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RESEARCH NOTE

Serum Biochemical Profile of Captive-bred Philippine Crocodiles (*Crocodylus mindorensis* Schmidt, 1935) Sub-adults

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The Philippine crocodiles *Crocodylus mindorensis* Schmidt, 1935 are a critically endangered species that necessitate minimally invasive diagnostic tools for their physiological state and health assessment. In the current study, we determined the reference ranges for the serum biochemistry of male and female captive-bred *C. mindorensis* sub-adults. We collected blood samples from the post-occipital venous sinus of six male and seven female captive-bred crocodile sub-adults at the Palawan Wildlife Rescue and Conservation Center and quantified the serum biochemical values for cholesterol, triglycerides, uric acid, glucose, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total protein, and globulin. We defined reference ranges through the central 95% of the concentration values obtained. The uric acid concentrations were significantly different between male and female *C. mindorensis* sub-adults. Uric acid levels were higher ($P = 0.035$) in male sub-adults because of their higher food intake resulting from dominance and aggression during feeding times. Serum biochemical values of *C. mindorensis* exhibited similarities with and variations from other crocodile species. We briefly discussed the differences with other species and the influence of factors such as field and laboratory methodologies, environmental conditions, nutritional status, and size class.

Keywords: captive-bred crocodiles, critically endangered species, Philippine crocodiles, serum biochemical profile, serum uric acid, sex difference

Crocodylus mindorensis Schmidt, 1935 is endemic to the Philippines (Manalo *et al.* 2016) and inhabits small lakes, riverine tributaries, and marshes. It was originally distributed in Mindoro, Masbate, Negros, Samar, Mindanao, and in the Sulu archipelago (van Weerd *et al.* 2016). Remnant viable wild populations are present in the foothills of Northern Sierra Madre Natural Park (van de Ven 2009), Ligawasan Marsh Game Refuge and Bird Sanctuary up to the highlands of Bukidnon (Pomares *et al.* 2008), and the population introduced for conservation purposes in Siargao Island Protected Landscapes and Seascapes (Manalo and Alcala 2015). Quite small in comparison to other crocodilian species, *C. mindorensis* can only grow up to three meters and has a golden-brown shade that deepens in color during maturation (van Weerd *et al.* 2016). The International Union for Conservation of Nature considers this endemic species as Critically Endangered in the Red List of Threatened Species.

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Albeit protected by the Philippine Wildlife Resources Conservation and Protection Act or Republic Act No. 9147 (Republic of the Philippines 2001), the species still faces threats such as habitat loss, persecution, and fishing net entanglement (van Weerd *et al.* 2016). Also, the United Nations-driven Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) highlighted Philippine crocodiles – among other ecologically important predators – as particularly threatened by extinction (Faridah-Hanum *et al.* 2018).

Other than threats coming from anthropological activities, zoonotic diseases or zoonoses also extend to reptilian vectors, including crocodiles (Johnson-Delaney 2006). Zoonotic diseases carried by crocodiles may be viral, bacterial, or parasitic in nature. The lack of clinical symptoms exhibited by crocodiles infected with zoonotic diseases emphasizes the importance of other diagnostic tools in their health assessment.

Numerous studies have determined the blood biochemical properties of different crocodilian species all over the world (Millan *et al.* 1997; Stacy and Whitaker 2000; Lovely *et al.* 2007; Padilla *et al.* 2011; Zayas *et al.* 2011; Amin and Shrivastav 2014; Scheelings *et al.* 2016). The biochemical and hematological values of crocodiles have been evaluated and related to various variables such as sex, size classes, and the environment to which the crocodiles are exposed (Millan *et al.* 1997; Lovely *et al.* 2007). Blood biochemical profiling is a minimally invasive method that can access the health and physiological state of animals (Diethelm and Stein 2006). For prophylaxis and investigation of disease outbreaks in crocodile farms, there is a need for an established database of standard and normal ranges for blood biochemistry and hematology of crocodiles (Millan *et al.* 2000).

