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Research article

Clinical haematology, biochemistry profiles and erythrocytic morphometry of confiscated Sunda pangolins (*Manis javanica*) in the Centre for Rehabilitation and Quarantine of Confiscated Animals, Wildlife and Alien Species, Thailand

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Abstract

Every year, ten thousand Sunda Pangolins (*Manis javanica*) are rescued and confiscated from the illegal trade around Southeast Asia, including Thailand. Most of them are usually moribund and need intensive veterinary interventions to recover their health before being released to the wild. However, a lack of reliable haematology and clinical biochemistry references for Thai confiscated Sunda pangolins is a major difficulty to reach this goal. The aim of this study was to establish a standard panel of haematology and serum biochemistry profiles used for confiscated pangolins in Thailand. Coccygeal venepuncture of 52 confiscated pangolins was performed. Data acquisitions of haematology and serum biochemistry were made using automated blood analysers. Reference intervals were determined following the ASVCP guidelines. Erythrocytic morphometry was performed with light and scanning electron microscopy. The lower and upper limits at 90% confidence interval were calculated. The means of all parameters were calculated and then compared with those in two previous studies. There were differences of haematologic and biochemical parameters among the studies in some aspects. Blood corpuscle morphometry was done manually. The eosinophils in females were bigger than males. Wide scientific discussion was made to explain these variations. Ultimately, a panel of haematology and serum biochemistry profiles for Thai confiscated Sunda pangolin has been established. Veterinarians can refer to all parameters to evaluate the animals' health and diseases.

Keywords: Blood, Electron microscopy, Morphology, Reference interval, Sunda pangolin.

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INTRODUCTION

Sunda pangolins *(Manis javanica)* are one of the most endangered species. They are critically prone to extinction because of illegal hunting for food and Asian traditional medicine. Although they are protected by the Wild Animal Reservation and Protection Act B.E. 2535 in Thailand (Clark et al., 2008; Shepherd, 2008; Challender et al., 2019), however, approximately thousand Sunda pangolins are still poached yearly. Of these, hundreds are confiscated and rescued throughout the country. Unfortunately, they usually require intensive veterinary interventions after confiscation, because of poor physical conditions and behavioural changes. These include dehydration, emaciation, stress, trauma, wounding, and infection.

In veterinary medicine, haematologic and biochemical profiling is always guided veterinarians to establish appropriate therapies for domestic animals. This is also true in wildlife medicine. However, the primary difficulty is a lack of haematology and biochemistry references, owing to the small number of wildlife specimens and improper health verification, particularly in confiscated Sunda pangolins. Although, many attempts have been made to set up their haematological and biochemical profiles in ASEAN countries so far, e.g., Singapore and Vietnam (Ahmad et al., 2018; Ahmad et al., 2021; Yu et al., 2021), but many laboratory parameters in those studies still vary from publication to publication, resulting in inappropriate application to confiscated Sunda pangolins in Thailand.

Since haematology and biochemistry profiling is imperative particularly for investigating and monitoring the health and illness of confiscated Sunda pangolins. Moreover, the differences among several evaluating parameters previously reported may cause inconsistent clinical diagnosis for confiscated Sunda pangolins in Thailand. Therefore, clinical haematology and biochemistry profiling of those pangolins in Thailand is still reasonable. In this study, we performed clinical haematological and biochemical profiling of the confiscated Sunda pangolins sheltered in the Centre for Rehabilitation and Quarantine of Confiscated Animals, Wildlife and Alien Species (CRQCA), Mahidol University, Kanchanaburi Campus, which is the primary rescuing centre for confiscated pangolins in Thailand. The study was established using the standard automated blood and biochemistry analysers to reduce any bias in specific instrumentation and human error. In addition, scanning electron microscopy (SEM) was performed in two cases to provide a preliminary database for further erythrocytic morphometry.

MATERIALS AND METHODS

Animals, Selection Criteria and Husbandry

This study was conducted in the Centre for Rehabilitation and Quarantine of Confiscated Animals, Wildlife and Alien Species (CRQCA), Mahidol University, Kanchanaburi Campus during July 2014 to August 2021. Ninety-nine confiscated Sunda pangolins with a complete physical examination on arrival, were given a health assessment on day 14 after entering the shelter. Common signalments including body weight, color, and sex were recorded,

except age because there have been no criteria available for age determination in this species so far. However, all pangolins that had the body weight over 2 kg, were considered to be adult (Challander et al., 2011).

Only the pangolins that could pass these selection criteria: 1) weight over 2 kg, 2) having normal appetite, 3) displaying normal vital physiology including defecation and urination with no serious clinical signs, 4) sustaining intact behaviours such as coiling during restraint, and 5) being disease-free, were assessed to be healthy. Finally, fifty-two adult pangolins; thirty-one males and twenty-one females, were selected to participate this study.

Four to five pangolins were then grouped and kept in 3.90 x 3.88 x 4.00 m^3 holding pens with 1.5 m high concrete walls, an unpolished concrete floor, furnished with large branches and sleeping burrows. Sand areas and water trays were provided for the pangolins to urinate and defecate (Heath and Vanderlip, 1988). The pangolins were fed once a day with Weaver ants *(Oecophylla smaragdina)* and ground termite mounds at the ratio 4:1 offered once a day during 20:00-21:00 hr. Drinking water was adequately provided and changed twice a day.

Blood collection and laboratory analysis

Before blood sampling, each pangolin was fasted for at least 12 hours. It was manually restrained during blood collection using the standard methods previously described for Indian pangolins (Perera et al., 2020). Approximately 4 ml of blood was collected aseptically by coccygeal venipuncture with a 23G needle and a 5 ml disposable syringe. Subsequently, 1.5 ml of blood was transferred into an EDTA collection tube (BD Vacutainer®, BD Diagnostics, Plymouth, UK) for a complete blood count (CBC), thin blood film evaluation and electron microscopy. The rest was then transferred to a plain collection tube (BD Vacutainer®, BD Diagnostics, Plymouth, UK) for serum biochemistry analysis. Blood collection and evaluation was done as quickly as possible to prevent any stress that may affect the haematology and biochemical profiles. All blood samples were analysed immediately otherwise they were kept in the iced box for any delayed test, but not over than 2 hours after collection.

