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Hematology and serum biochemistry of captive Sunda pangolin (*Manis javanica*) in Wildlife Reserves Singapore

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ABSTRACT. The Sunda pangolin (*Manis javanica*) faces threat of extinction due to illegal trafficking of its scales for the purpose of traditional medicine in the Asian region. *Ex-situ* captive breeding and reintroduction programs have been identified to be a key effort in the conservation of the species. The establishment of blood parameters for captive Sunda pangolins are vital to assess the health in these animals during health assessments. The objective of this study is to establish blood parameters for captive Sunda pangolins and compare the blood parameters with the established blood reference ranges for rescued wild Sunda pangolins in Singapore. Blood parameters for hematology and serum biochemistry were established from 13 clinically normal captive Sunda pangolins. Male captive Sunda pangolins were found to have significantly (P<0.05) higher potassium compared to the female captive Sunda pangolins. Captive Sunda pangolins were found to have significantly (P<0.05) lower white blood cell count (WBC), neutrophil counts, alanine phosphatase (ALP) and phosphorus and significantly (P<0.05) higher PCV, TP, globulin and blood urea nitrogen (BUN) when compared with rescued wild Sunda pangolins from the previous study.

KEY WORDS: captive, hematology, *Manis javanica*, serum biochemistry, Sunda pangolin

Pangolins or scaly anteaters are placental mammals from the order Pholidota. Pangolins are characterized by their unique overlapping epidermal scales [11]. Unfortunately, the pangolin's unique scales, composed of flattened, solid and keratinized cells similar to a primate's finger nails, are widely used in traditional medicine in Southeast and East Asia [2, 11]. The high demand of pangolin scales for traditional medicine and meat for the luxury meat market in both China and Vietnam drive poaching and trafficking of this species. Pangolins have been identified as the most trafficked mammals in the world [2].

Rapid population declines of wild Sunda pangolins (*Manis javanica*) due to overexploitation and low reproduction rate makes the species susceptible to extinction [10, 28]. Sunda pangolins had been listed as 'critically endangered' by the International Union for Conservation of Nature (IUCN) [7]. There is an increasing need for multi-disciplinary conservation strategies that include the integration of *in-situ* (in the wild) and *ex-situ* (in zoological facilities) management processes to save this threatened species [5, 21, 24].

Husbandry and health programs are important parts of *ex-situ* conservation programs to ensure the captive population remain in optimal health. Diagnostics such as radiographs and bloodwork are regularly used to assess health status and diagnose disease in unwell pangolins. Blood parameters of Sunda pangolins have been established and conducted for both confiscated and rescued wild Sunda pangolins [1, 25]. However, interpretation and use of reference ranges in the wild need to be done with caution as certain blood parameters may be altered due to the diet and stress of capture or restraining of the animal [17]. This study represents the first effort to establish both hematology and serum biochemistry for captive Sunda pangolins.

The objective of this study is to establish the hematology and serum biochemistry parameters for captive pangolins in Wildlife Reserves Singapore (WRS) and determine if there are sex related differences for these parameters. This study also compares the hematology and serum biochemistry of previous work on the rescued wild Sunda pangolin in Singapore [1].

The study was conducted in accordance with WRS's ethical and animal welfare principles. The study was approved by WRS's in-house research advisory panel.

From January 2011 to December 2018, 13 adult captive Sunda pangolins from WRS's collection were transferred from their exhibit to the veterinary hospital for routine health checks. The Sunda pangolins are kept in individual housing and the volume of the housing range from 8.88 to 42.74 m^3 [14]. The housings were all roofed and concreted with earth as the main form of substrate

