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ORIGINAL RESEARCH

Reference intervals for hematology, serum biochemistry, and basic clinical findings in free-ranging Chinese Pangolin (*Manis pentadactyla*) from Taiwan

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Key Words

Age group, Pholidota, seasonal, sex-related

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Background: There are 8 species of Pangolins found in Asia and Africa. Among them, the Chinese Pangolin (*Manis pentadactyla*) is an endangered insectivorous mammal found only in Asia. Hematology and serum chemistry reference intervals are critical for evaluating an animal's well-being and can be useful for clinical diagnostic purposes. Currently, there are no such reference intervals available for any Pangolin species.

Objective: The purpose of the present study was to establish reference intervals for hematology and serum biochemical analytes, and some basic clinical findings, in Chinese Pangolins.

Methods: Reference intervals for the hematology and serum chemistry variables, and basic clinical findings (body weight, heart rate, body temperature, blood oxygen saturation) were collected from 100 clinically healthy Chinese Pangolins (51 males and 49 females) using parametric and non-parametric percentile methods. In addition, seasonal, age-related, and sexual differences for all variables were statistically analyzed.

Results: No significant differences in the reference intervals were found between males and females, except for body weight. However, significant seasonal differences were observed for heart rate, body temperature, serum ALT and lipase activities, and phosphate concentrations. The variables, which were significantly different between adult and sub-adult Pangolins were heart rate, MCH, creatinine, total protein, phosphate, glucose, and potassium concentration, and amylase activity. Seasonal and age group differences should be taken into consideration when using these reference intervals.

Conclusions: The findings from the present study represent a valuable resource for assessing the health of Chinese Pangolins, and contribute toward the conservation of this endangered mammal.

Introduction

Pangolins (Pholidota: Manidae), also known as scaly anteaters, are nocturnal, insectivorous mammals found in several Asian and African countries.¹ There are 8 extant species, which inhabit tropical and subtropical forests, dry woodlands and open savannahs of the Old World.^{1,2} Pangolin biology and ecology has been poorly studied. Currently, the Chinese Pangolin (*Manis pentadactyla*) is listed as Critically Endangered

A2d+3d+4d on the IUCN (International Union for Conservation of Nature) Red List of Threatened Species and the population trend has been found to be decreasing.³

Chinese Pangolins are native to Taiwan and all *Manis* species have been protected since August 1990 under Taiwan's 1989 Wildlife Conservation Act.^{3,4} This species is found in a wide range of habitats, including primary and secondary tropical forests, limestone forests, bamboo forests, grasslands, and agricultural

fields.^{5,6} In Taiwan, hunting and destruction of its natural habitat have been the major cause of the declining wild populations of Chinese Pangolins.^{4,5}

Hematologic and biochemical studies are important to evaluate an animal's health and the physiologic status of various organs.⁷ Actual hematology and blood chemistry reference intervals (RI) are available for a wide range of domestic and wild animals that receive veterinary care. However, no RI are available for any of the Pholidota (Pangolin species). Although phylogenetically the group of Pholidota has been placed as the sister group to Carnivora based on chromosomal study⁸, the hematology and blood chemistry RI of carnivores cannot be applied to the Pangolin due to the significant differences in their morphology, life history, and physiology. Some hematology and serum biochemistry analytes have been reported from a small population of Chinese Pangolins managed in captivity⁹, but RI were not determined. The objectives of the present study were to establish the RI for select hematology and serum chemistry variables and basic clinical findings in the free-ranging Chinese Pangolins, and evaluate the effect of sex, age, and seasons on these variables.

Materials and Methods

Animal population

The Pangolins used in the present study were either caught in the wild or rescued from Taitung County and nearby locations in southern Taiwan, and brought to the Pingtung Rescue Center for Endangered Wild Animals (PTRC), National Pingtung University of Science and Technology (NPUST) for clinical examination and sample collection. The present study was done as a part of a "Pangolin biology and ecology project" with approval from the Taiwan Forestry Bureau. Samples were obtained from 180 Chinese Pangolins between 2010 and 2014, during the spring–summer (March to August) and autumn–winter (September to February) seasons. The captured or rescued Pangolins were placed in a cage provided with appropriate bedding and transported in a vehicle from the capture site to the PTRC. Upon arrival at the rescue center, they were housed in a Pangolin enclosure (1.2 m × 1.2 m × 2.4 m), and the sample collection was done on the next day. Inclusion and exclusion criteria of the Pangolins for the study were established on the basis of clinical examination. Pangolins showing clinical abnormalities (poor body condition, dull appearance, reduced or no appetite, diarrhea, wounds, fractures, injuries, abnormal respiratory signs, and radiographic

findings) and juveniles (< 1 kg body weight), pregnant, and recaptured Pangolins were excluded, and only the adult (≥ 2 kg body weight) and sub-adult ($\geq 1 \leq 2$ kg body weight) Pangolins found to be apparently healthy on clinical examination were included in the present study, they included 51 males and 49 females. Pangolins were fasted overnight before the clinical examination and sample collection.

