Yu et al. Zoological Studies 2013, 52:24
http://www.zoologicalstudies.com/content/52/1/24

Zoological Studies
a SpringerOpen Journal

RESEARCH Open Access

Hematologic and plasma biochemical reference values of the yellow pond turtle *Mauremys mutica* and the effects of sex and season

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Abstract

Background: The International Union for Conservation of Nature considers the yellow pond turtle *Mauremys mutica* to be an endangered species. Hematologic analyses are useful tools for monitoring the health, disease processes, and physiologic status of reptiles by clinicians and conservationists. The objectives of this study were to measure plasma biochemical values in healthy captive yellow pond turtles, determine reference values, and evaluate the effects of sex and season on the results. Blood samples were taken from the jugular vein of 53 adult captive individuals (18 males and 35 females) in four different months that represented summer, winter, fall, and spring in Taiwan. Plasma biochemical assays were performed using an automatic analyzer. Descriptive statistics and distributions of each data variable were analyzed using SAS software.

Results: Hematological and plasma biochemical reference values of the yellow pond turtle were determined in this study. There were no significant sex differences in hematological values; however, there were seasonal differences, and interactions between sex and season were observed. In females, lactate dehydrogenase, uric acid, calcium, cholesterol, and triglyceride concentrations were significantly higher than in males. There were seasonal differences but no sex and season interactions in serum biochemical values.

Conclusions: This information can serve as baseline reference data for future health assessment studies of free-ranging and captive *M. mutica*, and for epidemiologic, conservation, and captive-breeding studies.

Keywords: Blood test; *Mauremys mutica*; Reference values; Yellow pond turtle

Background

The yellow pond turtle *Mauremys mutica* is one of the four native species of turtle in Taiwan. It is also found in China, Japan, and northern and central Vietnam. However, populations are decreasing as a result of habitat loss and collection for the Chinese herbal medicine and pet markets. The International Union for Conservation of Nature considers *M. mutica* to be an endangered species (Asian Turtle Trade Working Group 2000).

Blood analyses are useful, widely used tools that aid in the diagnosis and monitoring of animal health and disease, and in the differentiation of physiologic processes. These techniques have been used with several wildlife species, especially with threatened or endangered populations, and may aid in evaluating ecosystem health (Kenichi et al. 2011). Animals have very complicated and delicate responses to stress that protect against environmental perturbations and which may be disadvantageous to their physiology, psychology, growth, and breeding (Bharath et al. 2012). The blood of reptiles contains nucleated erythrocytes, nucleated thrombocytes, heterophils, eosinophils, basophils, lymphocytes, and monocytes. Hematology is used to detect conditions related to these cells, such as anemia, inflammatory diseases, parasitemias, hematopoietic disorders, and hemostatic alterations (Terry 2006). Blood biochemical profiles are often used to assess the physiologic status of reptilian patients. Factors that affect the hematologic and plasma biochemical values of reptiles include environmental conditions, age, gender, nutrition, season, use of anesthetics, and the physiologic status of the reptile such as dehydration and estrus (Dessauer 1970; Samour et al.

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1986; Lawrence 1987; Terry 2006; Chung et al. 2009). However, baseline hematological and biochemical parameters of *M. mutica* have not yet been published. The purpose of this study was to establish accurate baseline values of clinical laboratory data for *M. mutica* with regard to sex and season. The data presented should be beneficial to the conservative medicine of this endangered species.

Methods

Subjects and husbandry

Clinically normal adult males (n = 18) and females (n = 35) of *M. mutica* were maintained together in an outdoor enclosure at the Laboratory Animal Facility, National Taiwan Univ. (Taipei, Taiwan). The enclosure measured 15 m^2, contained three basking areas, had sand as bedding material, and two 4.3-m^2 ponds. Fresh vegetables, fruit, liver, and commercial pellet food were provided at 2% of body weight (BW) every 2 to 3 days. The frequency of feeding depended on the weather and appetite. If the food was finished within 10 min, additional food was given. Differences in activity and behaviors like swimming and nesting were recorded, and blood samples were collected in April, August, and November 2010 and in February 2011. BW was measured to the nearest 5 g with an electric scale, and the maximum straight carapace length was measured to the nearest 1 mm with vernier calipers each time blood was collected.

