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Reference intervals for hematology, plasma biochemistry, and bone mineral density in captive *Ceratophrys cranwelli* (Anura: *Ceratophryidae*)

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Abstract

Hematology, plasma biochemistry, body composition, and bone mineral density (BMD) were analyzed for 30 captive *Ceratophrys cranwelli (C. cranwelli)* to establish the reference intervals. There was no significant difference between males and females in all blood routine tests. Blood biochemistry included 17 analytes, and only total bile acid (TBA), calcium (CA), and phosphorus (PHOS) showed significant differences. Male TBA levels were higher than females, while female CA and PHOS levels were higher than males. The body composition and BMD of males and females were similar, except for bone area, which showed a significant gender difference, with females having higher values than males. The data obtained in this study can help with the medical management of diseased individuals and serve as a reference for health assessments of future populations.

Keywords *Ceratophrys cranwelli*, Hematology, Biochemistry, Bone mineral density, Reference intervals

Introduction

Ceratophrys cranwelli (*C. cranwelli*) belongs to the order Anura, Ceratophryidae and genus Ceratophrys. Originally from South America, it is widespread in Argentina, Bolivia, and the Grand Canyon of Brazil (Reichle et al. 2004). As we all know, amphibians are often used as an important indicator of environmental quality (Hopkins 2007; Bancroft et al. 2008; Alton and Franklin 2017; Walls and Gabor 2019; Zhao et al. 2022). Amphibians are very sensitive to environmental influences such as changes in the aquatic environment and pollutants (Hopkins 2007). Artificial changes in biological habitats have had many negative impacts on amphibians. For example, the environment can affect the timing of the appearance of amphibian skeletons (Rose 2021), and environmental pollutants can affect reproductive success, leading to population decline (Walls and Gabor 2019). Amphibians are widely used as models to study environmental problems (Burlibaşa 2011). Therefore, establishing normal reference intervals for *C. cranwelli* hematology, plasma biochemistry, body composition, and bone mineral density (BMD) will help monitor population status and assess population health.

Additionally, *C. cranwelli* is popular with hobbyists for its unique large mouth and is currently one of the most common pet frogs in the world. As a pet frog, *C. cranwelli* is susceptible to various diseases common in amphibians, such as red leg syndrome, flavobacteriosis, mycobacteriosis and chlamydiosis, chytridiomycosis, parasitic disease,

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nutritional deficiency diseases (Densmore and Green 2007). It is not recommended to administer medication without checking for illness. Only a correct diagnosis can guide treatment, and a blood test is an important tool for an accurate diagnosis. Reptiles and amphibians are susceptible to metabolic bone diseases (Klaphake 2010), and checking body composition and BMD can monitor the nutritional status of frogs.

The reported blood reference intervals for frogs such as *X. laevis* (Anura: Pipidae) (Chang et al. 2015), *L. Caerulea* (Anura: Hylidae), and *L. infrafreta* (Anura: Hylidae) (Young et al. 2012) and *R. catesbeiana* (Anura: Ranidae) (Peng et al. 2016) are not only conducive for treating frog diseases but also add information to the amphibian hematology and its evolution and the development process. *C. cranwelli* hematology, plasma biochemistry, body composition, and BMD reference intervals have not been reported. Therefore, it is very important to establish normal reference intervals for these elements in *C. cranwelli* (Bentley 1984; Elmberg 1991; McWilliams 2008). As such, this study aimed to determine reference intervals for hematology, plasma biochemistry, body composition, and BMD of captive *C. cranwelli*.