Blood sampling procedure

Anesthesia was induced by inhalation in a gas chamber with 5% isoflurane and oxygen at a flow rate of 6 L/min. The Pangolins were removed from the chamber once they had lost their righting response, and were placed on a surgical table covered with a cloth. Supplemental heat was not provided during the sample collection. Body weight was determined and the anesthesia was maintained by introducing isoflurane (2–2.5%) and oxygen (2 L/min) applied by a face-mask. The clinical variables were recorded immediately and included heart rate, blood oxygen saturation (SpO₂), and rectal temperature. The SpO₂ was measured by placing the sensor of the pulse oximeter (Nonin model 9847 pulse oximeter; Nonin Medical, Inc., Plymouth, MN, USA) on the loose skin of the inguinal areas. Blood (3–5 mL) was collected from the tail vein by inserting a 23-Gauge 1¼-inch needle along the ventral midline of the tail (Figure 1), and was immediately placed in an EDTA tube and red top serum collection tube (BD Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for hema-



Figure 1. Blood collection from the tail vein of a Chinese Pangolin under general anesthesia. The animal was positioned in dorsal recumbency for blood collection. The site for needle insertion can be located under the scales along the ventral midline of the tail. Insertion site was swabbed with alcohol, and then the 23 G 1¼-inch needle was inserted at a slight angle to a depth of approximately 2 cm until some drops of blood were seen in the hub of the needle, after which the required volume of blood was aspirated.

tology and serum chemistry analysis, respectively. It took approximately 10 minutes for measuring the clinical variables and collecting the sample. After the sample collection, all the Pangolins were micro-chipped and allowed to recover. They were released back into the wild at the original capture site on the following day.

Laboratory analysis

A CBC was done using a Hemavet 950 Analyzer (Erba Diagnostics, Inc., Miami, FL, USA) and the serum chemistry analysis was conducted using a FUJI DRI-CHEM 4000i instrument and reagents (FUJIFILM Co., Minato-ku, Tokyo, Japan). The CBC included total WBC, RBC, and thrombocyte (PLT) counts, and HGB, HCT, MCV, MCH, and MCHC. The Hemavet system separates the cells by producing a stream of single cells through the aperture for accurate and precise analysis using a patented focus flow technology (Hemavet 950 Analyzer product manual). Spectrophotometry is used to measure the HGB concentration after cell lysis. The control reagent supplied with the analyzer along with the blank sample and cleaning cycle were run on a daily basis following the manufacturer's instruction and before processing new samples. Clotted EDTA tubes were not processed for the hematology study.

Serum chemistry variables included AST, ALT, amylase (AMY), and Lipase (LIPA) activities, and urea, creatinine (CRE), total protein (TP), albumin (ALB), total calcium (Ca), phosphate (Phos), glucose (GLU), creatine kinase (CK), uric acid (UA), sodium (Na), potassium (K), chloride (Cl), total cholesterol (T-CHO), total bilirubin (T-BIL), and direct bilirubin (D-BIL) concentrations. The FUJI DRI-CHEM 4000i uses a colorimetric method to measure the enzyme activities and chemical analytes, and a potentiometric method to measure the electrolytes (Table 1). Calibration was done using the quality control (QC) card supplied with the FUJI DRI-CHEM slides whenever slides from a new lot were used. Both hematology and serum biochemistry analyzers were routinely examined by the instrument supplier for maintenance. Findings from the visibly hemolyzed samples were not included in the statistical analysis.

Statistical analysis

Statistical analysis for RI was performed using MedCalc for Windows, version 12.5 (Medcalc software, Ostend, Belgium), RI, mean, median, SD, skewness, and kurtosis were calculated, and normal distribution was also determined for all the hematology, serum chemistry,

Table 1. Analytic methods for measurement of clinical biochemical analytes in serum of Chinese Pangolins.

Analyte	Method
Colorimetric method*	
AST	Amino transition reaction of L-aspartic acid and α -ketoglutaric acid catalyzed by AST to form oxalacetic acid, which is converted to pyruvic acid by oxaloacetic decarboxylase; subsequent reaction by hydrogen peroxidase (H_2O_2) produces a blue color dye.
ALT	Amino transition reaction of L-alanine and α -ketoglutaric acid catalyzed by ALT to form pyruvic acid; subsequent reaction by H_2O_2 produces a blue color dye.
Urea	Urea decomposed by urease to release ammonia (NH_3), which reacts with bromocresol green to produce a green color dye.
Creatinine	Creatinine is decomposed by creatinine deiminase releasing NH_3 , which reacts with bromphenol blue to produce a blue color dye.
Total protein	Biuret method (reaction of peptide bonds with copper ions in alkaline medium producing a red purple color)
Albumin	Albumin reacts with bromocresol green (BCG) to form an albumin-BCG complex, which is a blue color dye.
Calcium	Bound type Ca is dissociated to free-type Ca, which reacts with chlorophosphonazo III to produce a dye.
Phosphate	Phosphorous reacts with xanthosine by purine nucleoside phosphorylase, producing xanthine, which further reacts with xanthine oxidase to produce H_2O_2 ; imidazole blue color dye is formed when H_2O_2 and leuco dye react with peroxidase (POD).
Glucose	Glucose oxidase catalyzes the oxidization of glucose to produce H_2O_2 , which reacts with dye precursors in presence of POD to form a red color dye.
CK	CK catalyzes the reaction of creatine phosphate and ADP releasing ATP, which reduces nitroterazolium blue to form diformazan (purple color dye) by the action of other coexisting enzymes.
UA	UA is hydrolyzed by uricase to form H_2O_2 , which oxidizes diarylimidazole leuco dye by the action of POD to form a blue color dye.

