey” (on average 7.85 $\mu\text{mol/l}$) there were statistically higher levels in German tortoises ($p < 0.001$).

Also, there were statistically significant differences depending on season. The level was increasing ($p= 0.02$) from spring (21.25 $\mu\text{mol CI } 95$) into summer (38.81 $\mu\text{mol CI } 95$) and it was decreasing ($p < 0.001$) from summer into fall (15.29 $\mu\text{mol CI } 95$).

3.4. Manganese

For manganese there were statistically significant differences depending on sex. Female tortoises (on average 12.3 $\mu\text{mol/l}$) had statistically significant higher levels than male tortoises (on average 8.29 $\mu\text{mol/l}$) ($p= 0.049$). There were also statistically significant differences depending on season. The level in the spring (15.4 $\mu\text{mol CI } 95$) was statistically higher than in the fall (8.23 $\mu\text{mol CI } 95$) ($p < 0.001$).

3.5. Iodine

For iodine, differences were found in the feed rations. The content in the German feed rations of the reptile sanctuary (3.98 mg/kg dry matter) was ten times higher than in the French feed rations (0.33 and 0.46 mg/kg dry matter). In the feed rations of the private owner (50.6 mg/kg dry matter) it was more than ten times higher than in the German feed rations of the reptile sanctuary.

3.6. Iron

For iron there were statistically significant differences depending on the season. Iron levels showed statistically significant lower levels in spring (7.23 $\mu\text{mol CI } 95$) than in the fall (8.23 $\mu\text{mol CI } 95$) ($p=0.001$).

For iron there was not a statistically significant correlation

between blood and feed values.

4. Discussion

The health status was proven secure only for the tortoises from Germany and Turkey, as their blood samples served for medical check and blood values were in reference values. In France, blood samples were taken from apparently clinically healthy free- ranging tortoise specimens, but there was no proof of this by blood values.

Skin or shell diseases in tortoises are probably associated with deficiencies of trace elements [17]. Since no animals with diseases were examined in the present study, this relationship could not be verified.

Not all tested animals revealed the same age and weight. In whole body analysis of *Testudo* there were statistically positive correlations for iron and age [20]. In the present study, there were no statistically significant correlations between levels of trace elements in the blood serum and age of tortoises detected. Time of blood collection, body temperature of the animals, storage time of the blood serum samples, and time of feeding were not documented. Levels of blood parameters in *Testudo* can change by seasonal changes [23], blood storage time, and temperature [30]. Checking serum value of iodine in *Testudo spp.* there were seasonal differences for female species with an increasing level of iodine, which increases during the year. For male species, iodine levels decreased from spring to summer, and increase from summer to fall [23].

There were no statistically significant differences for iodine content in the present study. The present study shows statistically significant differences in the levels of trace elements in *Testudo spp.* due to seasonal influences only for the levels of zinc, iron, selenium, and manganese.

In cattle it is shown that food composition may also have an influence on levels of trace elements [36]. In cases of excessive raw fibre, there is lower caloric intake. This is due to lower digestibility, and inhibition of absorption of trace elements [6].

The food rations of the tested tortoises were analyzed, but showed only a current snapshot, as they were sampled in between May and September. Throughout the year, the animals find different species of plants depending on vegetation and season. Also, the composition of the plants differs in dry matter, protein, fiber content, and the content of trace elements [14].

The possible infection with gastrointestinal parasites may affect the level of trace elements in the serum. Tortoises infected by gastrointestinal parasites showed a larger intake of trace elements, higher levels of trace elements in their feces, and better digestibility of incoming trace elements [2]. For free ranging animals in France, parasite infection is probable.

In the reptile shelter, all tortoises underwent a parasitological examination and routine deworming. For Turkey the procedure of parasitological examination is unknown.

Blood samples were taken from dorsal tail vein for testing. Therefore it must be considered that the levels of trace

amounts in the blood may vary. This could depend on the site of blood collection, and also the dilution by lymph fluid as shown by Eatwell [9]. A dilution of the blood sample with lymph fluid in the tail vein samples in comparison to samples from the jugular vein was demonstrated. Eatwell [9] recommends, for these reasons, the withdrawal of blood from the *jugular vein*. Consequently a dilution of the blood samples with lymph fluid cannot be excluded.

There were no differences for levels of trace elements in blood serum between the probably ill tortoises [27] and the probably healthy tortoises of the present study. For zinc deficiency, correlations with shell deformations are known in practice [17] but have not yet been proven.

The serum levels of zinc, manganese, selenium, and copper in the blood were within the range from blood serum of probable diseased *Testudo* [27] and values from tissue of *Testudo* [20]. They were also comparable with other herbivores as they were compared with reference values in blood serum of horses [21].

