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Original article

REFERENCE VALUES FOR HAEMATO-BIOCHEMICAL PARAMETERS OF CASPIAN POND TURTLE (*MAUREMYS CASPICA*) IN THE CASPIAN SEA

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Summary

Adel, M., H. Abbasi, V. Arizza, S. P. H. Shekarabi & S. M. Al-Jufaili, 2023. Reference values for haemato-biochemical parameters of Caspian pond turtle (*Mauremys caspica*) in the Caspian Sea. *Bulg. J. Vet. Med.* (online first).

The Caspian pond turtle, *Mauremys caspica*, is an important reptile species in the southern basin of Caspian Sea. Haematological indices and serum biochemistry can be utilised as valuable screening tools for the health status of many domestic and wild animals. In this study, the blood parameters were measured in male and female turtles to establish physiologic reference ranges of selected haematological and serum biochemical parameters in *M. caspica*. For this purpose, blood samples were collected from 60 Caspian pond turtles (30 males and 30 females) from the southern basin of Caspian Sea. Significant differences were observed in some haematological indices (*i.e.* haemoglobin and haematocrit) and serum biochemical indices (*i.e.* alkaline phosphatase, aspartate aminotransferase, and creatine kinase) between the male and female turtles ($P<0.05$). However, further research is necessary to elucidate the influence of size, sex, season, and habitat variations on haemato-biochemical parameters in the Caspian pond turtle.

Key words: blood biochemistry, Caspian Sea, haematological parameters, *Mauremys caspica*

INTRODUCTION

Haematological and serum biochemistry parameters have been recognised as crucial tools to assess the health status of many domestic and wild animals. In fact, they can be used for monitoring health, physiological performance, stress res-

ponses, and diagnosing infectious and non-infectious diseases (Cerreta *et al*., 2020; Kophamel *et al*., 2022). There are many literature data available dealing with the haematological and biochemical values in chelonians (Sinaei *et al.,* 2019; Miguel *et al.,* 2020). For instance, Delgado *et al.* (2011) analysed the standard biochemical parameters of wild juvenile loggerhead sea turtle (*Caretta caretta*). Ehsanpour *et al.* (2015) determined the normal values of serum biochemical parameters in apparently healthy female hawksbill turtles (*Eretmochelys imbricata*) from the natural nesting habitat in Qeshm Island. The blood cells of Asian forest tortoise (*Testudo horsfieldii*) were evaluated based on the morphological features by Shadkhast *et al.* (2010). Javanbakht *et al.* (2013) also investigated the morphological analysis of blood cells in *Testudo graeca*, *Emys orbicularis*, and *Mauremys caspica* in Iran. Moreover, Arizza *et al.* (2014) characterised the haematological parameters of Sicilian pond turtles, *Emys trinacris*. Soltanian *et al*. (2021) also reported reference intervals for haematological and blood biochemical parameters in softshell turtles (*Rafetus euphraticus*).

The Caspian pond turtle, *Mauremys caspica*, belongs to the family Geoemydidae and inhabits the eastern Mediterranean region from north-western of Saudi Arabia, Bahrain, Caucasus, Turkey, Tbilisi, Iraq, and Iran (Vamberger *et al.,* 2013). Some toxicological assays were conducted on *M. caspica* due to some unique features of turtles in the environment (Soltanian *et al.,* 2018). However, there are currently no published data on the haematological and serum biochemical variables of the Caspian pond turtle in the southern basin of Caspian Sea as a scientific basis for other biological research.

The main purpose of this research is to distinguish the normal values of blood and serum biochemical parameters between male and female *M. caspica* from the southern basin of Caspian Sea.

MATERIALS AND METHODS

Sample collection and animal maintenance

Sixty *M. caspica* were sampled from Golestan and Mazandaran provinces (Caspian Sea, Iran) (Fig. 1). The permission of collecting *M. caspica* was issued by the Iranian Department of Environment (local No. 1184-N/14/2). They were transported alive in plastic boxes to the central laboratory of CSE Research Centre (Caspian Sea, Sari, Iran). The turtles were adapted to the new conditions for 14 days in seawater aquaria (200 L). The lab conditions were maintained similar to the natural environmental conditions (temperature 19.0±1.3 ºC, dissolved oxygen 7.8±0.5 mg/L, pH 7.5±0.3, and 14 h light:10 h dark photoperiod cycle) to minimise additional stress induced by environmental parameters. The turtles were fed three times per day at apparent satiety with minced Kilka fish (*Clupeonella* sp.; Clupeidae) and fresh vegetables (cucumber and lettuce). The water quality of each aquarium was monitored daily and regularly siphoned from the bottom and changed with fresh seawater. At first, the turtles were examined by external observation to ensure that they were healthy and the clovac was checked to distinguish sexes. Then, their total weight (g) and total length (cm) were measured (Table 1).