Hematologic and plasma chemical assays

Turtles were sedated with an intramuscular injection of 5 to 10 mg/kg tiletamine-zolazepam (Zoletil®, Virbac, Carros, France), and 1 ml of blood was obtained from the jugular vein using a 29-gauge × 12.7-mm needle. Blood was placed in two tubes of 0.25 and 0.75 ml containing lithium heparin, respectively: 0.75 ml of blood was centrifuged at 2,000×g for 3 min to separate the plasma used for biochemistry analysis, and 0.25 ml of blood was used to obtain data on the packed cell volume (PCV), hemoglobin (Hb) concentration, red blood cell (RBC) and white blood cell (WBC) counts, and WBC differential count.

Plasma biochemical assays were performed using an automatic analyzer (VITROS® 350 Chemistry System, Ortho Clinical Diagnostics, Johnson & Johnson, Melbourne, Australia). The assays included in the clinical chemistry profile were aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium, uric acid, cholesterol, creatinine, creatine kinase (CK), glucose, phosphorous, total protein (TP), triglyceride, lactate dehydrogenase (LDH), sodium, potassium, and chloride. Detailed techniques and reference methods applied for each analyte were according to the online datasheet of VITROS® instructions (available at http://www.orthoclinical.com).

An air-dried blood smear was stained with Liu's stain (Liu's stain A and B, ASK®, Taipei, Taiwan), and a manual 100-cell differential count was obtained. WBC and RBC counts were performed using a hemocytometer and Natt and Herick's solution (Terry et al. 2007). Microhematocrit tubes containing ammonium-heparin were filled with blood and centrifuged at 14,800×g for 3 min to determine the PCV. Hb was assayed by the cyanmethemoglobin method. The mean cell volume (MCV), mean cell Hb (MCH), and mean cell Hb concentration (MCHC) were calculated using the formulae: MCV = (PCV/RBC) × 10; MCH = (Hb/RBC) × 10; and MCHC = (Hb/PCV%) × 100.

Statistical analysis

Outliers were deleted if the difference between the outlying value and adjacent value exceeded one third of the total range of all values. In addition, values over three-times the standard deviation (SD) were deleted (Lumsden et al. 1978; Healy 1979; Harris et al. 1995; Solberg 1999).

Descriptive statistics and distributions for each data variable were examined using SAS, vers. 8.2 (SAS Institute, Cary, NC, USA). Mean values and the SD were calculated for males and females in each season. A Kolmogorov-Smirnov test ($p< 0.05$) was used to ascertain whether the data were from a Gaussian distribution (Lumsden et al. 1978). If the data were from a Gaussian distribution, a two-way analysis of variance (ANOVA) ($p< 0.05$) was used on all blood and biochemical variables to test for effects of season, sex, and the interaction of sex and season. If the data were not from a Gaussian population, the data were log-transformed before being analyzed. Scheffe's F test was used for *post hoc* group comparisons. For results that did not significantly differ between the sexes, reference values were determined from values pooled from male and female turtles in each season (Lumsden et al. 1978; Chung et al. 2009).

When the data were from a Gaussian distribution, reference intervals were defined by minimum and maximum values for groups of fewer than 40 samples and by central 95% percentiles (mean ± 2 SD) for groups of more than 40 samples (Solberg 1999).

Results

Weather, habitat, body condition, and reproductive performance

According to the Central Weather Bureau of Taiwan, monthly average temperatures in Taipei in April, August, and November 2010 and February 2011 were 20.7°C, 30.0°C, 21.5°C, and 16.9°C, respectively. Monthly average humidity levels were 78%, 72%, 75.4%, and 78%, respectively.