Even with extensive conservation efforts by the Philippine government and non-governmental organizations alike, there is still insufficient information about *Crocodylus mindorensis* and its comparative health status with other crocodilian species (Hinlo *et al*. 2014). In the current study, we aimed to determine the serum biochemical profile and compare the reference ranges for the standard or normal serum biochemistry of male and female captive-bred *C. mindorensis* sub-adults.

We captured and restrained captive-bred *Crocodylus mindorensis* sub-adults using the internationally accepted standards on the direct capture method for crocodilians described in Manolis and Webb (2016). In order to morphologically identify the captured individual, we selected only those *C. mindorensis* with six post-occipital scutes and counted the tail scute-clippings, which are used as a marking system. We measured the head length and total length and expressed the values as mean ± standard deviation. We performed digital cloacal palpation to determine sex (Scheelings *et al.* 2016). Sub-adult crocodiles were housed in partially shaded concrete tanks with a density of 1.5 individuals m^{-2} at sufficient land to water ratio of 60:40 and fed with chicken, pork, and fish twice a week.

In total, we collected blood samples from 13 sub-adults. Prior to blood collection, all individuals were fasted for 24 h to reduce the possibility of obtaining highly lipemic samples (Millan *et al.* 1997). We collected blood samples (5 mL from each individual) from the post-occipital venous sinus located at the dorsal midline and caudal to the base of the head (Millan *et al.* 1997) using a sterile 23 G needle and a 5-cc syringe and transferred the collected blood samples into vacutainers (without anticoagulant) on ice. Immediately after, we transported the samples to the Department of Biology, Ateneo de Manila University for further analysis.

After we had centrifuged the blood samples at 54,208 relative centrifugal force for 15 min, we transferred the serum into Eppendorf microtubes and stored them at –25 °C prior to biochemical analysis. We performed the biochemical tests for creatinine, glucose, ALT, AST, cholesterol, uric acid, triglycerides, total protein, albumin, and globulin using blood chemical analyzer kits (Stanbio Laboratory, Boerne, TX, USA) and a semi-automated chemistry analyzer (STATFAX Model 1904 plus, Awareness Technology, Inc., USA) – following the manufacturer’s protocols as described in Velasquez *et al.* (2016). We performed duplicate tests for every specimen in each parameter. We obtained globulin values through the difference of the total protein and albumin concentrations (Millan *et al.* 1997). In order to define reference ranges, we combined all the obtained values from both sexes for each serum biochemical parameter measured through the central 95% of the concentration values obtained (Boyd 1984).

We processed the data using IBM SPSS ver. 20 (IBM Corp. Armonk, NY, USA) and analyzed them for significant difference ($P \leq 0.05$) between sexes. For data with normal distribution and no observed outliers, we used the Independent Samples t-test to compare for any significant difference between sexes. For data with non-normal distribution and outliers, we used the Mann-Whitney test to compare for any significant difference between sexes.

We identified seven females and six males of *Crocodylus mindorensis* sub-adults. Their head length ranged from 17–29 cm (22.92 ± 1.57 cm in males; 21.64 ± 0.47 cm in females). The total length ranged from 127–212 cm (165.38 ± 11.4 cm in males; 151.93 ± 4.03 cm in females). There was no significant difference ($P > 0.05$) in terms of head length and total length between sexes.

We present the serum biochemical profile of *Crocodylus mindorensis* sub-adults in Table 1 and their reference ranges in Table 2. There were no significant differences ($P > 0.05$) in all serum biochemical values between sexes except uric acid. Male sub-adults exhibited significantly higher (P = 0.035) uric acid concentrations than female sub-adults.

This is the first report of ALT, AST, creatinine, triglycerides, and uric acid in *Crocodylus mindorensis*. Albumin, cholesterol, globulin, glucose, and total protein were once previously determined for samples of this species (n = 7–45) by Goh *et al.* (1991) wherein the variables such as size class, environment, methodology, and nutrition were not specified. Our data show similar ranges for albumin, globulin, and total protein; however, cholesterol and glucose values are distinctly higher in the initial study (cholesterol, 13.99 ± 4.05 µmol L^{-1}; glucose, 6.33 ± 3.00 µmol L^{-1}), suggesting the individuals tested by Goh *et al.* (1991) were subjected to more recent food intake or were most likely not fasted for 24 h before blood collection.