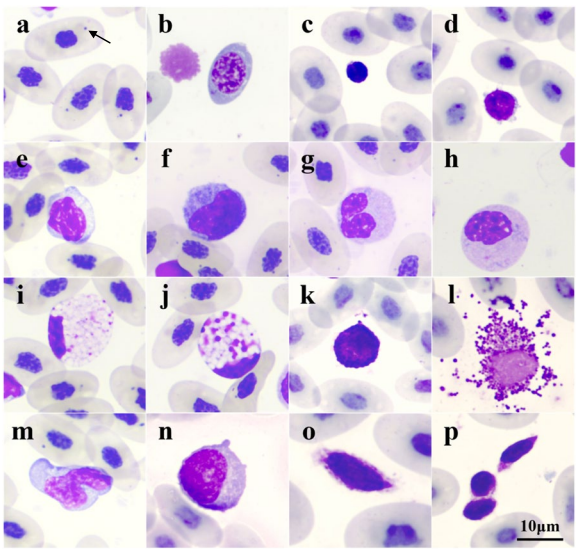

Fig. 1 Blood cell types in *C. cranwelli*. **a** Erythrocytes containing strongly basophilic particles (arrow). **b** Immature erythrocyte. **c**, **d** Small lymphocyte. **e** Large lymphocyte. **f** Lymphocytes with coarse particles. **g**, **h** Neutrophil. **i**, **j** Eosinophil. **k**, **l** Basophil. **m**, **n** Monocyte. **o**, **p** Thrombocyte

Results

We sampled 30 individuals of *C. cranwelli*, 10 males, and 20 females. Gender identification was determined by the presence or absence of nuptial pads on the thumbs of the forelimbs. Males possessed black nuptial pads, while females did not. Both body weight and Snout-vent length (SVL) were measured. The data revealed that females were larger and heavier than males. The body weight of the males averaged 108.70 ± 21.75 g (Mean ± SD), while that of the females averaged 115.40 ± 21.61 g. The SVL measured 8.48 ± 0.56 cm and 9.01 ± 0.61 cm in males and females, respectively.

Hematology

Regarding hematology, erythrocytes were observed to be oval with oval nuclei. The margins of the nuclei were irregular. The nuclei stained dark blue-purple using Wright-Giemsa, while the cytoplasm stained a very light pink. Occasionally, a strong basophilic granule was visible in the cytoplasm of erythrocytes (Fig. 1a). The length of the erythrocyte was measured to be 18.36 ± 1.19 μm, the width 11.09 ± 0.81 μm, and the length/width (L/W) ratio1.66 ± 0.15. No significant gender difference was found in terms of erythrocyte length (P = 0.371), width (P = 0.095), and L/W ratio (P = 0.096). The length and width of the nucleus were 5.85 ± 0.56 μm and 4.02 ± 0.37 μm, respectively. The sizes of the erythrocytes and nuclei were measured as 159.58 ± 17.22 μm^2 and 18.45 ± 2.33 μm^2, respectively, resulting in a nucleoplasmic ratio of 0.117 ± 0.016. Specific data can be found in Table 1.

Leukocytes were also observed to be similar to other amphibians, including neutrophils, lymphocytes, eosinophils, basophils, and monocytes. Among them, lymphocytes were the most numerous leukocytes, accounting for 71.33 ± 6.81%. Basophils accounted for 9.41 ± 3.24%, neutrophils 8.62 ± 3.32%, eosinophils 8.01 ± 3.30%, and monocytes the least at 2.46 ± 1.12%. There was no significant gender difference in the WBC differential counts. The neutrophil to lymphocyte ratio (N: L) was 0.12 ± 0.06.

Lymphocytes can be divided into small and large lymphocytes. The most common type is small lymphocytes, characterized by round nuclei stained dark bluish-purple with Wright-Giemsa. Small lymphocytes have a high nucleocytoplasmic ratio and minimal cytoplasm, and often exhibit outward projections of the cell membrane resembling pseudopodia (Fig. 1c, d). Large lymphocytes are less common and have oval or irregularly shaped nuclei that appear dark purple. The cytoplasm lymphocytes are looser than that of small lymphocytes and appear abundant and light blue (Fig. 1e). Occasionally, large granular lymphocytes with eccentric nuclei, dense dark purple-stained chromatin, darker blue-purple, and rough cytoplasm can be observed (Fig. 1f).

Neutrophils have round nuclei, often divided into 2–3 lobes and stained purple. Occasionally, the nuclei are oval (Fig. 1h). The cytoplasm of neutrophils is stained light bluish violet interspersed with some violet granules.