A complete blood count was performed using an automated haematology analyser (Vet abc™ 16-P, ABX Diagnostics, France). The red blood cells (RBC), white blood cells (WBC), and platelets were counted. Mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), and red blood cell distribution width (RDW) were calculated and reported. Microhematocrit centrifugation was done at 10,000 rpm for 5 minutes to assess the packed cell volume (PCV). An air-dried blood film was prepared using a wedge technique and stained with Wright Giemsa (Thrall et al., 2012). Erythrocytic indices, leukocytic differential count, and blood parasites other than microfilaria were acquired under light microscopy (Axiostar™ plus, Carl Zeiss, Germany) by two experienced pathologists. Morphology and diameter of the erythrons, leucons, and thrombocytes were also recorded. All blood smear parameters were measured on 200 cells/HPF and presented as mean ± SD. In case of disagreement, a discussion was done between the pathologists for the final decision. Microfilariasis was inspected from the buffy coat (Collins, 1971).

Serum pancreatic amylase (P-amylase), amylase, cholesterol, creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were assessed using a dry biochemistry analyser (Reflovet™ Plus, Roche Diagnostics GmbH, Mannheim, Germany). Blood glucose was measured with a portable blood glucometer (Accu-check® Performa, Roche Diagnostics GmbH, Mannheim, Germany), and the plasma protein was measured by refractometry. All analytical parameters of haematology and biochemistry analysers were set routinely using canine profiles. Our preliminary study had shown that this setting gave rise consistent results without error in Sunda pangolin haematology and biochemistry (unpublished data). Internal quality controls of the analysers were performed based on manufacture recommendations daily and annually.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed in the last two cases after complete blood count and clinical biochemistry. Briefly, 0.5 ml of the whole blood was washed with 1X phosphate-buffered saline (PBS) and centrifuged at 1500 rpm for 3 min, twice, to separate the blood corpuscles from the plasma. The blood cells were then re-suspended in 1 ml Hank's balanced salt solution (HBSS) and fixed with 1.2% glutaraldehyde in 0.1 M sodium cacodylate (CAC) buffer pH 7.3 for one hour. Thereafter, they were washed twice with 0.16 M CAC buffer and incubated in 1% osmium tetroxide (OsO_4) in 0.1 M CAC buffer, pH 7.3 for 1 h. The blood cells were dehydrated with a series of graded ethanol (25%, 50%, 75%, 95% and 100%), followed by a series of ethanol-isoamyl acetate mixtures (ratio 3:1, 1:1, 1:3 and pure isoamyl acetate), with 10 min for each. The cell suspension was dropped on a 10 mm aluminium membrane. After 2 minutes, the cells were dried in a CO2 critical point dryer. The cell-containing membrane was then glued onto the aluminium stubs. The blood corpuscles were coated with a gold sputtering device. Finally, scanning electron microscopy was performed using a JSM-6610LV electron microscope (JEOL Ltd., Tokyo, Japan) with an acceleration voltage of 15 kV (Ketpun et al., 2017).

Data analysis

Haematological results and morphometric measurements were entered into a computerized database (Microsoft Excel 2010). Statistical analyses were performed using Reference Value Advance V 2.1 freeware (National Veterinary School of Toulouse, France) (Geffré et al., 2011). Not all values were assessed for every pangolin. Non-quantitative values reported from Reflovet® plus were not included in the analysis. The reference interval was calculated following the ASVCP guidelines (Friedrichs et al., 2012). Values in each parameter were plotted in a histogram and normality (Gaussian) was determined with the Anderson-Darling test. Outliers were identified with Horn's algorithm using Tukey's interquartile fences. All values that were identified as outliers were then re-examined in the histogram. Values that were aberrant observations were excluded from the data set and values that were not were retained. The upper and lower reference limits were calculated using robust methods for Gaussian and nonparametric methods for parameters with the absence of Gaussianity. The 90% confidence interval (CI) of the limits was determined using the

bootstrap method. The 90% Cl was not calculated for the parameters with a sample size of less than 20. The mean, median, SD, variance, maximum and minimum values were calculated with Microsoft Excel 2010 and reported.

Prior to developing the RI for each parameter, an independent t-test was performed to compare the data from males and females. Statistical significance was assessed at a level of $p < 0.05$. Data were pooled for both sexes for the parameters with no significant difference. Parameters that were significantly different between sexes were reported as RI for each gender. Notably, each morphometric parameter of the erythrocytes investigated by light and scanning electron microscopy morphometry were reported as the mean ± S.D.

RESULTS

Blood cell Morphology

The light-microscopic morphometry and morphology of erythrocytes, platelets and leukocytes are shown in Table 1 and Figure 1, respectively. Of these data, erythrocytes and most leukocytes were sex-independent except band neutrophil and basophil. Moreover, their sizes and shapes were similar to other mammalian blood corpuscles.

Microscopically, the erythrocytes were round. Their cytoplasmic membranes were smooth. The cytoplasm was homogeneously eosinophilic, and some cells had an obscured biconcave region. Three types of granulocytes were present in the blood films, neutrophils, eosinophils, and basophils. Mature (segmented) neutrophils were round. They contained multilobular nuclei with pale eosinophilic cytoplasm. Numerous secondary granules admixed with the larger azurophilic granules were interspersed throughout the cytoplasm. The other population of neutrophils was immature (band) neutrophils. They were round cells and slightly larger than their mature counterparts, as in other mammals. The nuclei were unsegmented and horseshoe-shaped. Their cytoplasm was also pale and basophilic, containing a moderate amount of azurophilic granules.