Blood sampling

The turtles were subjected to food deprivation for 24 h before blood sampling and

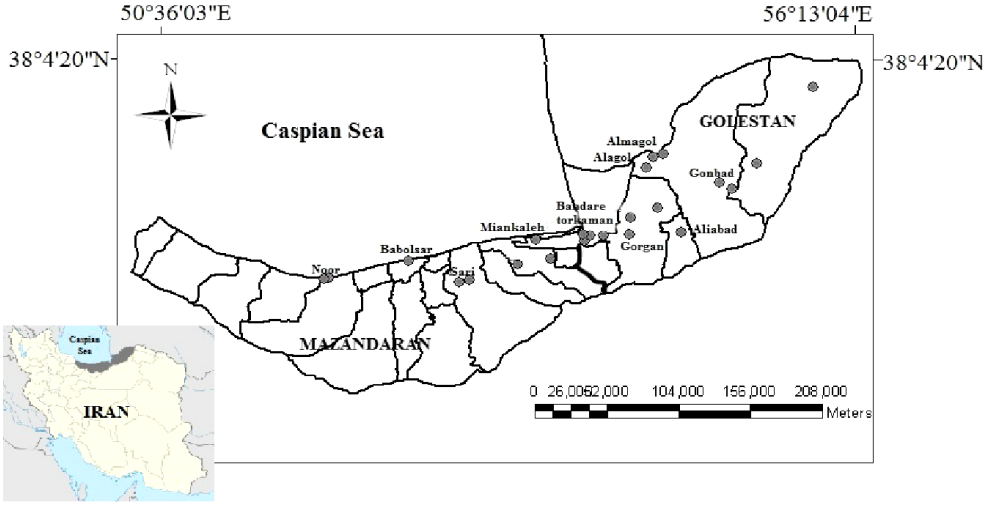

Fig. 1. Sampling sites of *Mauremys caspica* from Golestan and Mazandaran provinces, Iran (southern basin of the Caspian Sea).

Table 1. Biometric data of *Mauremys caspica* from the southern basin of Caspian Sea

Sex	Length (cm)	Weight (g)	Age (years)
Male (n = 30)	11.30 ± 3.8 [a]	277.3 ± 14.2[a]	3 ± 1 [a]
Female (n = 30)	10.84 ± 2.6[a]	271.8 ± 10.6[a]	3 ± 1 [a]

were anaesthetised with ketamine hydrochloride 5% (Alfasan Company, Iran) and diazepam 2% (Caspian Tamin Company, Iran) at 120 mg/kg and 2 mg/kg, respectively (by intramuscular injection). Then, 0.5 mL blood sample was collected from the dorsal coccygeal vein and transferred to tubes containing heparin as an anticoagulant and used for complete blood count tests. Also, 1 mL blood sample was moved to plain tubes and left to clot at refrigerator temperature for 1 h. Finally, the sera were separated by centrifugation at 3000×g for 15 min and the supernatants were stored at –80 ^{o}C until use.

Haematology parameters

The counting of red (RBC) and white blood cells (WBC) was performed by an improved Neubauer haemocytometer chamber using 1:200 Hayem's and Natt-Herrick fluids (Shadkhast *et al.,* 2010). Haematocrit (HCT, %) was determined by the standard microhaematocrit procedure (Ehsanpour *et al.,* 2015). Haemoglobin (Hb, g/L) was measured based on the cyanomethemoglobin method. The differential leukocyte counts were also computed based on the cells' morphology (*i.e.* heterophils, lymphocytes, monocytes, eosinophils, and basophils) from previously prepared Wright-stained slides under a light microscope (Nikon, Japan) (Shadkhast *et al.,* 2010).