Profiling of the blood or serum biochemistry is an effective diagnostic tool to evaluate the physiological status of reptiles (Campbell 2006). The profiles vary greatly among crocodilian species and are affected by environmental factors to which these reptiles are exposed. The reference ranges computed in the current study can be utilized as an effective diagnostic tool for the health assessment of critically endangered *Crocodylus mindorensis* sub-adults, considering that the individuals in this study were declared healthy through visual inspection of body condition by the assisting wildlife veterinarian. The neck, abdomen, and base of the tail have sufficient fat stores that are rounded and not sunken.

Table 1. Serum biochemical profiles of male and female *Crocodylus mindorensis* sub-adults. Values are expressed as mean ± standard deviation (SD) and as a range. Asterisk (*) indicates a significant difference between the sexes ($P \leq 0.05$).

Serum biochemical parameters	Males		Females	
	Mean ± SD	Range	Mean ± SD	Range
Creatinine (µmol L^{-1})	55.28 ± 25.71	27.65–91.97	62.11 ± 18.11	42.52–89.79
Glucose (mmol L^{-1})	3.68 ± 1.44	1.92–5.50	3.14 ± 0.77	2.43–4.50
ALT (U L^{-1})	31.78 ± 17.24	19.18–61.63	28.21 ± 9.87	14.27–41.19
AST (U L^{-1})	10.14 ± 11.74	0–28.71	20.26 ± 13.44	8.71–44.00
Total protein (g L^{-1})	95.50 ± 56.28	57.89–193.25	81.07 ± 40.20	58.15–190.20
Albumin (g L^{-1})	19.91 ± 3.80	15.38–25.44	17.86 ± 4.10	11.25–22.98
Globulin (g L^{-1})	75.58 ± 53.51	37.19–168.25	63.21 ± 36.72	25.30–128.50
Cholesterol (mmol L^{-1})	4.10 ± 0.78	2.86–4.74	4.61 ± 1.13	3.33–6.37
Uric acid (µmol L^{-1})	908.14 ± 317.10*	437.90–1282.14	439.58 ± 221.58*	144.10–794.65
Triglycerides (mmol L^{-1})	6.08 ± 8.38	0.83–20.51	1.40 ± 1.25	0.52–3.72

Table 2. Normal range for serum biochemical values of captive-bred *Crocodylus mindorensis* sub-adults from the PWRCC. Test method used per serum biochemical parameter is also shown.

Serum biochemical parameters	Test method	Reference range (95% of concentration values)
Creatinine (µmol L^{-1})	Kinetic: alkaline picrate	28.98–94.16
Glucose (mmol L^{-1})	Oxidation of glucose	1.98–5.43
ALT (U L^{-1})	Kinetic: L-aspartate and 2-oxoglutarate	14.89–40.34
AST (U L^{-1})	Kinetic: L-aspartate and 2-oxoglutarate	0–42.23
Total protein (g L^{-1})	Biuret reaction	38.01–195.15
Albumin (g L^{-1})	Bromocresol green	11.85–25.25
Globulin (g L^{-1})	Equation	24.85–169.90
Cholesterol (mmol L^{-1})	Uricase	2.90–6.19
Uric acid (µmol L^{-1})	Cholesterol esterase	173.84–1256.78
Triglycerides (mmol L^{-1})	Glyceryl-phosphate oxidase	0.53–17.66

In the current study, only serum uric acid exhibited a significant difference between sexes. The lack of significant difference in blood or serum biochemistry between male and female individuals was also reported in other crocodilian species such as *Crocodylus palustris* and *C. johnstoni* (Stacy and Whitaker 2000; Scheelings *et al.* 2016).