(continued)

Table 1 (continued)

Analyte	Method
Amylase	AMY reacts with substrate (4,6-ethylidene-4-nitrophenyl- α -D-maltoheptaoside) and further decomposition by α -glucosidase releases p-nitrophenol.
Lipase	Lipase catalyzes the hydrolysis of triolein, 2-monoglyceride lipase, and the product formed is further decomposed to glycerol by monoglyceride lipase; glycerol generates L- α -glycerophosphate by glycerol kinase in presence of ATP and Mg^{2+} , which further produces H_2O_2 with the action of glycerol-3-phosphate oxidase; finally H_2O_2 oxidizes diarylimidazole leuco dye by the action of POD to produce a blue color dye.
Total cholesterol	Lipoprotein is decomposed to cholesterol, cholesterol ester, and protein by surfactant; cholesterol ester is hydrolyzed to produce free cholesterol by cholesterol esterase; free cholesterol and endogenous cholesterol generate H_2O_2 by reaction with cholesterol oxidase; finally H_2O_2 and POD oxidize leuco dye to form a blue color dye.
Direct bilirubin	Diazo reaction (D-BIL react with diazonium salt of benzenesulfonic acid to form diazo dye)
Total bilirubin	Indirect bilirubin is dissociated with dyphylline and undergoes diazo reaction together with direct bilirubin to form diazo dye.
Potentiometric method Sodium, potassium, chloride	The concentrations of the electrolytes are measured by the change in potential between the specimen and an electrolyte reference solution with a fixed concentration of electrolytes.

*The optical reflection density of the dye measured is converted into the concentration of the analyte by using a calibration curve preinstalled in the analyzer.

and clinical variables studied. Outliers were identified by the Tukey's test (for Gaussian data distribution) and Reed test (for non-Gaussian data distribution) twice, once on the original data and again after identified outliers were eliminated. Upper and lower reference limits were calculated from the central 95% interval (between the 2.5 and 97.5 percentiles) of each variable according to the International Federation of Clinical Chemistry (IFCC) protocol using a nonparametric percentile method (for non-Gaussian data) and

parametric methods (for Gaussian data) following the CLSI Guidelines C28-A3.^{10–12} The parametric method also determined the 90% confidence intervals (CI) around the limits. The bootstrap method was used to determine 90% CI around the limits calculated by nonparametric percentile method by the use of R Statistical software for windows, version 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria). RI for the sub-adult Pangolins ($n = 23$) were calculated by the robust (for non-Gaussian data) and parametric method (for Gaussian data). Significance of differences between independent groups (males and females, adults and sub-adults, and spring–summer and autumn–winter) was determined by Mann–Whitney *U* test for non-Gaussian and Student's *t*-test for Gaussian data distribution using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA).

Results

Of 180 Chinese Pangolins (wild captured and rescued) brought to the PTRC during the study period, findings from 80 captured Chinese Pangolins were excluded from the present study, as they exhibited various signs of illness such as injuries, fractures, wounds, poor body condition, abnormal breathing, etc. Of the rescued Pangolins, 57 showing other signs of illness were also excluded from the analysis. Only 23 rescued Pangolins were found to be apparently healthy on clinical examination and were included in the analysis. No significant differences in the variables studied were found between rescued and captured Pangolins.

All Pangolins

Reference intervals of the clinical variables (heart rate, rectal temperature, and SpO_2), hematology, and serum chemistry analytes along with the mean, median, and SD are shown in Tables 2–4, respectively.

Males and females

Overall, there was no significant difference in the variables studied between males and females ($P < .05$), except for body weight (Tables 5 and S1, Figure 2A), with females weighing less than males.

Data collected in spring–summer and autumn–winter

The comparisons of studied analytes of the Chinese Pangolin in different seasonal groups are shown in

Table 2. Reference intervals (RI) from the central 95th percentile for clinical variables in anesthetized Chinese Pangolins.