Biochemical assays

The serum alanine aminotransferase (ALT, U/L), aspartate aminotransferase

Table 2. Mean haematological parameters in both sexes of *Mauremys caspica*. Different superscript letters denote significant differences (P<0.05)

Parameter	Female	Male
	Mean ± SD	Mean ± SD
Red blood cells ($\times 10^{12}$ /L)	0.38 ± 0.04[a]	0.44 ± 0.05[a]
Haematocrit (%)	28. 2 ± 1.5[a]	30.6 ± 2.1[b]
Haemoglobin (g/L)	62.4 ± 11.4[a]	74.2 ± 16.4[b]
White blood cells ($\times 10^{9}$ /L)	5.28 ± 1.06[a]	5.46 ± 1.3[a]
Heterophils (%)	68.4 ± 3.38[a]	70.4 ± 2.8[a]
Lymphocytes (%)	24.3 ± 2.42[a]	24.6 ± 1.03[a]
Basophils (%)	2.14 ± 0.85[a]	1.36 ± 0.74[a]
Eosinophils (%)	4.14 ± 0.38[a]	4.86 ± 0.28[a]
Monocytes (%)	0.52 ± 0.12[a]	0.39 ± 0.09[a]

(AST, U/L), alkaline phosphatase (ALP, U/L), lactate dehydrogenase (LDH, U/L), creatine kinase (CK, U/L), triglycerides (mmol/L), cholesterol (mmol/L), glucose (mmol/L), total protein (g/L), albumin (g/L), and globulin (g/L) levels were measured spectrophotometrically (Shadkhast *et al.,* 2010) by a biochemical auto-analyzer device (Eurolyser, Belgium) with relevant Pars Azmoon kits (Pars Azmoon, Tehran, Iran) based on the manufacturer's guidelines.

Blood electrolyte tests

Calcium, phosphorus and magnesium values (mmol/L) were assessed by a colorimetric technique using the biochemical auto-analyzer based on Delgado *et al.* (2011) method. The sodium and potassium concentrations (mmol/L) were determined using a photoelectric flame photometer (Jenway, England).

Statistical analysis

The data were subjected to Student's *t*-test after being tested by the Shapiro-Wilk test to determine the significant differences between haematological and serum biochemical indices between the sexes. A significance level of P<0.05 was used and the results were expressed as means ± standard deviation (SD). All the statistical analyses were performed with the SPSS software package ver. 22.

RESULTS

The results of haematological parameters for *M. caspica* from the southern basin of Caspian Sea are given in Table 2. No significant differences were obtained in WBC and RBC counts in both sexes (P > 0.05). The higher values of Hb (74.2±16.4 g/L) and HCT (30.6±2.1 %) were observed in male turtles (P<0.05). In addition, Hb and HCT values in this study were within the range 51.2–73.4 g/L and 25.7–34.8 %, respectively. The mean Hb values were 62.4±11.4 and 74.2±16.4 g/L in both sexes (P=0.007), respectively. Also, the mean HCT values were 28.2± 2.5 and 30.6±3.1% in female and male brood stocks, respectively (P=0.004).

The normal biochemical and electrolytes values in male and female *M. cas-*

pica are illustrated in Table 3. From the results, a significant difference was found out between ALP, AST, and CK values in both sexes. The average values of AST in male and female *M. caspica* were 348.62±58.6 and 274.28±43.8 U/L (P=0.009), and CK varied from 364.3± 54.3 to 436.5±68.7 U/L in female and male turtles, respectively (P=0.018). Also, the ALP level in females was 96.2±14.3 U/L and in the male turtles – 72.6±17.6 U/L (P=0.004). No significant difference was measured in the calcium, phosphorus, magnesium, sodium, and potassium concentrations between the male and female turtles (P>0.05). Also, no significant differences were obtained for ALT, LDH, triglycerides, cholesterol, glucose, total protein, albumin, and globulin levels (P>0.05).

DISCUSSION

Measurement of blood parameters and their reference ranges in *M. caspica* play a crucial role in determining the relationships between the health status and the ecosystem. Haematological parameters of chelonians are affected by age, sex, diet, diseases, maturation stage, environmental conditions, water quality parameters, and sampling methods (Pagano *et al.,* 2019; Rodríguez-Almonacid *et al.,* 2022).