The male *Crocodylus mindorensis* sub-adults exhibited a range of 437–1282 µmol L^{-1} whereas females exhibited a range of 144–795 µmol L^{-1} serum uric acid. The normal range of serum uric acid in crocodiles is less than 884 µmol L^{-1}, with values greater than 1326 µmol L^{-1} indicative of hyperuricemia (Campbell 2006). Uric acid is the main catabolic end-product of protein, purines, and non-protein nitrogen in reptiles that makes up around 80–90% of the total nitrogen waste excreted by terrestrial reptiles (Campbell 2006). Although the uric acid levels in male sub-adults are near the threshold value, we attribute the difference in uric acid concentrations to the higher protein intake of the male sub-adults that were slightly bigger in size (although not statistically significant). Because of their generally heightened aggression, male sub-adults outperform females during food acquisition (Morpurgo *et al.* 1993). This dominant behavior leads to higher food intake in male sub-adults. Hence, a more efficient and balanced feeding protocol between male and female captive-bred crocodiles is recommended.

The serum biochemical profile of *Crocodylus mindorensis* showed similarities with and deviations from the profiles of other crocodilian species. Differences can be influenced by variations in the field and laboratory methodologies, such as the site of blood collection and the analytical methods used (Padilla *et al.* 2011). The time at which the blood was collected also affects the blood biochemical profile. Albeit the crocodiles fasted for 24 h before blood collection as recommended by previous studies (Zayas *et al.* 2011), a duration of at least 36 h is needed for the stomach to empty after feeding (Huchzermeyer 2003). Moreover, a certain species may also be exposed to and adapted to particular environmental factors. Dissimilarities in nutrition, population dynamics, and environmental quality can also result in changes in reference ranges and profiles among species (Stacy and Whitaker 2000).

The ranges of creatinine (29–94 µmol L^{-1}) and of total protein (38–195 g L^{-1}) in *Crocodylus mindorensis* are higher than in C. *palustris* (27–53 µmol L^{-1} and 27–35 g L^{-1}, respectively) (Stacy and Whitaker 2000), *C. niloticus* (29–40 µmol L^{-1} and 41–57 g L^{-1}, respectively) (Lovely *et al.* 2007), and *C. porosus* (20–51 µmol L^{-1} and 41–70 g L^{-1}, respectively) (Millan *et al.* 1997). Creatinine is the waste product of the breakdown of creatine phosphate and is filtered by the kidney (Mayer and Donnelly 2013). The high creatinine values of the *C. mindorensis* sub-adults could be attributed to possible dehydration, which reduces overall tubular secretion and promotes tubular reabsorption of the kidneys, leading to retention and elevated levels of creatinine in the blood (Campbell 2006). The dehydration could have also caused a reduction in plasma volume or hemoconcentration, resulting in elevated levels of total protein in the serum. Moreover, the usual diet composition of chicken, pork, and fish fed to the captive-bred *C. mindorensis* sub-adults could have contributed to the higher total protein level. In addition, poorer nutritional plane or parasitism (Campbell 2006) in wild or free-ranging crocodiles reduces the total protein concentrations. Farmed *C. niloticus* individuals have exhibited higher serum total proteins compared to their wild counterparts (Lovely *et al.* 2007). This could explain why the total protein values for free-ranging *C. niloticus* were lower than those of the captive-bred *C. mindorensis*.

The higher dietary fat intake in captive-bred *C. mindorensis* sub-adults could also account for the higher triglyceride levels in the serum.

Likewise, globulin levels were elevated in *C. mindorensis*. The variation in globulin levels among populations and species of crocodiles could be because of differences in antigenic stimulation. Alpha, beta, and gamma globulins are said to increase with pathogen presence (Campbell 2013). Although not directly tested, *C. mindorensis* sub-adults may have had an immune response against environmental pathogens, leading to differences in globulin concentrations. On the other hand, *C. mindorensis* sub-adults exhibited low AST values (0–42.23 U L^{-1}), suggesting no apparent organ damage. The normal range of values of AST in reptiles is less than 250 U L^{-1}, with higher values indicative of damage to the skeletal muscles, liver, or myocardium (Campbell 2006).

The resulting normal reference ranges for serum biochemistry for captive-bred sub-adults of *Crocodylus mindorensis* can be utilized for health assessments of captive crocodiles and, thus, aid in future conservation efforts of the critically endangered Philippine Crocodiles.

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