Table 1 Sex and seasonal differences and the interaction of sex with season in the hematology of captive adult *Mauremys mutica*

			Seasonal differences				Sex differences				Sex × season	
Analyte	Spring [a]	Summer [a]	Fall [a]	Winter [a]	*F* value [b]	*p* value	Male	Female	*F* value	*p* value	*F* value	*p* value
RBCs (10^6/μl)	-	H	-	-	15.164[c]	**	-	-	nsd	nsd	nsd	nsd
PCV (%)	H	-	-	-	22.34	**	-	-	nsd	nsd	nsd	nsd
Hb (g/dl)	H	-	-	-	14.809	**	-	-	nsd	nsd	nsd	nsd
WBCs (μl)	-	-	-	H	10.366	**	-	-	nsd	nsd	nsd	nsd
Heterophils (μl)	-	H	-	-	3.084	**	-	-	nsd	nsd	nsd	nsd
Lymphocytes (μl)	-	H	-	L	6.469	**	-	-	nsd	nsd	nsd	nsd
Basophils (μl)	H	-	-	-	11.576	**	-	-	nsd	nsd	nsd	nsd
Eosinophils (μl)	-	-		-	nsd	nsd	-	-	nsd	nsd	nsd	nsd
Monocytes (μl)	-	-	L	H	3.147	**	-	-	nsd	nsd	3.108	*

[a] Means significantly higher (H) or lower (L) than for the other sex or the other seasons. [b] Two-way ANOVA ($p < 0.05$). [c] nsd, no significant difference. ** $p < 0.01$.
RBCs, red blood cells; PCV, packed cell volume; Hb, hemoglobin; WBCs, white blood cells.

Table 2 Hematological and plasma biochemical reference values for male captive adult *Mauremys mutica* in February, April, August, and November

Variable	February	April	August	November
Hb (g/dl)	4.1 to 7.6	4.9 to 8.8	2.7 to 7.8	4.7 to 7.2
PVC (%)	20 to 46	26 to 40	15 to 36	22 to 30
RBCs (10^6/μl)	0.33 to 0.83	0.55 to 1.11	0.15 to 1.31	0.4 to 0.91
WBCs (μl)	2,220 to 15,400	2,220 to 27,800	3,400 to 11,600	2,000 to 14,000
Heterophils (μl)	38 to 82	37 to 87	35 to 77	41 to 76
Lymphocytes (μl)	4 to 41	7 to 33	12 to 38	13 to 37
Basophils (μl)	0 to 37	1 to 30	0 to 29	5 to 34
Eosinophils (μl)	0 to 1	0 to 2	0 to 1	0
Monocytes (μl)	3 to 15	1 to 12	1 to 12	0 to 9
AST (U/l)	39 to 229	58 to 332	60 to 230	41 to 133
LDH (IU/l)	759 to 4,265	870 to 8,102	730 to 4,956	787 to 3,385
BUN (mg/dl)	1 to 16	3 to 16	1 to 19	1 to 11
Calcium (mg/dl)	8.8 to 14.6	8.7 to 16.2	8.5 to 12.5	8.3 to 12.1
Phosphorus (mg/dl)	1.9 to 3.4	1.7 to 4.9	2.8 to 4.3	2.1 to 3.1
CK (U/l)	153 to 1,240	230 to 691	97 to 1426	119 to 587
Uric acid (mg/dl)	0.3 to 1.1	0.5 to 1	0.7 to 1.7	0.4 to 0.8
Creatinine (mg/dl)	0.1 to 0.2	0.1 to 0.4	0.1 to 0.3	0.1
Glucose (mg/dl)	34 to 87	52 to 125	41 to 102	32 to 89
Total protein (g/dl)	3 to 5.9	3.5 to 6.3	2.1 to 7	2.1 to 4.4
Triglyceride (mg/dl)	37 to 464	33 to 913	27 to 304	5 to 542
Cholesterol (mg/dl)	80 to 224	83 to 286	61 to 149	62 to 131
Sodium (mmol/l)	131 to 137	131 to 137	113 to 137	122 to 134
Potassium (mmol/l)	2.9 to 3.8	3.5 to 4.6	4.5 to 8.9	3.2 to 5.8
Chloride (mmol/l)	101 to 110	101 to 110	102 to 111	98 to 107

Hb, hemoglobin; PCV, packed cell volume; RBCs red blood cells; WBCs, white blood cells; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; CK, creatine kinase.