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used in all the enclosures, pine bark is also made available as an added option for the Sunda pangolins. Climbing structures and nest boxes were also furbished in the enclosure setup. The temperature within the enclosure setup ranges from 24 to 30°C and a relative humidity between 77 and 98%. They were also fed specially formulated diet consisting of ants, ant eggs, mealworms, insectivore pellets, silkworms, chicken eggs, chitin and calcium powder, and Vitamin B and K, presented in a blended form. Captive Sunda pangolins were categorized as adult animals based on the criteria of previous study by Ahmad *et al.* 2018 [1]. Sunda pangolins were considered adult when their body weight was above 4.0 kg in females and above 6.0 kg in males. The age range of the animals in the study were between one year to ten years old. Animal was placed in a gas chamber and anesthetized with 5% isoflurane (Attane, Piramal Healthcare, Bethlehem, PA, USA) with an oxygen flow rate of 3.0 l/min. Once the animal had been deeply sedated, it was removed from the gas chamber and placed on a mask. Animals were then maintained on 3% isoflurane with flow rate of 1.5 l/min.

Health assessments were then conducted on the anesthetized animals. Physical examination, blood work, radiography and ultrasonography assessment were conducted on the animals. Bloods for in-house hematology and serum biochemistry were collected from the coccygeal vein along the ventral midline of the tail. The skin between the scale was cleaned of soil and other debris before disinfection with 70% ethanol. A total of 1–3 ml of whole blood was then collected using a 3 ml syringe (Terumo Co., Tokyo, Japan) with a 21G × 1.5” needle (Terumo Co.). Five hundred µl of blood was then place in MiniCollect® blood collection tubes (Greiner Bio-One, Kremsmünster, Upper Austria, Austria) containing ethylene-diaminetetraacetic acid (EDTA) for hematology and 0.5 to 2.5 ml of blood was placed into plain blood collection tubes (Greiner Bio-One, Kremsmünster, Upper Austria, Austria) for serum biochemistry studies. The samples indicating signs of lipemia or hemolysis were removed from the study to control interferences. The samples were processed within 1 to 2 hr from the time of collection.

Once the health assessment and blood collection had been concluded, the Sunda pangolins were placed on 100% oxygen at a flow rate of 1.5 l/min for 3 to 5 min. Individuals were subsequently placed in individual pet carriers for recovery. Once each animal had fully recovered from anesthesia, it was returned to the section to be placed back in its exhibit.

The laboratory analysis procedure was carried out based on the procedure by Ahmad *et al* [1]. The hematology analyses were carried out on EDTA blood. The analyses were run on a VetScan HM5 (Abaxis, San Francisco, CA, USA) automated hematology analyzer using the canine setting. Analyses include red blood cell count (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), white blood cell count (WBC), lymphocytes (LYM), monocytes (MON), neutrophils (NEU), eosinophils (EOS) and basophils (BASO). Packed cell volume (PCV) was determined by micro-hematocrit centrifugation at 14,000 rpm for 5 min (Hettich Centrifuge Mikro 200, Andreas Hettich GmbH & Co., KG, Tuttlingen, Germany).

Blood samples used for serum biochemistry analyses were left to clot for a minimum of 1 hr. Once the blood had clotted, it was centrifuged at 3,750 rpm (4222 MKII, ALC International, Cologno Monzese, Italy) for 5 min to obtain the serum. All serum biochemical analyses were run with 100 µl of serum using the VetScan 2 analyzer (Abaxis). The following analytes were measured: total protein (TP), globulin (GLOB), albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), blood urea nitrogen (BUN), creatinine (CREA), glucose (GLU), calcium (Ca), phosphate (PHOS), sodium (Na^+) and potassium (K^+).