4.1. Copper

There were two times higher values for copper in the French forage plants than in the German food ration. These differences were also detected in the blood serum levels for copper. There was a statistically significant difference for copper in the blood serum between German and French tortoises. In German tortoises the values for copper were significantly lower, here there seems to be an influence of diet on blood serum levels.

4.2. Zinc

Comparing the values for zinc in the blood serum, there was a statistically significant difference between the two groups of free ranging tortoises in France. "France 1" turtles showed higher levels of zinc in their food plants and higher levels of zinc in blood serum. This would support the thesis that there is an influence from zinc in the food on the level of zinc in the serum. Another reason for lower zinc levels in Group "France 2" could be a higher content of calcium in their food plants as this group originated from a limestone rich area. A high content of calcium causes a worse absorption of zinc into the blood [22]. Reasons for statistically significant differences for zinc levels regarding sex, could be the deposit in the eggs of female tortoises and processes of fertility. The content of zinc in the content of eggs of tortoises (*Testudo spp.*) was 73.31 ± 5.76 milligrams per kilogram of dry matter [13]. This suggests a release of zinc into the eggs.

4.3. Iron

The whole-body study of Kopsch [20] yielded very high amounts of iron (234.4 ± 63.4 mg per kilogram dry matter). Especially contents in the liver ($6,507 \pm 3,569$ mg/kg dm) and shell (555.7 ± 169.7 mg/kg dm) were extraordinarily high, and much higher than in birds or mammals. However, values for iron in the blood serum of the tortoises in this study ($1.2 - 22.9$ $\mu\text{mol/l}$) were lower than the reference values for horses ($17.9 -$

64.5 $\mu\text{mol/l}$, [21]). Although tortoises contain large amounts of iron in their body, they seem to store iron especially in their liver and shell but not in their blood.

Another critical consideration is the type of measurement of iron in blood serum. Iron in blood serum can be bound by absorbed tannins from food rations.

The binding of iron in the blood would explain the tendency of increasing iron levels in the food and low iron levels in the serum. In other literature, content of trace elements in the liver of sea turtles was measured as an indicator of environmental pollution [1]. With 1180 mg/kg dry mass [d. m.] there was also prominent levels for iron in the liver of sea turtles [11].

4.4. Iodine

Iodine levels in blood serum were for all four groups very low, ($1 - 41.2$ $\mu\text{g/l}$) with the exception of the group of private owners ($24.7 - 100.5$ $\mu\text{g/l}$). The iodine values were lower than in probably ill tortoises ($1 - 2710$ $\mu\text{g/l}$, [27]) or horses ($50 - 120$ $\mu\text{g/l}$, [21]).

The value of iodine in the food rations of the captive tortoises from a private owner in Germany was ten times higher than in food ration from the reptile rescue station in Germany, and a hundred times higher than in the plants from France. A reason for general higher iodine level in German food rations probably is the addition of sepia to German food plants (reptile shelter, private owner), and sea weed (private owner). An iodine deficiency or iodine consumption can also be influenced by the presence of dietary goitrogens [6]. For free-ranging tortoises it cannot be ascertained which plant species they have actually consumed. A recording of dietary goitrogens can therefore not be ruled out.

5. Conclusion

Pet tortoises of the genus *Testudo* have a high value for their owners, which is why the diagnostic possibilities are becoming increasingly important. Despite significant differences, the values in blood serum of all groups in the present study showed a wide range and were very similar in all groups including pet tortoises in Germany as well as free-ranging tortoises in their natural habitat. This indicates a positive evaluation of the diet and husbandry of pet turtles in Germany, despite differences in diet composition - since the food plants of the natural habitats are not available to the owners - and despite differences in climatic conditions.

The contents of iodine, zinc and copper in diet seem to have an impact on the blood serum levels of *T. hermanni*. Zinc could also be influenced by calcium intake, as animals from the lime-rich habitat in France had lower blood zinc levels than the comparison groups. Preliminary reference values for trace elements can be a useful tool for diagnosing nutrient deficiencies, poisoning, and deficiency symptoms in *Testudo hermanni* and may serve as orientation for other tortoise species. For checking nutritional imbalances, the blood serum values of zinc, copper and iodine are appropriate. Serum levels of manganese, selenium, and iron

appear to be of limited value. For these trace elements, poor correlation was shown between dietary and blood serum levels. It does not seem possible to influence them by diet, but they showed seasonal variations (selenium, manganese, iron) and differences by gender (manganese) and by habitat (selenium).

Animal Welfare Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

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