All turtles had overwintered from December to late February, spending most of their time in the water. But during very cold periods, they would leave the water and 'huddle' together in a gap and sometimes bury themselves in the sand. At such times, their activity and appetite decreased but had returned to normal by March, at the onset of spring. Some minor wounds were discovered during the mating season between late March and September, attributable to copulation and fighting activities; however, no major injuries were found. During the study period, all females were gravid. Gravid females reproduce normally between May and early October according to a previously described timeline (Dessauer 1970). The average BW of all turtles was 790.6 (range, 1,160to 575) g. The average carapace length was 17.7 (range, 20.3 to 15.7) cm.

Hematology

There were no significant differences in hematological values between males and females. For both sexes, values of the PCV, Hb, and basophil differential count were significantly higher in spring. Values of RBCs, and heterophil and lymphocyte differential counts were significantly higher in summer. Values of the monocyte differential count were significantly lower in fall. Values for WBCs and the monocyte count were significantly higher in winter. Values for the lymphocyte count were significantly lower in winter (Table 1). There were interactions between sex and season for the monocyte count. Hematological reference values for males and females of *M. mutica* in spring, summer, fall, and winter are respectively given in Tables 2 and 3.

Clinical chemistry

In females, LDH, uric acid, calcium, cholesterol, and triglyceride concentrations were significantly higher than those in males. Males had significantly higher CK and AST concentrations. Both sexes showed significantly higher concentrations of AST, BUN, calcium, cholesterol, triglyceride, glucose, and total protein in spring; concentrations of phosphorus, uric acid, creatinine, potassium, and chloride were significantly higher in summer.

Table 3 Hematological and plasma biochemical reference values for female captive adult *Mauremys mutica* in February, April, August, and November

Variable	February	April	August	November
Hb (g/dl)	3.3 to 8.2	3.8 to 9.4	3.7 to 7.2	3.9 to 8.2
PVC (%)	19 to 41	21 to 47	18 to 42	21 to 34
RBC (10^6/μl)	0.34 to 0.84	0.38 to 1.2	0.41 to 1.16	0.24 to 0.84
WBC (μl)	3,330 to 26,400	3,552 to 21,200	2,200 to 26,400	2,600 to 15,400
Heterophils (μl)	34 to 74	29 to 75	41 to 92	36 to 76
Lymphocytes (μl)	8 to 34	1 to 41	6 to 39	12 to 30
Basophils (μl)	0 to 35	0 to 36	0 to 25	0 to 35
Eosinophils (μl)	0 to 2	0 to 3	0 to 2	0 to 1
Monocytes (μl)	2 to 12	1 to 22	1 to 10	1 to 11
AST (U/l)	42 to 147	53 to 213	65 to 186	48 to 116
LDH (IU/l)	823 to 2,658	744 to 8,468	635 to 2,474	759 to 3,327
BUN (mg/dl)	1 to 14	2 to 22	1 to 17	1 to 9
Calcium (mg/dl)	7.5 to 15.5	6.6 to 23	7.7 to 19.7	7.7 to 14.8
Phosphorus (mg/dl)	1.9 to 3.8	1.8 to 5.3	2.7 to 5.2	2.3 to 3.3
CK (U/l)	74 to 745	176 to 654	71 to 417	57 to 713
Uric acid (mg/dl)	0.3 to 1.2	0.6 to 1.5	0.5 to 1.3	0.4 to 1
Creatinine (mg/dl)	0.1	0.1 to 0.4	0.1 to 0.3	0.1
Glucose (mg/dl)	28 to 71	42 to 116	32 to 126	37 to 86
Total protein (g/dl)	2 to 4.4	2.1 to 6.1	1.7 to 5.6	1.9 to 3.7
Triglyceride (mg/dl)	65 to 785	63 to 877	41 to 409	78 to 549
Cholesterol (mg/dl)	71 to 167	66 to 285	113 to 223	70 to 170
Sodium (mmol/l)	127 to 136	121 to 136	112 to 143	122 to 133
Potassium (mmol/l)	2.7 to 5.7	3.3 to 5.3	4.4 to 6.2	3.3 to 6.2
Chloride (mmol/l)	98 to 107	96 to 109	96 to 112	98 to 107