Analytes	<i>n</i>	Outliers*	Mean	Median	SD	Lower limit (90% CI)	Upper limit (90% CI)	Dist.	M
Body weight (kg)†	100	0	3.15	2.70	1.37	1.19 (1.07–1.54)	6.00 (5.77–6.42)	NG	NP
Heart rate (beats/min)†	99	1	57	57	14	30 (27–34)	84 (80–88)	G	P
SpO ₂ (%)	99	1	96.44	97.00	3.11	89.00 (88.45–90.00)	100.00 (100.00–100.00)	NG	NP
Rectal temperature (°C) †	100	0	31.56	31.60	1.34	28.15 (27.84–29.43)	34.10 (33.40–34.81)	NG	NP

n indicates number of animals used to calculate the RI; Dist., data distribution; NG, non-Gaussian; G, Gaussian; M, method; NP, nonparametric; P, parametric.

*Outliers identified and removed by Tukey's test (G dist.) and Reed test (NG dist.).

†See Table 5 for subgroup-specific RI for these variables.

Table 3. Reference intervals (RI) from the central 95th percentile for the hematologic variables in anesthetized Chinese Pangolins.

Analytes	<i>n</i>	Outliers*	Mean	Median	SD	Lower limit (90% CI)	Upper limit (90% CI)	Dist.	M
WBC (10 ⁹ /L)	96	4	5.46	5.40	1.59	2.34 (1.88–2.80)	8.58 (8.12–9.04)	G	P
RBC (10 ¹² /L)	99	1	5.47	5.55	1.13	2.66 (2.33–3.60)	7.73 (6.96–8.22)	NG	NP
HGB (g/L)	96	4	130.85	130.50	25.12	81.83 (74.53–89.13)	179.88 (172.58–187.18)	G	P
HCT (L/L)	100	0	0.37	0.38	0.08	0.18 (0.15–0.24)	0.53 (0.49–0.58)	NG	NP
MCV (fL)	100	0	68.94	67.40	6.45	58.50 (57.56–61.08)	83.59 (80.37–84.64)	NG	NP
MCH (pg)†	100	0	23.38	23.35	2.69	18.36 (17.05–20.34)	31.61 (27.22–34.24)	NG	NP
MCHC (g/L)	100	0	339.77	339.50	41.80	254.35 (247.00–278.23)	463.20 (387.28–508.88)	NG	NP
PLT (10 ⁹ /L)	100	0	233.52	214.50	108.96	63.82 (42.30–111.00)	530.93 (429.88–630.68)	NG	NP

n indicates number of animals used to calculate the RI; Dist., data distribution; NG, non-Gaussian; G, Gaussian; M, method; NP, nonparametric; P, parametric.

*Outliers identified and removed by Tukey's test (G dist.) and Reed test (NG dist.).

†See Table 5 for subgroup-specific RI for these variables

Tables 5 and S2. Significantly different variables between seasons included lower heart rate and body temperature in autumn–winter than in spring–summer (Figures 2B,C, respectively), higher serum ALT and lipase activities in autumn–winter than in spring–summer (Table S2), and lower serum phosphate concentration in autumn–winter than in spring–summer (Figure 2D).

Adult and sub-adult groups

The comparisons of the clinical findings, hematology, and serum chemistry analytes for adult and sub-adult Pangolins are shown in Tables 5 and S3. Significantly different variables between adult and sub-adult groups included higher heart rate, phosphate, and potassium concentration (Figures 3A,E,G, respectively), and lower MCH, creatinine, total protein and glucose concentration, and amylase activity in sub-adults than in adults (Figure 3B–D,F,H).

Discussion

Reference intervals represent an important source of information for laboratory diagnosis and have gained universal acceptance as one of the most powerful tools

in laboratory medicine to aid in the clinical decision-making process.^{10,11,13} The RI for hematology and serum chemistry variables along with body weight, heart rate, body temperature, and SpO₂ reported herein will be a valuable resource for monitoring and assessment of the health of both captive and free-ranging Chinese Pangolins.

The Pangolin habitat where the present study was conducted is fragmented due to human settlement, farmland, and roads. All the healthy rescued Pangolins included in the present study were free-ranging, presented to the rescue center for further examination by our research staff. Moreover, the handling, transportation, housing, and sample collection method used for both rescued and wild caught animals was similar, and no significant differences were found between these groups.

Previous observations in 4 captive Chinese Pangolins reported the range for heart rate 80–86 beats/min and their body temperature between 33.0 and 34.5°C.¹⁴ However, in the present study the lower limit of both heart rate and body temperature was lower than in previous studies. The clinical variables in the present study were recorded under general anesthesia, whereas the previous study was done without anesthesia, which might be the reason for the observed difference.

Table 4. Reference intervals (RI) from the central 95th percentile for serum biochemistry analytes in anesthetized Chinese Pangolins.