Hematology and serum biochemistry datasets were studied in separate analyses between male and female Sunda pangolins. All independent variables were first tested for normality using the Shapiro-Wilks test and Anderson-Darling test. Datasets that were found to be parametric was then subjected to a Fisher test (F-test) to determine if variance in the data was equal. Two-tailed t-tests were then conducted for datasets that are parametric and for which the variance between the male and female Sunda pangolins were found to be equal. Datasets that were non-parametric were analyzed using the Mann-Whitney U test. Statistical significance was set to $P<0.05$ for all statistical analyses. Descriptive statistics including the mean, median and standard deviation were reported for the hematology and serum biochemistry. Minimum and maximum values were presented as the range for the data. The overall dataset for captive Sunda pangolins in WRS was then analyzed to determine if it significantly differed from the findings of previous work on rescued adult Sunda pangolins in Singapore. The two-tailed t-test was used to test for statistically difference between the normally distributed datasets from both studies. The Mann-Whitney U test was used to test for statistically significant differences between non-parametric datasets from both studies. Cohen D test were conducted on all the dataset to determine the effect size of the populations and its relationship between different populations. All analyses were performed using the statistical software R Core Team (version R. 3.6.0) [23].

A total of 13 (7 males and 6 females) blood samples from clinically normal adult Sunda pangolins were collected during routine health assessment. Potassium levels were significantly ($P<0.05$) different between adult male and female Sunda pangolins. Hematological and serum biochemistry variables of the captive adult Sunda pangolins can be seen in Tables 1 and 2.

The comparison between captive and rescued wild Sunda pangolins showed that PCV, TP, globulin and BUN to be significantly higher ($P<0.05$) in captive Sunda pangolins. On the other hand, WBC counts, neutrophil counts, ALP and phosphorous were found to be significantly lower ($P<0.05$) in captive Sunda pangolins compared to rescued wild Sunda pangolins.

Results of the hematological and serum biochemistry comparisons between captive and rescued wild Sunda pangolins in Singapore [1] can be seen in Tables 3 and 4.

This study provides important hematology and serum biochemistry parameters of captive Sunda pangolins. As pangolin species are known to be difficult to keep in captivity, most zoological institutions and rescue centers only keep small number of pangolins in their collection [8, 28]. The small population of captive Sunda pangolins in zoological institutions prevent the establishment of reference ranges or parameters for the species. WRS's successful Sunda pangolin rehabilitation and captive breeding program allowed us to establish the hematology and serum biochemistry parameters of captive Sunda pangolins. These parameters provide

Table 1. Hematology parameters from 13 captive Sunda pangolins in Wildlife Reserves Singapore

Parameters	n	Mean	Median	Standard deviation	Range	Cohen D test	*P* value between male and female
PCV (%) [a]	13	46.7	45.0	5.0	37.0–54.5	0.025	0.9293
Hemoglobin (g/l) [a]	13	154.0	150.0	22.5	118.0–203.0	0.3716	0.5273
RBC ($\times 10^{12}$/l) [a]	13	6.98	6.74	1.56	3.09–9.89	0.1710	0.7767
MCV (fl) [a]	13	65.8	66.0	4.6	54.9–73.0	0.5044	0.3961
MCH (pg) [b]	13	23.4	21.1	8.2	18.5–50.3	0.5309	0.7188
MCHC (g/l) [b]	13	304.3	328.0	72.0	68.4–343.0	0.2601	0.0735
WBC Count ($\times 10^9$/l) [a]	13	5.32	5.31	1.98	2.20–8.57	0.6713	0.2946
Lymphocytes ($\times 10^9$/l) [a]	13	1.07	0.74	0.70	0.23–2.35	0.099	0.8801
Monocytes ($\times 10^9$/l) [a]	13	0.33	0.29	0.25	0.03–0.82	0.4662	0.4163
Neutrophils ($\times 10^9$/l) [a]	13	3.80	3.42	1.57	1.37–7.28	0.8639	0.1433
Eosinophils ($\times 10^9$/l) [b]	13	0.11	0.06	0.11	0.02–0.40	0.6128	0.5222
Basophils ($\times 10^9$/l) [b]	13	0.01	0.00	0.02	0.00–0.05	0.5000	0.7188
Platelets ($\times 10^9$/l) [a]	13	140.8	120.0	51.1	79.0–237.0	0.6523	0.2168

PCV, packed cell volume; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC Count, white blood cell count. [a] Parametric distribution of the data. [b] Non parametric distribution of the data.