Based on our results, higher values of HCT and Hb were obtained in the male turtles than in the females. This can be probably attributed to the higher mobility and metabolic activity of the male turtles compared to the females. Another possible explanation would be a different hormonal status during the breeding season

Table 3. Serum biochemical parameters and electrolytes in male and female *Mauremys caspica*. Different superscript letters denote significant differences (P<0.05)

Parameter	Female	Male
	Mean ± SD	Mean ± SD
AST (U/L)	274.28 ± 43.8[a]	348.62 ± 58.6[b]
ALT (U/L)	12.26 ± 1.8[a]	12.68 ± 1.6[a]
CK (U/L)	364.3 ± 54.3[a]	436.5 ± 68.7[b]
ALP (U/L)	96.2 ± 14.3[a]	72.6 ± 17.6[b]
LDH (U/L)	1024.8 ± 284.5[a]	1316.2 ± 339.1[a]
Total protein (g/L)	37.8 ± 4.6[a]	41.2 ± 5.8[a]
Albumin (g/L)	12.9 ± 1.8[a]	12.4 ± 5.8[a]
Globulin (g/L)	24.2 ± 2.8[a]	28.2 ± 3.4[a]
Triglycerides (mmol/L)	2.75 ± 0.86[a]	3.14 ± 1.05[a]
Glucose (mmol/L)	4.68 ± 0.69[a]	5.47 ± 0.93[a]
Cholesterol (mmol/L)	3.32 ± 0.21[a]	3.69 ± 0.25[a]
Potassium (mmol/L)	3.12 ± 0.56[a]	3.04 ± 0.65[a]
Sodium (mmol/L)	120.4 ± 12.6[a]	132.5 ± 14.3[a]
Magnesium (mmol/L)	0.44 ± 0.15[a]	0.47 ± 0.19[a]
Calcium (mmol/L)	0.76 ± 0.04[a]	0.82 ± 0.06[a]
Phosphorus (mmol/L)	4.15 ± 0.39[a]	4.28 ± 0.45[a]

(Janzen *et al.,* 1998). There is a tendency amongst all animals that estrogens and progesterone suppress erythropoiesis and androgens enhance it (Nikinmaa, 1990). The HCT level is highly varied depending on species, age, nutrition status, water quality, and habitat. HCT value is varied in the range of 20 to 45% (Perpiñán *et al.,* 2008). High oxygen is needed for most of the reactive species such as marine teleost and hence, they have a greater HCT value compared with the sedentary turtle such as freshwater turtle species and desert tortoises (Ahmed *et al.,* 2020).

Among the WBC, the most and the lowest representative cells were heterophils and monocytes respectively, with similar results reported by Pires *et al.* (2006) in *Caretta caretta*; Tavares-Dias *et al.* (2009) in freshwater turtles (*Podocnemis expansa*), Metin *et al.* (2008) in *Mauremys caspica* and *Mauremys rivulata* and Hidalgo-Vila *et al.* (2007) in Mediterranean pond turtles (*Mauremys leprosa*). From the results, no significant differences were obtained in the leukocyte differential counts between both sexes, in line with the findings of Hidalgo-Vila *et al.* (2007) in *M. leprosa* and Yu *et al.* (2013) in yellow pond turtle (*Mauremys mutica*). This result was different from leukocyte differential counts of African side neck turtle (*Pelusios sinuatus*) where female turtles had higher mean values for lymphocytes and heterophils than male turtles (Omonona *et al.,* 2011).

In the present study, low percentage of basophils (< 5%) was observed in both sexes of Caspian pond turtles. An absolute percentage of basophils has also been reported in loggerhead sea turtle (*Caretta caretta*) by Keller *et al.* (2004) and green sea turtle (*Chelonia mydas*) by Samour *et al.* (1998). On the other hand, high numbers of basophils were observed in the South Asian box turtle, *Cuora amboinensis* (Perpiñán *et al.,* 2008). This difference may be related to gender, age, size, season, environmental parameters, environmental conditions, and different stainings (Perpiñán *et al.,* 2008). In turtles, the eosinophils are responsible for phagocytic activity, especially involved in the destruction of parasites. Based on the results, no significant differences in eosinophils' number were observed between the two sexes. Similar observations were reported in Mediterranean pond turtles by Hidalgo-Vila *et al.* (2007). The average amount of eosinophils in *M. caspica* was 4.86 ± 0.28 % and 4.14 ± 0.38 % in males and females, respectively. These values were lower than those reported in other species of turtles (Hidalgo-Vila *et al.,* 2007). In the study of Muñoz & De la Fuente (2004), female *M. caspica* had a lower heterophil percentage than males, although no differences were observed between the sexes in monocyte and eosinophil percentages. Season, age, and species are some main variables affecting peripheral eosinophil concentrations in various chelonians (Yu *et al.,* 2013).