Analytes	n	Outliers*	Mean	Median	SD	Lower limit (90% CI)	Upper limit (90% CI)	Dist	M
AST (U/L)	99	1	24.40	20.00	15.43	11.00 (11.00–13.00)	87.00 (46.40–94.00)	NG	NP
ALT (U/L)	100	0	154.86	129.00	81.98	48.05 (43.70–66.00)	395.83 (271.00–471.43)	NG	NP
Urea (mmol/L)	100	0	12.96	12.17	3.84	7.38 (7.05–8.60)	23.73 (19.86–24.78)	NG	NP
CRE (μmol/L)†	100	0	20.51	17.68	9.39	8.84 (8.84–8.84)	48.40 (35.36–53.04)	NG	NP
TP (g/L) †	97	3	61.75	61.00	6.25	49.50 (47.68–51.31)	74.00 (72.19–75.82)	G	P
ALB (g/L)	100	0	35.18	35.00	3.79	27.00 (26.48–29.48)	42.95 (40.53–45.05)	NG	NP
Ca (mmol/L)	100	0	2.53	2.44	0.30	1.96 (1.91–2.10)	3.10 (3.03–3.13)	NG	NP
Phos (mmol/L) †	99	1	1.97	1.97	0.40	1.18 (1.16–1.34)	2.84 (2.68–2.91)	NG	NP
GLU (mmol/L) †	99	1	5.04	4.66	1.38	2.30 (1.77–3.33)	8.63 (7.33–8.84)	NG	NP
CK (U/L)	100	0	887.46	751.00	559.73	349.70 (330.8–395.55)	2768.00 (1956.00–3313.48)	NG	NP
UA (μmol/L)	100	0	47.72	47.20	15.18	23.60 (11.21–29.5)	91.30 (68.00–97.50)	NG	NP
Na (mmol/L)	99	1	137.04	138.00	6.31	124.66 (122.85–126.48)	149.42 (147.60–151.23)	G	P
K (mmol/L) †	100	0	4.50	4.40	0.75	3.41 (3.25–3.60)	6.64 (5.70–6.90)	NG	NP
Cl (mmol/L)	100	0	92.46	93.00	6.18	80.05 (78.48–84.00)	105.43 (101.53–107.00)	NG	NP
AMY (U/L) †	99	1	201.88	189.00	99.31	50.50 (38.15–79.00)	475.00 (384.20–475.00)	NG	NP
Lipase (U/L)	99	1	50.94	50.00	13.96	25.00 (17.65–36.00)	86.50 (74.00–90.85)	NG	NP
T-CHO (mmol/L)	99	1	6.62	6.42	2.37	1.97 (1.29–2.65)	11.27 (10.59–11.95)	G	P
D-BIL (μmol/L)	100	0	3.38	3.42	2.15	1.71 (1.71–1.71)	10.26 (6.84–10.26)	NG	NP
T-BIL (μmol/L)	100	0	10.52	8.55	6.57	3.42 (2.52–5.13)	30.69 (18.00–38.95)	NG	NP

n indicates total number of animals used to calculate the RI; Dist., data distribution; NG, non-Gaussian; G, Gaussian; M, method; NP, nonparametric; P, parametric.

*Outliers identified and removed by Tukey's test (G dist.) and Reed test (NG dist.)

†See Table 5 for subgroup-specific RI for these variables

Table 5. Comparison of clinical, hematologic and serum biochemistry variables that were significantly different between males and females, autumn–winter and spring–summer seasons and adults and sub-adults.

Variable	Dist.	M	n	Outlier†	Mean ± SD	RI	Dist.	M	n	Outlier†	Mean ± SD	RI
Sex	Male			Female								
Body weight (kg)	NG	NP	51	0	3.41 ± 1.42	1.36–6.56	NG	NP	49	0	2.88 ± 1.28*	0.95–6.00
Season	Spring–summer			Autumn–winter								
Heart rate (b/min)	G	P	44	1	61 ± 14	34–88	NG	NP	55	0	56 ± 13**	33–84
Rectal temperature (°C)	G	P	43	2	32.08 ± 0.99	30.14–34.01	NG	NP	54	1	31.13 ± 1.33**	27.81–33.78
Phos (mmol/L)	G	P	44	1	2.09 ± 0.47	1.18–3.01	G	P	53	2	1.85 ± 0.28**	1.31–2.39
Age group	Sub-adult			Adult								
Heart rate (b/min)	NG	R	23	0	68 ± 11	44–93	G	P	76	1	53 ± 12**	29–78
MCH (pg)	G	P	23	0	22.40 ± 1.84	18.79–26.01	NG	NP	77	0	23.67 ± 2.85*	18.00–32.57
Creatinine (μmol/L)	NG	R	23	0	15.37 ± 6.65	8.84–26.52	NG	NP	77	0	22.10 ± 10.61**	8.84–53.04
Total protein (g/L)	G	P	22	1	58.00 ± 7.04	44.19–71.81	G	P	76	1	62.57 ± 6.09**	50.63–74.50
Phosphate (mmol/L)	G	P	23	0	2.14 ± 0.35	1.45–2.83	G	P	72	5	1.87 ± 0.36**	1.18–2.57
Glucose (mmol/L)	NG	R	23	0	4.49 ± 1.26	1.52–6.93	NG	NP	76	1	5.21 ± 1.39**	2.09–8.68
Potassium (mmol/L)	G	P	22	1	4.66 ± 0.49	3.70–5.61	NG	NP	77	0	4.43 ± 0.77*	3.30–6.90
Amylase (U/L)	G	P	23	0	123.70 ± 54.96	15.98–231.41	NG	NP	76	1	225.54 ± 97.76**	89.50–475.00

n indicates the number of animals used to calculate the RI; Dist., data distribution; NG, non-Gaussian; G, Gaussian; M, method; NP, nonparametric; P, parametric; R, robust.