Table 1 Mean diameters of erythrocytes and leukocytes of male and female Sunda pangolins

Parameter	Diameter (µm)		T score	P-value (P<0.05)
	Male (Mean ± SD)	Female (Mean ± SD)		
Erythrocyte	7.38 ± 0.43 (n=10621)	7.41 ± 0.43 (n=7356)	-3.016	0.002565
Neutrophil	13.76 ± 1.35 (n=723)	14.24 ± 1.65 (n=469)	-6.540	0.000010
Band Neutrophil	14.20 ± 1.34 (n=52)	14.82 ± 1.73 (n=22)	-1.918	0.059023
Lymphocyte	11.76 ± 1.53 (n=726)	11.59 ± 1.59 (n=486)	2.3179	0.020621
Monocyte	15.28 ± 1.99 (n=65)	15.96 ± 1.97 (n=83)	-2.916	0.004065
Eosinophil	12.72 ± 1.33 (n=13)	14.98 ± 1.70 (n=14)	-4.777	0.000061
Basophil	13.83 ± 1.38 (n=13)	14.32 ± 1.15 (n=21)	-1.221	0.230738

n=number of blood cells

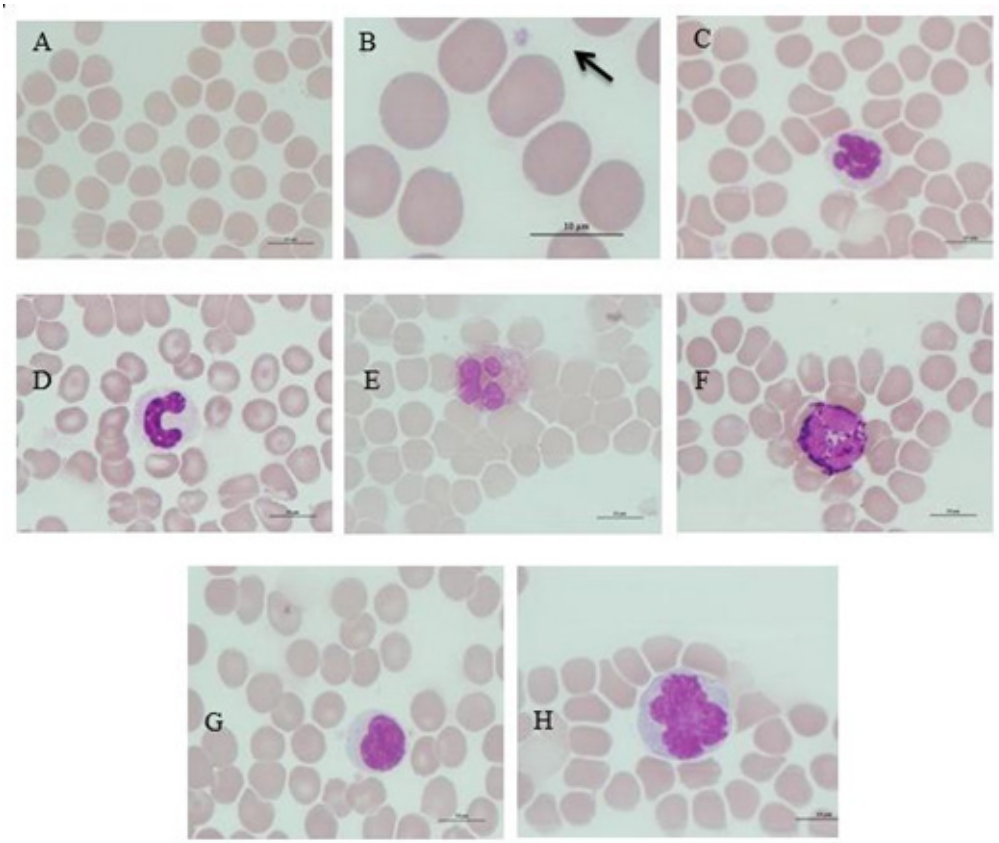

Figure 1 Morphologic description of the blood corpuscles of the confiscated Sunda Pangolins in this study. A) Erythrocytes; B) Platelet (Arrow); C) Segmented neutrophils; D) Band neutrophils; E) Eosinophils; F) Basophils; G) Lymphocytes and H) Monocytes.

Eosinophils were the round cells in which their nuclei are lobulated. There were numerous round-to-oval eosinophilic granules in the cytoplasm. However, few eosinophils may contain vacuolar cytoplasm. On the contrary, basophils were the granulocytes whose nuclei were divided into several large lobes. They always contained a large number of the intensely basophilic granules in the cytoplasm. For the platelets, they were the small cell fragments that stained basophilic and did not contain a nucleus. Their shape may be variably round to oval.

For mononuclear cells, lymphocytes were round to slightly oval with large round nuclei. The cytoplasm was scant, leading to a high nuclear-to-cytoplasm (N/C) ratio in these cells. Some lymphocytes may have small azurophilic granules in the cytoplasm. The shape and nuclei of monocytes varied tremendously depending on their biological activities. Inactive monocytes had round-to-oval nuclei, which could change to cloverleaf in activated cells. The cytoplasm was basophilic and contained a variable size of vacuoles.

SEM

Scanning electron microscopy (SEM) revealed that healthy erythrocytes were biconcave discoid cells (discocytes) (Figure 2). The concavity was shallow and circular. The microscopy also unveiled abnormal erythrocytes. Echinocytes (burr cells), characterized by many thorny projections in the cell

membranes, were readily observed throughout the blood film in this study (Figure 3). In addition, leptocytes (Figure 4, Panel A), stomatocytes (Figure 4, Panel B), and crenation (Figure 5) were remarkably observed.

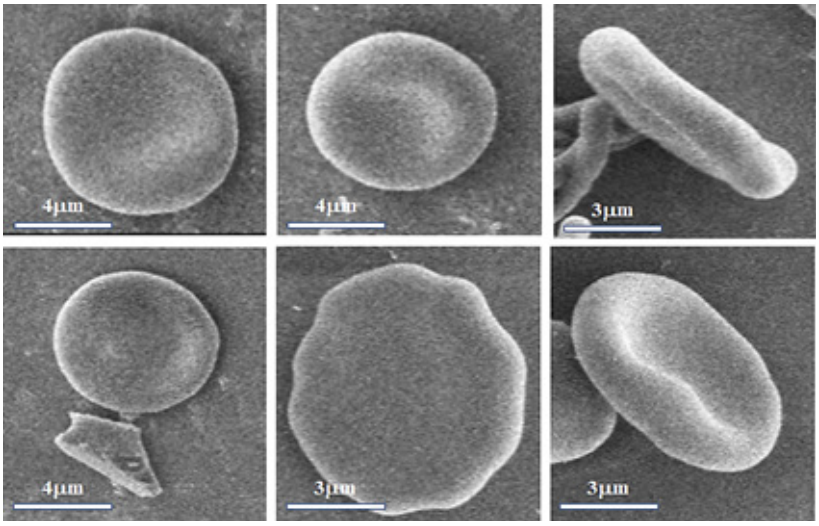

Figure 2 The figure panel illustrates the morphology of healthy erythrocytes under scanning electron microscopy. Notably, the erythrocytes vary both in size and shape. The cells are usually thin and flat. There is no membrane protrusion observed in normal erythrocytes. Also, the concaves are relatively shallow.