Abbreviations of variables are defined in the footnotes of Table 2.

The concentration of total protein was significantly lower in fall. The concentration of sodium was significantly lower in winter and was significantly higher in winter (Table 4). The plasma biochemical reference values for males and females of *M. mutica* in spring, summer, fall, and winter are respectively given in Tables 3 and 4.

Discussion

Weather patterns in Taiwan can be divided into two seasons: summer and winter. Therefore, we chose the months with the highest and lowest average temperatures to investigate seasonal variations in blood parameters. In addition, spring was included in order to determine the effect of reproduction on reference values, and fall refers to the specific time period 2 to 3 months before turtles began overwintering.

AST, glucose, total protein, cholesterol, and triglyceride values peaked in spring, which may have been due to higher copulation activity and egg production during this period (Cheng et al. 2010). Tissue injury and mating stress caused by copulation may have resulted in increased AST and glucose values (Terry et al. 2007). There were significant differences in calcium, cholesterol, and triglyceride values between the sexes and among seasons. Higher values in females than in males, and in mating than in non-mating seasons are consistent with the occurrence of egg production and vitellogenesis (Dessauer 1970; Jackson et al. 1974; Anderson et al. 1997; Christopher 1999; Zaias et al. 2006), because higher circulating calcium will support the demand for egg-shell production, and circulating protein, cholesterol, and triglyceride are main materials for follicular development.

Increased metabolic activity may be reflected in increased enzyme activity in plasma (Christopher et al. 1999) and vice versa. The significantly higher heterophil count in summer may have been due to increased metabolic activity, fighting, feeding, and copulation behaviors which cause tissue damage and inflammation (Terry 2006). In addition, the higher AST and CK values may indicate muscle and tissue injury resulting from the above-described activities and the higher metabolic rate of males than females of *M. mutica*. (Dickison et al. 2002; Chung et al. 2009). The significantly lower total protein value in fall and significantly lower glucose value in winter may have been due to lower activity levels and metabolic rate.

Given that proteins may serve as an energy source during hibernation, chelonians will produce and store nitrogenous wastes in the bladder. During this time, BUN values are elevated, and osmolarity increases to prevent water loss. After hibernation, the BUN value of chelonians decreases as water intake increases (Dessauer 1970; Wallace et al. 1970; Christopher et al. 1994; Chung et al. 2009). This may explain the elevated BUN observed in *M. mutica* in early spring. In addition, elevated BUN values in spring and high phosphorus and uric acid levels in summer may be explained by increased food consumption and a higher level of nitrogenous waste being discharged during warmer seasons (Terry 2006).

Table 4 Sex and seasonal differences and interaction of sex with season in the plasma biochemistry of captive adult *Mauremys mutica*