*P < .05 and **P < .01 vs male, spring–summer and sub-adult groups respectively by Mann–Whitney U test for non-Gaussian and Student's t-test for Gaussian distribution.

†Outliers identified and removed by Tukey's test (G dist.) and Reed test (NG dist.)

The low HCT (0.18 L/L) found in the lower limit of the RI may be attributed to age-related differences and/or sub-clinical anemia associated with endoparasite load.¹⁵ The higher urea concentrations in the

upper limit of the RI might be due to dehydration and/or increased muscle metabolism. Muscle damage associated with the physical/metabolic stresses of capture and transport can also elevate the CK activity in

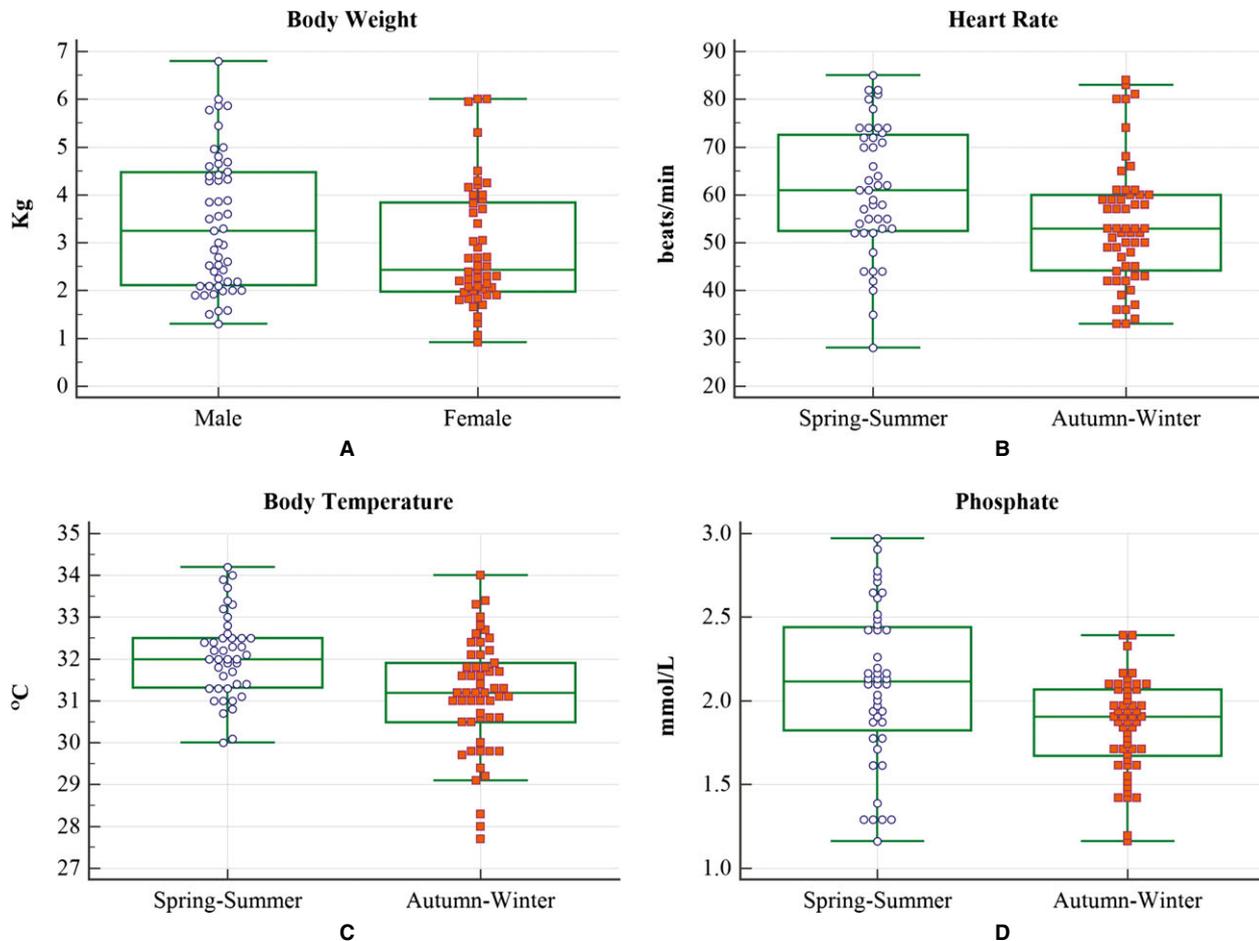


Figure 2. Combined box-and-whisker and dot plot for the variables that were significantly different between male and female Pangolins, and between seasons (spring–summer and autumn–winter). The central box represents the values from the lower to upper quartile (25–75 percentile). The middle line represents the median. The vertical line extends from the minimum to the maximum value, excluding outside and far out values, which are displayed as outliers (not eliminated). The whiskers represent the 1.5× the interquartile range and the dots on the plot represent individual data.