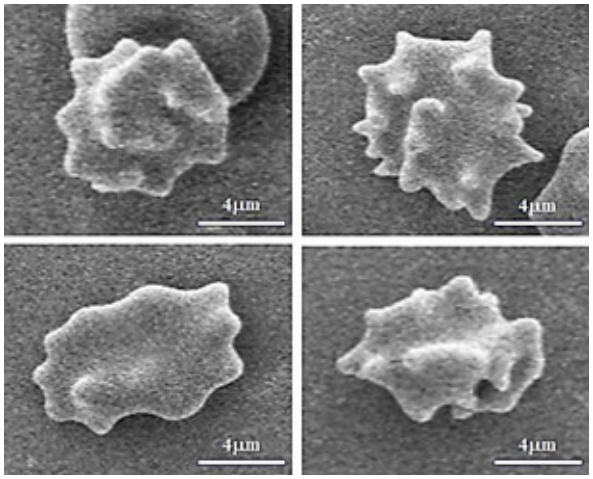

Figure 3 The morphology of echinocytes. Remarkably, the cell membranes are uniformly spiculated.

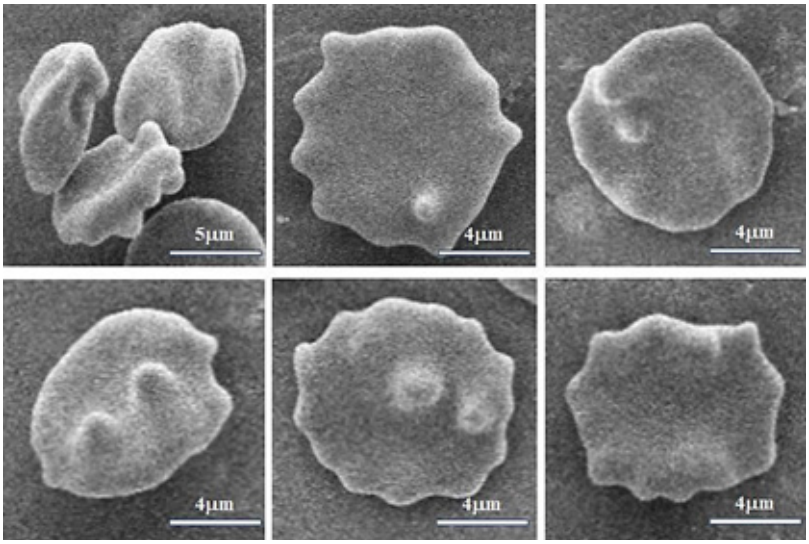

Figure 4 The upper panel (A1, A2, A3) exhibits leptocytes characterized by unilateral membrane folding to the concave centre. This morphological abnormality must be distinguished from stomatocytes (B1, B2, B3), in which the latter has the bilateral crescentic folding of the membrane, causing the deep invagination of the concavity.

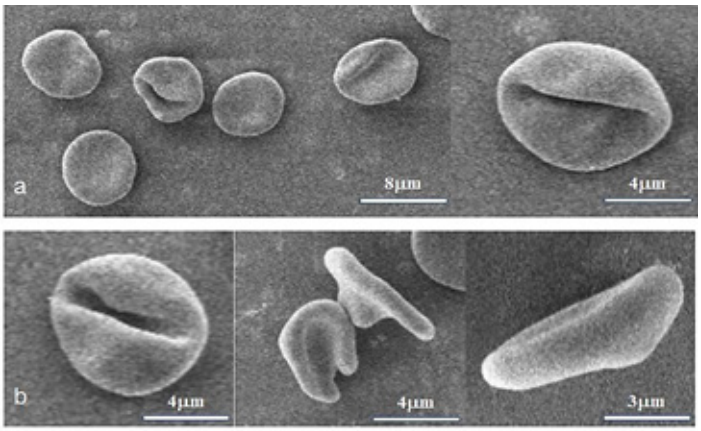

Figure 5 The figure panel demonstrates the artefactual morphology alteration referred to as crenation of the erythrocytes. Remarkably, the cell shape is consistently standard, but the cell membranes are notched or spiculated. This change must be differentiated from the early stage of echinocytosis.

Haematological Profile

The haematological parameters of the clinically healthy confiscated Sunda pangolins are shown in Table 2. Many parameters varied between those described by Ahmad et al. 2018 and 2021 (Ahmad et al., 2018; Ahmad et al., 2021). The different parameters were haemoglobin, platelets, neutrophils and lymphocytes. Of these, the haemoglobin level and neutrophil number were lower against their means in both Ahmad's publications. However, the numbers of platelets and lymphocytes were higher when compared to those in Ahmad' studies. Notably, microfilariasis was not detected in all specimens.

Table 2 Haematologic profiling of the 52 confiscated Sunda pangolins in this study