Table 2. Serum biochemistry parameters from 13 captive Sunda pangolins in Wildlife Reserves Singapore

Parameters	n		Mean	Median	Standard deviation	Range	Cohen D test	*P* value between male and female
Total Protein (g/dl) [a]	13		8.39	8.30	0.47	7.60–9.00	0.2880	0.6128
Globulin (g/l) [a]	13		47.4	46.0	5.9	37.0–57.0	0.9940	0.1057
Albumin (g/l) [a]	13		36.5	36.0	4.7	28.0–46.0	0.9993	0.0735
ALT (U/l) [b]	13		114.3	108.0	75.3	10.0–149.0	0.3872	0.8337
ALP (U/l) [a]	13		234.0	236.0	84.9	109.0–442.0	0.2281	0.6847
Total Bilirubin (μmol/l) [a]	13		8.2	8.0	1.6	6.0–11.0	0.5890	0.1531
Glucose (mmol/l) [a]	13		5.0	5.2	1.2	3.2–6.5	0.9740	0.1527
Blood Urea Nitrogen (mmol/l) [b]	13		17.2	12.2	14.8	6.4–63.0	0.3383	0.9442
Creatinine (μmol/l) [a]	13		41.6	38.5	25.0	11.0–98.0	0.3900	0.5366
Sodium (mmol/l) [a]	13		143.3	142.0	4.5	136.0–151.0	0.1412	0.8127
Potassium (mmol/l) [a]*	13	Combined	4.5	4.4	1.1	2.9–6.4	1.4599	0.0300
	7	Male	5.2	5.0	1.0	4.1–6.4		
	6	Female	3.8	3.9	0.8	2.9–4.7		
Calcium (mmol/l) [a]	13		2.58	2.61	0.18	2.38–2.79	0.4310	0.5079
Phosphorous (mmol/l) [a]	13		1.8	1.8	0.4	1.27–2.65	0.0718	0.1277
Amylase (U/l) [a]	13		361.8	336.5	97.9	255.0–597.0	0.3452	0.0796

ALP, alkaline phosphatase; ALT, alanine aminotransferase. *The mean between the male and female Sunda pangolins is significantly different ($P<0.05$). [a]Parametric distribution of the data. [b]Non parametric distribution of the data.

important data for facilities such as rescue centers that hold pangolins in their collection to conduct health checks, diagnose diseases and properly provide to treatment. However, the limitation of this study is that the small sample size of the captive Sunda pangolins. The small population size of the captive Sunda pangolins are subjected to type II errors when administering the statistical tests.

This study represents an expansion of the work previously conducted on rescued wild Sunda pangolins in Singapore [1]. In this study we compared the hematology and serum biochemistry dataset between the two studies to determine if there are significant differences between rescued wild and captive Sunda pangolins.

The potassium is significantly higher in the male captive Sunda pangolins compare to the females. Significantly elevated potassium between sexes among healthy individual are infrequently reported. Two studies of wild canvasback (*Aythya valisineria*) and in human reported significantly higher serum potassium in male but it did not explain the cause of the difference between the sexes in their studies [22, 29]. Further study required on captive Sunda pangolins to explain the significantly higher potassium in male Sunda pangolins.

WBC counts, neutrophil counts, phosphorus and ALP in captive Sunda pangolins were found to be significantly lower than those in rescued wild Sunda pangolins. The lower neutrophil and WBC counts of the captive Sunda pangolins could be due to the absence of physiological excitement, which would be present in rescued wild pangolins. Physiological excitement can result in the migration of marginal neutrophils into the bloodstream for circulation. This would lead to the increase of neutrophil and WBC count [4, 12]. As captive pangolins are accustomed to handling of their caretakers, it is possible the animals did not experience the same physiological stress as in rescued animals.