the blood, which might have resulted in concentrations of CK in the upper limit of the RI. Muscle exertion and dehydration during transportation may also be a cause for the K concentrations at the upper limit of the RI. Hemolysis of red blood cells can result in increased serum K concentration, even if the serum or plasma may not appear hemolyzed.¹⁶ In the present study, hemolyzed serum samples were excluded from analysis.

Seasonal differences

The reduced heart rate and body temperature observed in the Chinese Pangolins during the autumn–winter season could be for metabolic energy conservation. To achieve an overall reduction in thermoregulatory cost at cold ambient temperature, reduction in both basal metabolic rate and the set

point of core body temperature is needed.¹⁷ Pangolins are less active in winter and therefore, a physiologic mechanism to reduce the body temperature and heart rate and thus the energy expenditure for coping with a negative energy balance.

A long-term study examining seasonal variation in the serum enzyme activities in people reported that the AST and ALT activities tended to be higher in winter than in summer.¹⁸ A similar seasonal difference was observed in the present study, where the ALT and AST activities in autumn–winter were higher than in spring–summer. However, only ALT activity was significantly different in the present study. The seasonal differences in serum ALT and lipase activities in the present study have little clinical relevance as they were of low magnitude.

Phosphorous content in the diet can directly affect blood phosphate concentration. Chinese Pangolins