	Seasonal differences						Sex differences				Sex × season	
Analyte	Spring [a]	Summer [a]	Fall [a]	Winter [a]	*F* value [b]	*p* value	Male	Female	*F* value	*p* value	*F* value	*p* value
AST (U/l)	H	-	-	-	47.805 [c]	**	H	-	11.197	0.01	nsd	nsd
LDH (IU/l)	-	-	-	-	nsd	nsd	-	H	6.556	0.01	nsd	nsd
BUN (mg/dl)	H	-	-	-	42.358	**	-	-	nsd	nsd	nsd	nsd
Calcium (mg/dl)	H	-	-	-	5.584	***	-	H	18.037	0	nsd	nsd
Phosphorus (mg/dl)	-	H	-	-	72.936	**	-	-	nsd	nsd	nsd	nsd
CK (IU/l)	-	-	-	-	nsd	nsd	H	-	20.478	0	nsd	nsd
Uric acid (mg/dl)	-	H	-	-	41.521	**	-	H	7.878	0.005	nsd	nsd
Creatinine (mg/dl)	-	H	-	-	41.603	**	-	-	nsd	nsd	nsd	nsd
Glucose (mg/dl)	H	-		L	6.954	**	-	-	nsd	nsd	nsd	nsd
Total protein (g/dl)	H	-	L	-	28.378	**	H	-	7.791	0.006	nsd	nsd
Triglyceride (mg/dl)	H	-	-	-	53.135	**	-	H	28.053	0	nsd	nsd
Cholesterol (mg/dl)	H	-	-	-	31.098	**	-	H	12.034	0.01	nsd	nsd
Sodium (mmol/l)	-	-	L	H	6.293	***	-	-	nsd	nsd	nsd	nsd
Potassium (mmol/l)	-	H	-	-	59.339	**	-	-	nsd	nsd	nsd	nsd
Chloride (mmol/l)	-	H	-	-	24.152	**	-	-	nsd	nsd	nsd	nsd

[a] Means significantly higher (H) or lower (L) than for the other sex or the other seasons. [b] Two-way ANOVA ($p < 0.05$). [c] nsd, no significant difference. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$. Abbreviations of analytes are defined in the footnotes of Table 2.

Dietary sodium is absorbed from the intestines and transported to the kidneys where it is excreted or resorbed depending on a reptile's need for sodium. Reptilian sodium and potassium metabolism involves an active renin-angiotensin system with direct action on osmoregulation (Terry 2006). The significantly higher sodium level in winter may be explained by greater water loss or lower water intake during the winter because turtles spend less time in the water and more time on land in very cold periods. Serum potassium levels can be affected by dietary potassium intake, gastrointestinal potassium loss, and renal secretion (Terry 2006). In this study, significantly higher potassium levels in summer may have been due to greater food intake in the warmer time period. Blood chloride concentrations provide the least clinically useful information about electrolytes (Terry 2006). Thus, significantly higher chloride concentrations during summer may be of little use for the indicating an animal's condition.

In the authors' experience, blood sampling from the jugular vein of *M. mutica* was relatively difficult, compared to other chelonians of the same size. Identification of the jugular vein was complicated by the thick, wrinkled, green skin. Therefore, the use of a 29-guage needle increased the likelihood of successful venipuncture. Hemolysis was rarely encountered because the blood-drawing process was performed with care. However, the difficulty of sampling limited the size of *M. mutica* individuals that could be used for blood sampling and reduced the available sample size.

Conclusions

The hematologic and blood biochemical data from this study provide useful ranges for evaluating the health status of *M. mutica*. The data reported here were found to be comparable to previously published data for other native turtle species in Taiwan (Chung et al. 2009). Reference values for *M. mutica* obtained in this study should be of benefit to future clinical and conservation work on the endangered yellow pond turtle.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PHY participated in the design of the study and drafted the manuscript. PYY participated in the design of the study and performed the statistical analysis. YSC participated in the sequence alignment. CHC conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Acknowledgements

The authors acknowledge the Forestry Bureau, Council of Agriculture, Executive Yuan and National Taiwan Univ. Veterinary Hospital for supporting this research.

Received: 1 March 2013 Accepted: 5 July 2013
Published: 30 September 2013

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doi:10.1186/1810-522X-52-24
Cite this article as: Yu *et al.*: Hematologic and plasma biochemical reference values of the yellow pond turtle *Mauremys mutica* and the effects of sex and season. *Zoological Studies* 2013 **52**:24.