Parameter	N	Dis	Min	Max	Mean ± SD	Median	Lower limit (90% CI)	Upper limit (90% CI)
PCV (%)	52	G	27.00	59.00	43.80±7.40	43.00	27.30 (23.40-30.80)	58.00 (55.40-60.30)
HB (g/dl)	52	G	8.80	19.60	13.20±2.24	13.24	8.59 (7.85-9.51)	17.67 (16.74-18.69)
RBC (10^6/mm^3)	51	G	3.96	9.76	6.57±1.40	6.33	4.02 (3.62-4.38)	9.71 (9.00-10.48)
MCV (fl)	52	G	57.00	73.00	65.80±3.80	65.50	57.30 (57.00-58.70)	73.00 (72.70-73.00)
MCH (pg)	48	NG	15.20	24.70	21.11±1.64	21.30	15.40 (15.20-18.98)	24.43 (23.24-24.70)
MCHC (g/dl)	45	NG	29.90	34.40	32.05±0.87	31.90	30.04 (29.90-31.10)	34.30 (33.69-34.40)
RDW (%)	52	NG	11.30	16.40	13.07±1.29	12.80	11.33 (11.30-11.63)	16.27 (15.70-16.40)
Platelet (10^3/mm^3)	52	NG	47.00	390.00	199.30±99.50	192.00	49.30 (47.00-67.20)	385.80 (366.00-390.00)
WBC (10^3/mm^3)	51	G	1.8	10.7	5.358±2.10	5.20	0.95 (0.36-1.80)	9.50 (8.51-10.45)
Male (10^3/mm^3)	31	G	1.8	7.7	4.55±1.58	4.60	1.23 (0.55-2.03)	7.81 (6.93-8.63)
Female (10^3/mm^3)	20	G	2.1	10.7	6.60±2.23	6.55	1.83 (0.44-3.44)	11.45 (9.89-12.77)
Neutrophil (10^3/mm^3)	48	G	0.49	6.31	2.71±1.41	2.60	0.56 (0.41-0.79)	6.21 (5.44-7.05)
Lymphocyte (10^3/mm^3)	47	G	0.64	3.05	1.70±0.57	1.56	0.47 (0.27-0.71)	2.87 (2.56-3.12)
Monocyte (10^3/mm^3)	44	NG	0.00	0.19	0.05±0.05	0.05	0.00 (0.00-0.00)	0.19 (0.15-0.19)
Eosinophil (10^3/mm^3)	51	NG	0.00	0.46	0.11±0.12	0.08	0.00 (0.00-0.00)	0.45 (0.29-0.46)
Basophil (10^3/mm^3)	52	NG	0.00	0.32	0.02±0.05	0.00	0.00 (0.00-0.00)	0.25 (0.09-0.32)
Band Neutrophil (10^3/mm^3)	52	NG	0.00	0.11	0.01 ± 0.02	0.00	0.00 (0.00-0.00)	0.10 (0.05-0.11)

N = sample size, Dis = distribution, G = Gaussianity, NG = non-Gaussianity

Biochemistry Profile

Table 3 summarizes the serum biochemistry parameters tested in this study. As in the haematologic study, most of the biochemical parameters fell in the comparative ranges of the anesthetized pangolins, although, glucose, amylase, creatinine and serum potassium seemed to be changed. However, they were still in the normal referral intervals of most mammalians.

Table 3 Blood chemistry parameters and reference interval of clinically healthy adult Sunda pangolins

Parameter	N	Dis	Min	Max	Mean ± SD	Median	Lower limit (90% CI)	Upper limit (90% CI)
Total Protein (g/dl)	52	G	6.00	10.40	8.31±0.98	8.30	6.33 (5.98-6.70)	10.28 (9.90-10.66)
Glucose (mg/dl)	38	NG	45	101	68.53±15.36	64.50	44.50 (41.60-48.20)	108.40 (95.00-121.90)
ALP (U/L)	32	G	61.2	519	319.38±116.80	319.50	78.64 (24.98-136.51)	564.57 (503.60-623.60)
AST (U/L)	39	G	15.40	72.20	40.39±11.86	39.10	19.05 (15.47-23.61)	67.47 (59.58-75.85)
ALT (U/L)	48	G	28.70	256.00	113.69±52.83	114.00	23.13 (12.33-37.99)	238.98 (215.09-270.13)
BUN (mg/dl)	24	NG	11.30	48.10	29.91±9.03	26.30	12.20 (9.33-16.41)	52.22 (41.60-59.01)
Amylase (U/dl)	29	G	92	926	545.80±200.30	528.00	128.80 (24.70-239.50)	969.20 (851.20-1075.40)
Male (U/dl)	18	G	92	794	487.72±196.55	474.50	-	-
Female (U/dl)	11	G	444	926	640.73±175.14	543.00	-	-
Creatinine (mg/dl)	38	NG	<0.5*	1.22	0.61±0.18	0.51	-	-
Cholesterol (mg/dl)	43	NG	<100*	222	141.35±46.98	128.00	-	-
P-Amylase (mg/dl)	13	G	204.00	1300.00	942.54±278.43	952.00	-	-
pH (pH units)	7	-	7.42	7.77	7.58±0.14	7.59	-	-
PCO2 (mmHg)	8	-	24.00	123.00	51.13±31.28	45.50	-	-
PO2 (mmHg)	8	-	35.00	241.00	109.38±75.35	92.50	-	-
BE (mEq/L)	7	-	7.20	77.40	22.77±24.72	15.70	-	-
HCO3 (mEq/L)	7	-	31.90	115.40	48.40±29.87	36.80	-	-
SO2 (%)	6	-	78.00	100.00	92.00±9.03	95.00	-	-
Na (mmol/L)	7	-	140.00	151.00	144.86±3.29	145.00	-	-
K (mmol/L)	8	-	4.70	5.70	5.11±0.36	5.15	-	-
Cl (mmol/L)	8	-	105.00	113.00	109.63±2.62	110.00	-	-

N = sample size, Dis = distribution, G = Gaussianity, NG = non-Gaussianity
*The lowest value the biochemistry analyzer can provide

DISCUSSION

This study reports the first complete haematology and biochemistry profiling of un-anesthetized confiscated Sunda pangolin in Thailand. It will provide the crucial data for health and disease investigations of this endangered species in CRQCA and other rescuing sanctuaries around the country. In this study, it is noteworthy that the similarity of each CBC and serum biochemistry parameter to that reported in both primary references was swayed. Some studied parameters had the means close to their responsible parameters in the confiscated pangolins, while others shifted closer to those in the captive pangolins. The remaining parameters fell in the middle between both. However, more is needed to know about the governing factors of these.

To the best of our knowledge, the influential factors affecting haematology and serum biochemistry profiles of Sunda pangolins are sampling time, specimen collection technique, assessment method, laboratory equipment, personal competence, age, gender, animal maturity, environment, nutrition, physiological condition, stress, subclinical diseases and immobilisation (Weiser et al., 2007). All pangolins in this study were conscious; hence a strong

biological reaction to stimuli during specimen collection probably occurred, when compared to their anaesthetized counterparts in Ahmed's studies. This difference may lead to a deviation of some haematology and serum biochemistry parameters distinctly.