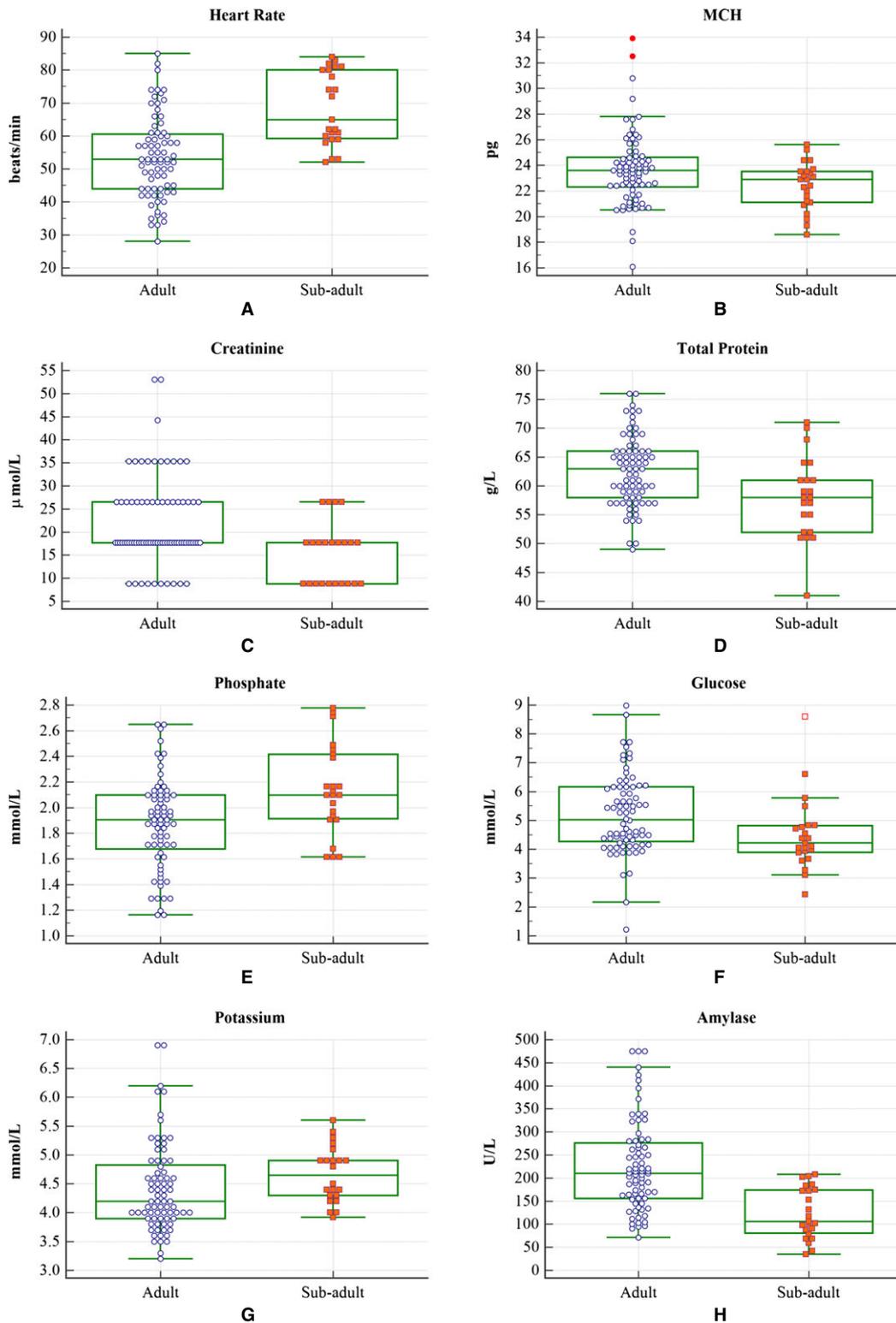


Figure 3. Combined box-and-whisker and dot plot for the variables that were significantly different between adult and sub-adult Pangolins. The central box represents the values from the lower to upper quartile (25–75 percentile). The middle line represents the median. The vertical line extends from the minimum to the maximum value, excluding outside and far out values, which are displayed as outliers (not eliminated). The whiskers represent the 1.5× the interquartile range and the dots on the plot represent individual data.

consume mostly ants during summer and mostly termites during winter.¹⁹ Studies have shown that the phosphorus content in ants is higher than in termites^{20,21}, which might be the reason for the higher serum phosphate concentration during spring–summer in Chinese Pangolins.

Age group differences

The higher heart rate in sub-adult Pangolins is consistent with similar findings in other animals. In general, smaller animals have a higher metabolic rate than the larger ones in the same taxonomic group. They have a larger body surface area and greater need of oxygen by unit of body mass. This greater oxygen demand is covered by increased heart rates.²²

Previous studies in bears have found a significantly lower MCH in young bears than in adults, with similar trends in MCHC, HGB, HCT, and RBC counts, although not statistically significant.^{15,23} The erythrocyte variables, RBC counts, HGB, HCT, MCV, MCH, and MCHC were lower in sub-adult Pangolins than in adult Pangolins in the present study. However, only MCH was found to be significantly lower. Lower erythrocyte variables in younger animals may be related to plasma expansion associated with development and accelerated body weight gain exceeding the rate of RBC production.²⁴ Thus, it is important to use age-specific RI for these variables in Pangolins to avoid an incorrect interpretation.

The adult Pangolins have a higher muscle mass than sub-adult Pangolins, which may explain the higher serum creatinine concentrations in the adult Pangolins compared to the sub-adults, as serum creatinine concentrations are directly related to the muscle mass of an animal.²⁵

The significant differences observed in the total serum protein concentration between adult and sub-adult Pangolins could be explained as an age-related phenomenon as similar findings have also been reported in carnivores.^{26,27}

Serum phosphate concentration is higher in the young growing animals compared to the adults in many species studied so far.²⁸ Physiologically increased serum and plasma phosphate concentrations are seen in young and growing animals due to enhanced intestinal phosphorous uptake and decreased renal phosphorus excretion, presumably to facilitate bone mineralization.²⁹

Likewise, higher serum K concentrations in the sub-adult Pangolins could be related to metabolic differences as previously reported in young dogs and cats compared to the adults.^{30,31}

In contrast, amylase activity was lower in young Pangolins, as previously reported in kittens, which might be related to animal development.³²

The differences in serum glucose concentrations between adult and sub-adult Pangolins in the present study could be associated with the handling and transport stress.³³ Although identical methods were used in handling, transportation, and housing of all Pangolins, different age groups may have responded differently to the stressors. However, the serum concentrations of stress hormones in the Pangolins were not evaluated in the present study. It should be noted that the sample size of sub-adult Pangolins in the present study was small, which might have resulted in some of the variations in the studied variables.

Conclusions

The present study calculated the RI for hematology, serum chemistry, and some clinical variables from free-ranging Chinese Pangolins. There were no significant differences in clinical, hematology, and serum chemistry variables between male and female Chinese Pangolins, except in the body weight. Therefore, separate RI for males and females are not necessary, and the RI determined for the whole population studied can be used as the RI for the species. However, significant seasonal and age group differences in some of the variables were observed, which should be taken into consideration when making comparisons for health evaluation purposes.

These RI determined in this study will be useful to zoos, animal rescue centers, and other facilities that handle and provide health care to Pangolins, and therefore will contribute toward the conservation of this endangered mammal. In addition, these findings may act as a guide for expected values in the other 7 Pangolin species as currently there are no available RI on hematology and serum chemistry variables for these species. Further research is necessary to collect and compare the findings between these 8 Pangolin species to determine if RI are similar or different, and then determine whether universal RI are appropriate.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Comparison of clinical, hematologic, and serum biochemistry variables between anesthetized male and female Chinese Pangolins.

Table S2. Comparison of clinical, hematologic, and serum biochemistry variables of anesthetized Chinese Pangolins between spring–summer and autumn–winter.

Table S3. Comparison of clinical findings, hematologic, and serum biochemistry variables between sub-adult and adult anesthetized Chinese Pangolins.