Table 3. Hematology parameters of captive Sunda pangolins compared to other published parameters for rescued wild Sunda pangolins in Singapore [1]

Parameters	n	Captive Sunda pangolins (Current study) Mean ± SD	n	Rescued Wild Sunda pangolins [b] (Previous study) Mean ± SD	Cohen D Test	*P* value between captive and wild rescued pangolins
PCV (%) [a]	13	46.7 ± 5.0	16	38.8 ± 6.6	1.3493	0.0074
Hemoglobin (g/l) [a]	13	154.0 ± 22.5	16	128.1 ± 18.4	1.2602	0.0500
RBC ($\times10^{12}$/l)	13	6.98 ± 1.56	16	5.81 ± 1.13	0.8516	0.6200
MCV (fl)	13	65.8 ± 4.6	16	66.4 ± 3.8	0.1422	0.3077
MCH (pg)	13	23.4 ± 8.2	15	22.9 ± 6.8	0.0664	0.2584
MCHC (g/l)	13	304.3 ± 72.0	15	325.65 ± 143.4	0.1882	0.5754
WBC Count ($\times10^9$/l) [a]	13	5.32 ± 1.98	16	8.77 ± 3.36	1.2510	0.0013
Lymphocytes ($\times10^9$/l)	13	1.07 ± 0.70	14	1.32 ± 0.82	0.3279	0.3222
Monocytes ($\times10^9$/l)	13	0.33 ± 0.25	16	0.40 ± 0.27	0.2690	0.3953
Neutrophils ($\times10^9$/l) [a]	13	3.80 ± 1.57	15	6.62 ± 3.41	1.0623	0.0198
Eosinophils ($\times10^9$/l)	13	0.11 ± 0.11	15	0.14 ± 0.15	0.2281	0.9522
Basophils ($\times10^9$/l)	13	0.01 ± 0.02	15	0.01 ± 0.02	0.0000	0.8887
Platelets ($\times10^9$/l)	13	140.8 ± 51.1	14	116.6 ± 37.8	0.5384	0.0970

PCV, packed cell volume; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC Count, white blood cell count. [a]Significant differences ($P<0.05$) were present between the current study on captive Sunda pangolins in Wildlife Reserves Singapore and the previous study on rescued wild Sunda pangolins in Singapore [1]. [b]Hematology parameters of clinically healthy adult rescued wild Sunda pangolins from the previous study [1].

Table 4. Serum biochemistry parameters of captive Sunda pangolins compared to other published parameters for rescued wild Sunda pangolins in Singapore [1]

Parameters	n	Captive Sunda pangolins (Current study) Mean ± SD	n	Rescued wild Sunda pangolins [c] (Previous study) Mean ± SD	Cohen D Test	*P* value between captive and rescued wild pangolins
Total Protein (g/dl) [b]	13	8.39 ± 0.47	16	7.65 ± 0.82	1.1073	0.0003
Globulin (g/l) [b]	13	47.4 ± 5.9	16	37.9 ± 13.2	0.9292	0.0001
Albumin (g/l)	13	36.5 ± 4.7	14	38.2 ± 8.1	0.2567	0.7718
ALT (U/l)	13	114.3 ± 75.3	15	126.6 ± 51.5	0.1907	0.2380
ALP (U/l) [b]	13	234.0 ± 84.9	14	444.3 ± 172.3	1.5483	0.0001
Total Bilirubin (µmol/l)	13	8.2 ± 1.6	16	8.9 ± 2.8	0.2938	0.2187
Glucose (mmol/l)	13	5.0 ± 1.2	16	5.5 ± 1.7	0.3398	0.72634
Blood Urea Nitrogen (mmol/l) [b]	13	17.2 ± 14.8	15	9.81 ± 5.18	0.6665	0.0074
Creatinine (µmol/l)	13	41.6 ± 25.0	12	34.2 ± 19.4	0.3320	0.4654
Sodium (mmol/l)	13	143.3 ± 4.5	16	146.1 ± 5.3	0.5695	0.4771
Potassium (mmol/l) [a]	13	4.5 ± 1.1	15	4.6 ± 0.5	0.7647	0.6892
Calcium (mmol/l)	13	2.58 ± 0.18	16	2.46 ± 0.13	0.7643	0.0561
Phosphorous (mmol/l) [b]	13	1.8 ± 0.4	16	2.3 ± 0.4	1.2500	0.0002
Amylase (U/l)	13	361.8 ± 97.9	15	340.0 ± 49.6	0.280916	0.9063