In line with this study, the number of neutrophils was lower than in Ahmad's studies. A possible explanation might be due to the anaesthetic effect. Isoflurane anaesthesia can inhibit the activation of adhesion molecules, including L-selectin, β2-integrins, CD11a, and CD11b. This alteration attenuates neutrophil tethering, rolling, firm adhesion, and trans-endothelial emigration. Hence, neutrophils will be shifted from the marginal to circulating pools, causing an increased blood neutrophils (de Rossi et al., 2002; Carbo et al., 2013; Szrama et al., 2021). Although unknown the exact mechanism, isoflurane anaesthesia can cause lymphopenia but not monocytopenia in anesthetized subjects (Kim et al., 2011). Taken together, the numbers of neutrophils and lymphocytes in this study may reflect the natural levels of neutrophils and lymphocytes in conscious Sunda pangolins more properly than the isoflurane anesthetized pangolins in Ahmad's studies.

Another explanation of the fluctuation of several haematology means, even though lesser possible, may result from the fact that not all pangolins in this study were fully recovered from their unhealthy states at the time of blood collection. Consequently, several leukocytic parameters were wavered. Subclinical injuries or chronic active inflammation might remain in some pangolins. Therefore, their leukocytes were gradually utilized in the tissues. In addition, increased lymphocyte number might be another indicator of body need for interacting with injuries. The lower neutrophils in this study may also involve the oestrous cycle causing an increased neutrophil immigration into the reproductive tract (Hussain and Daniel, 1991; Williams et al., 1992; Quartuccio et al., 2020).

Furthermore, oestrogen also affects leukopoiesis and eosinophil development. Hematopoietic stem cells and eosinophils express a high number of oestrogen receptors-α (ERα) on their membranes. Without oestrogen, the self-renewal of hematopoietic stem cells and terminal leukocytic development are down-regulated, as seen in female mice and humans (Nakada et al., 2014; Fañanas Baquero et al., 2021). Since female mammals including Sunda pangolins have a higher circulating oestrogen; therefore, the turnover rate of myelopoiesis in female pangolins would be greater than in males, leading to an increase in all blood leukocytes. Oestrogen is also responsible for eosinophil trafficking in the uterus during the oestrous cycle to prevent uterine infections (Rothenberg and Hogan, 2006). As a result, eosinophils in females would be more differentiated and mature. They could deposit many subcellular organelles, causing an enlargement of the cells. Fortunately, during CCTV surveillance in February, several female pangolins were in oestrous cycle showing the natural copulatory behaviour. Therefore, this clue may indicate that the larger eosinophils in the female pangolins in this study were influenced by the oestrus cycle.

Haematologic features of pangolins in this study may not be affected by stress in all certain aspects. Basically, acute stress can lead to catecholamine release from the adrenal medulla. It can increase circulating erythrocytes and haemoglobin concentration by splenic contraction (Pohlin et al., 2020; Yu et al., 2021). However, the hemogram in this study has not demonstrated acute

stress from improper animal handling or personal incompetence since erythrocyte and haemoglobin levels were not increased. Furthermore, catecholamine can increase the blood glucose transiently but the glucose level in this study was in the mammalian referral interval, approximately 3.5-7.2 mmol/L). Prolonged catecholamine discharging under subacute stress can lead to persistent IL-6-dependent neutrophil trafficking in the tissues (Kim et al., 2014). However, neutrophil concentration in this study was not increased, particularly when compared to the anesthetized pangolins. Therefore, the effect of acute stress and sub-acute stress could be neglected in this study.

The stress leukogram that often develops within 4 hours after acute stress (Moore et al., 1992). Prolonged glucocorticoid release during subacute to chronic stress can cause neutrophilia, lymphopenia, and increased serum alkaline phosphatase (Ahmad et al., 2021; Yu et al., 2021). However, no evidence was observed for stress leukogram in this study because of the lower neutrophil number and the greater lymphocyte concentration compared the anesthetized pangolins in Ahmad's studies. Nonetheless, the alkaline phosphatase was still normal in mammalian ranges (Ochi et al., 2013). Bone marrow suppression can be ruled out as a cause of the lowering of neutrophils and monocytes, since the level of erythrocytes and their hematologic indices still fit into the referral ranges of the other mammals and anesthetized Sunda pangolins. However, further study should be done to clarify the exact levels of neutrophils, monocytes and lymphocytes in absolutely disease-free Sunda pangolins.

Apart from the above factors, in-house automated blood analysers may cause a minor deviation in the results (Phillips et al., 1988; Kropf et al., 1991; Lopes-Pereira et al., 1996). For example, both Vet abc™ in this study and VetScan™ HM5 in the references work based on impedance principles, but the equipment design, piezoelectric sensor, aperture size, and electrolytic medium may vary between the manufacturers, although all automated blood analysers must have their accuracies calibrated with a gold standard method such as hemocytometry before marketing. Accordingly, a measured value of each parameter may deviate but within a minimal range. Therefore, most haematologic parameters in this study deviated slightly from the references. As in the case of haematology analysers, all automatic blood chemistry analysers marketed today have been developed based on various standard biochemistry methods. However, biochemistry analysers from different manufacturers frequently employ a different mode of biochemical reactions to evaluate each biochemical parameter. This causes a deviation of the test results among the analysers.

For instance, creatinine and serum potassium in this study were slightly higher than those reported in the references. According to the manufacturers' disclosures, Reflovet™ plus uses the 5-step creatinine iminohydrolase reaction, while VetScan® 2 uses the 4-step creatinine amidohydrolase reaction to evaluate creatinine concentration. In addition, Reflovet® plus also engages the valinomycin reaction to measure serum potassium, whereas VetScan™ 2 engages phosphoenolpyruvate dephosphorylation. These dissimilarities, of course, can cause the deviation in the test results, even though they measure the same substrates. Similarly, lower serum glucose in this study may result from the usage of a handheld glucometer.

This study also reveals the SEM morphology of Sunda pangolin erythrocytes for the first time. Scanning electron microscopy has consistently exhibited that the erythrocytes were biconcave discocytes. The concavity was shallow and obscured, and its shape circular. Moreover, echinocytes seemed to be a regular blood figure in this animal species. The presence of these cells in the film may indicate that the erythrocytes of Sunda pangolin might be sensitive to osmotic alteration and one probable cause in this study was a delayed blood preservation with SEM fixatives since the fixation for

SEM was performed after routine haematology and clinical biochemistry. Leptocytes and stomatocytes were also present in the study.