Despite that measurements of blood biochemical and physiological parameters are commonly used as diagnostic tools in aquatic toxicology and bio-monitoring (Kophamel *et al.,* 2022), to the best of our knowledge, there are no references for Caspian pond turtle from the southern basin of Caspian Sea. Liver enzymes in aquatic animals are considered indicators of hepatic activity and changes in the secretion of liver enzymes can be affected by physical and chemical parameters of water, density, cultivation conditions, type of diet, age, gender, and health status (Omonona *et al.,* 2011). LDH and CK are crucial enzymes to evaluate the antioxidant capacity and general health status of

fish, while the ALT, ALP, and AST values are often measured to determine the liver damage in animals (Wagner & Congleton, 2004). Their activity is modulated by sex, salinity, nutrition, sampling season, health status, growing conditions, stress factors, habitat, and sex hormones (Yu *et al.,* 2013). Based on our results, male turtles had higher mean values for AST, ALP and CK than female turtles. Similar findings were reported in *M. mutica* by Yu *et al.* (2013) although, as reported by Omonona *et al.* (2011) in *P. sinuatus*, Hidalgo-Vila *et al.* (2007) in Mediterranean pond turtles and Yilmaz and Tosunoglu (2010) in *Emys orbicularis* and *Mauremys rivulata,* no significant difference was observed in liver enzyme activity.

In accordance with the studies of Hidalgo-Vila *et al.* (2007), Metin *et al.* (2008), and Yu *et al.* (2013), our results showed no significant sex differences in triglycerides, cholesterol, glucose, total protein, albumin, and globulin levels. Glucose and triglyceride levels were strongly influenced by handling, different seasons and sexes, age variations, nutrition status, temperature, metabolism, migration, reproductive cycle, and maturation stages (Satheeshkumar *et al.,* 2011).

The serum total protein can be used as a bioindicator to analyse the health status and nutrition condition of aquatic animals as well as a diagnostic tool for diagnosing infectious and non-infectious diseases. Based on our results, serum protein concentration varied from 32 to 48 g/L and no significant differences were observed between both sexes. Similar results were observed by Metin *et al.* (2008) and Omonona *et al.* (2011). A lower level of serum protein was observed during foraging seasons in *E. imbricata* and in poor feeding conditions in *P. expansa* (Tavares-Dias *et al.,* 2009). In general, changes in the serum total protein levels are highly dependent on the animal's age, species, nutrition, and immune system status.

Serum electrolytes as a part of the biochemical composition of blood are changed and affected in some bacterial infections, stress, environmental condition, nutrition, and season. In this research, no differences were obtained in calcium, phosphorus, magnesium, sodium, and potassium concentration between both sexes. Similar results were pointed out by Yu *et al.* (2013) in *M. leprosa* and Hidalgo-Vila *et al.* (2007) in *M. mutica*. The plasma electrolytes concentrations of Caspian pond turtle were consistent with those reported for the *M. caspica* (Metin *et al.,* 2008), Pig-nosed turtles, *Carettochelys insculpta*, Loggerhead sea turtles, *Caretta caretta* and *C. caretta* (Delgado *et al.,* 2011). On the other hand, in the study pf Metin *et al.* (2008), the value of calcium in female *M. caspica* was significantly higher than that in male turtles.

The changes observed in the haematological and biochemical variables of the Caspian pond turtle between males and females may reflect the impact of sex differentiation in the physiological blood indices. Therefore, the results of this study provide knowledge of the characteristics of haematological indices of the Caspian pond turtle from the southern basin of Caspian Sea.

Taken together, serum biochemical indices are potential biomarkers in diagnosing and evaluating maturation and health status in Caspian pond turtle. It is suggested to perform regularly haematological studies to monitor the physiological performance and health status of the Caspian pond turtle. Further investigations are needed to elucidate the impact of size, sex, season, and habitat variations on

haemato-biochemical parameters in Caspian pond turtles.

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