ALP, alkaline phosphatase; ALT, alanine aminotransferase. [a]Significant differences ($P<0.05$) were present on the current study on captive Sunda pangolins in Wildlife Reserves Singapore for the analyte between male and female Sunda pangolins. [b]Significant differences ($P<0.05$) were present between the current study on captive Sunda pangolins in Wildlife Reserves Singapore and the previous study on rescued wild Sunda pangolins in Singapore [1]. [c]Serum biochemistry parameters of clinically healthy adult rescued wild Sunda pangolins from the previous study [1].

The lower phosphorus and ALP level could also be due the absence of physiological excitement in the captive animals as they were brought to the hospital for anaesthesia and blood sampling. The rescued wild pangolins may be physiological excitement and exertion during its rescue that resulted in elevation of phosphorus [19, 27]. ALP elevation in rescued wild pangolins could be due to the stress of capture resulting in the release of cortisol. This cortisol will then result in the release of ALP [27].

The PCV, TP, globulin and BUN levels of the captive animals were found to be significantly higher compared to that in rescued wild Sunda pangolins. The significantly higher PCV in captive Sunda pangolins could be related to the better nutrition that the animal in the captivity received compared to the free ranging animals [13, 26]. Seasonal increase level of PCV had been recorded in wild western grey kangaroo (*Macropus fuliginosus*) that related to higher availability of protein source of the grazing area [3].

This study shows globulin levels in captive pangolins to be significantly higher than in rescued wild Sunda pangolins. Higher globulin can be indicative of immunological response to an infection or allergy [18]. However, all the Sunda pangolins during the health assessment did not show signs of infection or allergy reaction. The animals also had no history of infection or allergy prior to the health assessment. The globulin levels in the captive animals could be due to the higher protein diet and better living conditions. These levels could be indicative of increased protein synthesis due to the higher protein diet [20]. It is also possible that better nutrition with higher protein assists with building up immunity of the captive animals compare to in the wild [15]. The significantly higher TP in captive animals were be probably related to the significantly higher globulin in captive Sunda pangolins. Elevation of globulin resulted in the increase serum protein concentrate that leads to higher TP value in the captive Sunda pangolins [12, 18].

The higher BUN in the captive pangolins could also be due to the higher proportion of protein in the captive Sunda pangolin diet compare to that in the rescued wild or free ranging Sunda pangolins. High protein diets are known to cause elevation in the BUN [9, 17, 19]. A study published on Asian pangolin captive diet indicates it is similar to the crude protein of wild pangolin diets which consist mainly of invertebrate [6]. However, the diet did not factor the high amount of soil and leaf matter consumed by wild pangolins as they feed. The consumption of such matter may dilute the overall protein intake [16]. Hence it is possible with the captive diet, pangolins receive higher proportions of crude protein compared to in the wild diet.

Hematology and serum biochemistry parameters established in this study are important measures for clinical assessments of captive Sunda pangolins. Establishment of blood parameters for captive animals are important as the hematology and serum biochemistry parameters will be influenced by excitement or stress during the handling or capture of wild animals, as well as differences in nutrition compared to captive animals.

CONFLICT OF INTEREST. Authors declare no conflicts of interest.

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