These abnormalities may result from plasma membrane folding due to an osmotic alteration from specimen preparatory protocols or subclinical diseases. Hence, any further study of Sunda pangolins erythrocytes with SEM should be done with caveat. The authors strongly recommend a mass study to verify that whether sample collection, storage condition, and pathophysiologic states will impact on morphological changes of Sunda pangolin erythrocytes under SEM.

Intriguingly, the hematologic features in this study were consistent with those previously described in various Felidae (Prihirunkit et al., 2007; Salakij et al., 2008; Salakij et al., 2010a; Salakij et al., 2010b; Hon et al., 2020). Thence, this may imply that Sunda pangolins may have evolved from the same ancestor as other felids.

CONCLUSIONS

In conclusion, the first complete haematology and serum chemistry profiles of conscious Thai confiscated Sunda pangolins have been established. Veterinarians and others can use all parameters to investigate and monitor the health and diseases of Thai Sunda pangolins confidently. However, we recommend a further large-scale study on absolutely disease-free Sunda pangolins to improve the validity of all parameters. In addition, SEM and morphometry also provide a powerful tool for any future study on erythrocytic and leukocytic morphometry of Sunda pangolins and other animal species, especially of diseased blood corpuscles.

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AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: study conception and design: Marnoch Yindee, Warisara Thomas; data collection: Marnoch Yindee, Warisara Thomas, Wantida Horpiencharoen, Nuttapon Bangkaew, Nicharee Income, Worapong Kosaruk, Wasinee Thepapichaikul, Witsanu Wongsawang, Wallaya Manatchaiworakul; analysis and interpretation of results: Marnoch Yindee, Warisara Thomas, Dettachai Ketpun; draft manuscript preparation: Marnoch Yindee, Warisara Thomas, Wallaya Manatchaiworakul, Dettachai Ketpun, and Worawidh Wajjawalku. Finally, all authors reviewed the results and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors would like to declare that there is no commercial or academic competing interest of any company, or third party named in the context of this literature.

REFERENCES

Ahmad, A.A., Samsuddin, S., Oh, P.Y., Martinez-Perez, P., Rasedee, A., 2018. Hematological and serum biochemical parameters of rescued Sunda pangolins (*Manis javanica*) in Singapore. J. Vet. Med. Sci. 80(12), 1867-1874.

Ahmad, A.A., Sekar, S., Oh, P.Y. Samsuddin, S., 2021. Hematology and serum biochemistry of captive Sunda pangolin (*Manis javanica*) in Wildlife Reserves Singapore. J. Vet. Med. Sci. 83(2), 309-314.

Carbo, C., Yuki, K., Demers, M., Wagner, D.D., Shimaoka, M., 2013. Isoflurane inhibits neutrophil recruitment in the cutaneous Arthus reaction model. J. Anesth. 27, 261-268.

Challender, D.W.S., Thai, N., Van Jones, M., May, L., 2011. Time-budgets and activity patterns of captive Sunda pangolins (*Manis javanica*). Zoo. Biol. 31, 206–18.

Challender, D., Willcox, D.H.A., Panjang, E., Lim, N., Nash, H., Heinrich, S., Chong, J., 2019. *Manis javanica*. The IUCN Red List of Threatened Species 2019. Available online: https://dx.doi.org/10.2305/IUCN.UK.2019-3.RLTS.T12763A123584856.en (Accessed on December 5, 2022).

Clark, L., Thai, N.V., Phuong, T.Q., 2008. A long way from home: the health status of Asian Pangolins confiscated from the illegal wildlife trade in Viet Nam. In Proceedings of the Workshop on the Trade and Conservation of Pangolin Native to South and Southeast Asia, Singapore Zoo, Singapore, 30 June - 2 July 2008, pp. 111-131.

Collins, J.D., 1971. The detection of microfilariae using the capillary haemotocrit tube method. Trop. Anim. Health. Prod. 3(1), 23-25.

de Rossi, L.W., Horn, N.A., Buhre, W., Gass, F., Hutschenreuter, G., Rossaint, R., 2002. The effect of isoflurane on neutrophil selectin and beta (2)-integrin activation in vitro. Anesth. Analg. 95(3), 583-587.

Fañanas-Baquero, S., Orman, I., Aparicio, F.B., Bermudez de Miguel, S., Merino, J.G., Yañez, R., Sainz, Y.F., Sánchez, R., Dessy-Rodríguez, M., Alberquilla, O., Alfaro, D., Zapata, A., Bueren, J.A., Segovia, J.C., Quintana-Bustamante, O., 2021. Natural estrogens enhance the engraftment of human hematopoietic stem and progenitor cells in immunodeficient mice. Haematologica. 106(6), 6591670.

Friedrichs, K.R., Harr, K.E., Freeman, K.P., Szladovits, B., Walton, R.M., Barnhart, K.F., Blanco-Chavez, J., 2012. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet. Clin. Pathol. 41(4), 441-453.

Geffré, A., Concordet, D., Braun, J.P., Trumel, C., 2011. Reference value advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel. Vet. Clin. Pathol. 40(1), 107-112.

Heath, M.E., Vanderlip, S.L., 1988. Biology, husbandry, and veterinary care of captive Chinese pangolins (*Manis pentadactyla*). Zoo. Biol. 7, 293-312.

Hon, M., Thomovsky, E.J., Brooks, A.C., Johnson, P.A., 2020. Cryopreservation of feline red blood cells in liquid nitrogen using glycerol and hydroxyethyl starch. J. Feline. Med. Surg. 22(4), 366-375.

Hussain, A.M., Daniel, R.C.W., 1991. Studies on some aspects of neutrophil functions and uterine defences in Cows during the estrous cycle. Reprod. Domest. Anim. 26(6), 290-296.

Ketpun, D., Sailasuta, A., Suwannaphan, T., Bhanpattanakul, S., Pimpin, A., Srituravanich, W., Sripumkhai, W., Jeamsaksiri, W., Piyaviriyakul, P., 2017. The Viability of single cancer cells after exposure to hydrodynamic shear stresses in a spiral microchannel: a canine cutaneous mast cell tumor model. Micromachines. 9(1), 9.

Kim, M.H., Gorouhi, F., Ramirez, S., Granick, J.L., Byrne, B.A., Soulika, A.M., Simon, S.I., Isseroff, R.R., 2014. Catecholamine stress alters neutrophil trafficking and impairs wound healing by β2 adrenergic receptor-mediated upregulation of IL-6. J. Invest. 134(3), 809-817.

Kim, W.H., Jin, H.S., Ko, J.S., Hahm, T.S., Lee, S.M., Cho, H.S., Kim, M.H., 2011. The effect of anesthetic techniques on neutrophil-to-lymphocyte ratio after laparoscopy-assisted vaginal hysterectomy. Acta. Anaesthesiol. Taiwan. 49(3), 83-87.

Kropf, J., Marx, A.M., Hildebrandt, J., Gressner, A.M., 1991. Practical implications of coexistent different technologies in clinical chemical laboratories. Solid phase chemistry and conventional analysis. Eur. J. Clin. Chem. Clin. Biochem. 29(10), 675-683.

Lopes-Pereira, C.M., Harun, M., Schmidtova, D., Timane, I., Mabasso, M., Otto, F., Bogin, E., 1996. Use of the dry chemistry "Reflotron" blood analyzer under outdoor-field conditions in veterinary medicine. Eur. J. Clin. Chem. Clin. Biochem. 34(3), 231-235.

Moore, G.E., Mahaffey, E.A., Hoenig, M., 1992. Hematologic and serum biochemical effects of long-term administration of anti-inflammatory doses of prednisone in dogs. Am. J. Vet. Res. 53(6), 1033-1037.

Nakada, D., Oguro, H., Levi, B.P., Ryan, N., Kitano, A., Saitoh, Y., Takeichi, M., Wendt, G.R., Morrison, S.J., 2014. Oestrogen increases haematopoietic stem-cell self-renewal in females and during pregnancy. Nature. 505(7484), 555-558.

Ochi, T., Nishiura, I., Tatsumi, M., Hirano, Y., Yahagi, K., Sakurai, Y., Sudo, Y., Koyama, H., Hagita, Y., Fujimoto, Y., Kitamura, S., Hashimoto, H., Nakamura, T., Yamada, A., Tanimoto, M., Nishina, N., 2013. Effects of transport stress on serum alkaline phosphatase activity in beagle dogs. Exp Anim. 62(4), 329-332.

Perera, P., Algewatta, H.R. Karawita, H., 2020. Protocols for recording morphometric measurement of Indian Pangolin (*Manis crassicaudata*). MethodsX. 7, 101020. Phillips, S., Wyndham, L., Shaw, J., Walker, S.F., 1988. How accurately does the Reflotron drychemistry system measure plasma total cholesterol levels when used as a community-screening device. Med. J. Aust. 149(3), 122-125.

Pohlin, F., Hooijberg, E.H., Buss, P., Huber, N., Viljoen, F.P., Blackhurst, D., Meyer, L.C.R., 2020. A Comparison of hematological, immunological, and stress responses to capture and transport in wild white rhinoceros bulls (*Ceratotherium simum simum*) supplemented with azaperone or midazolam. Front. Vet. Sci. 20(7), 569576.

Prihirunkit, K., Salakij, C., Apibal, S., Narkkong, N.A., 2007. Hematology, cytochemistry and ultrastructure of blood cells in fishing cat (*Felis viverrina*). J. Vet. Sci. 8(2), 163-168.

Quartuccio, M., Cristarella, S., Medica, P., Fazio, E., Mazzullo, G., Rifici, C., Liotta, L., Satué, K., 2020. Endometrial cytology during the different phases of the Estrous Cycle in Jennies: new evidences. Animals (Basel). 10(6), 1062.

Rothenberg, M.E., Hogan, S.P., 2006. The eosinophil. Annu. Rev. Immunol. 24, 147-174. Salaki,j C., Prihirunkit, K., Salakij, J., Narkkong, N.A., Thongthainun, D., 2010a. Characterisation of blood cells in jungle cat, Felis chaus (Carnivora, Felidae). Comp. Clin. Pathol. 20, 319-326.

Salakij, C., Salakij, J., Prihirunkit, K., Apibal, S., Narkkong, N.A., Thongthainun, D., 2008. Hematology, cytochemistry and ultrastructure of blood cells in Clouded leopard (*Neofelis nebulosa*). J. Anim. Vet. Adv. 7(7), 847-853.

Salakij, C., Salakij, J., Prihirunkit, K., Narkkong, N.A., Pitakkingthong, D., 2010b. Characterization of blood cells in the Leopard Cat (*Prionailurus bengalensis*). Vet. Clin. Pathol. 39(2), 193-198.

Shepherd, C.R., 2008. Overview of pangolin trade in Southeast Asia. Pangolin capture and trade in Malaysia. In Proceedings of the Workshop on the trade and conservation of pangolins native to South and Southeast Asia, Singapore Zoo, Singapore, 30 June-2 July 2008, pp. 6-9.

Szrama, J., Kusza, K., Sobczyński, P., Molnar, Z., Siemionow, M., 2021. The effect of volatile anesthetics on cellular responses in the microcirculation of free tissue transfers. Adv. Interv. Cardiol. 4 (66), 1-6.

Thrall, M.A., Weiser, G., Allison, R.W., Campbell, T.W., 2012. Veterinary hematology and clinical chemistry, 2nd edition. John Wiley & Sons, New Jersey.

Weiser, M.G., Vap, L.M., Thrall, M.A., 2007. Perspectives and advances in in-clinic laboratory diagnostic capabilities: hematology and clinical chemistry. Vet. Clin. North. Am. Small. Anim. Pract. 37(2), 221-36.

Williams, E.S., Thorne, E.T., Kwiatkowski, D.R., Lutz, K., Anderson, S.L., 1992. Comparative vaginal cytology of the estrous cycle of black-footed ferrets (Mustela nigripes), Siberian polecats (*M. eversmanni*), and domestic ferrets (*M. putorius furo*). Small. Anim. Pract. 37(2), 221-236.

Yu, J.H., Huong Nguyen, N.D., Lam, H.K., Jimerson, J., Hayek, L.C., Raphael, B.L., Nguyen, V.T., Valitutto, M.T., 2021. Serum biochemistry and select mineral parameters of pre-release Sunda pangolins (*Manis Javanica*) following rehabilitation in Vietnam. J. Zoo. Wildl. Med. 52(1), 241252.

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