Table 1 Descriptive statistics and significant analysis in erythrocytes and nuclei

Variable	n	Mean ± SD	Median	Min	Max	RI (95% CI)	*P* value	*P* value (sex)
L	**500**	**18.36 ± 1.19**	**18.31**	**14.29**	**23.11**	**18.32–18.41**	**< 0.001**	**0.371**
♂	250	18.38 ± 1.20	18.37	14.29	23.11	18.30–18.45	0.100	
♀	250	18.36 ± 1.18	18.28	14.64	22.99	18.31–18.41	< 0.001	
W	**500**	**11.09 ± 0.81**	**11.06**	**8.00**	**13.00**	**11.06–11.11**	**0.010**	**0.095**
♂	250	11.05 ± 0.78	11.01	8.50	12.97	11.00–11.10	0.200	
♀	250	11.11 ± 0.82	11.08	8.01	12.99	11.07–11.14	0.020	
L/W	**500**	**1.66 ± 0.15**	**1.65**	**1.21**	**2.40**	**1.66–1.67**	**< 0.001**	**0.096**
♂	250	1.67 ± 0.16	1.66	1.28	2.27	1.66–1.68	0.005	
♀	250	1.66 ± 0.15	1.65	1.21	2.40	1.65–1.67	< 0.001	
NL	**500**	**5.85 ± 0.56**	**5.81**	**4.33**	**7.75**	**5.80–5.90**	**0.004**	**0.991**
♂	250	5.86 ± 0.57	5.84	4.49	7.75	5.79–5.93	0.004	
♀	250	5.84 ± 0.56	5.80	4.33	7.53	5.77–5.91	0.358	
NW	**500**	**4.02 ± 0.37**	**4.02**	**3.07**	**5.27**	**3.99–4.05**	**0.074**	**0.088**
♂	250	4.02 ± 0.36	4.02	3.19	5.27	3.98–4.06	0.053	
♀	250	4.02 ± 0.38	4.03	3.07	5.07	3.97–4.06	0.334	
NL/NW	**500**	**1.47 ± 0.21**	**1.44**	**1.01**	**2.36**	**1.45–1.49**	**< 0.001**	**0.677**
♂	250	1.471 ± 0.21	1.44	1.04	2.31	1.44–1.50	< 0.001	
♀	250	1.469 ± 0.21	1.45	1.01	2.36	1.45–1.50	0.001	
S	**500**	**159.58 ± 17.22**	**157.93**	**117.35**	**220.36**	**158.06–161.09**	**< 0.001**	**0.740**
♂	250	160.13 ± 16.9	157.40	117.35	220.36	158.02–162.24	< 0.001	
♀	250	159.01 ± 17.55	158.43	118.67	210.32	156.83–161.20	0.330	
NS	**500**	**18.45 ± 2.33**	**18.37**	**13.13**	**26.32**	**18.25–18.66**	**0.001**	**0.982**
♂	250	18.48 ± 2.25	18.37	13.56	25.27	18.20–18.76	0.002	
♀	250	18.42 ± 2.42	18.39	13.13	26.32	18.12–18.72	0.103	
NS/S	**500**	**0.117 ± 0.016**	**0.116**	**0.07**	**0.17**	**0.115–0.118**	**0.404**	**0.637**
♂	250	0.117 ± 0.017	0.116	0.07	0.16	0.115–0.119	0.509	
♀	250	0.116 ± 0.015	0.116	0.07	0.17	0.114–0.118	0.924	

Length and width in μm and size in μm^2

Abbreviations: *CI* Confidence interval, *L* Erythrocyte length, *W* Erythrocyte width, *L/W* Length/width, *NL* Nucleus length, *NW* Nucleus width, *S* Erythrocyte size, *Max* Maximum, *Min* Minimum, *NS* Nucleus size, *NS/S* Nucleocytoplasmic ratio, *n* Cell number, *SD* Standard deviation, *RI* Reference interval

Eosinophils are round, usually larger than neutrophils, and easily distinguished from other leukocytes. The nuclei are round or oval, often located on one side of the cell, and are purple. Coarse eosinophilic granules are found in the cytoplasm, but these do not stain well and are often stained pale pink (Fig. 1i). Vacuolated eosinophils, or eosinophils with both vacuoles and eosinophilic granules, are also commonly seen in the blood smear (Fig. 1j).

Basophils are dark blue-purple, and the nucleus is in the center of the cell, which is round or oval. Nuclei are often covered with aggregated basophilic granules (Fig. 1k). It was also observed that the basophilic particles spread out like a network (Fig. 1l).

There are few monocytes with kidney-shaped or horseshoe-shaped nuclei. The nucleus is looser than other leukocytes and is stained purple, while the cytoplasm is more uniform and delicate and stained blue-gray (Fig. 1m, n). Monocytes are sometimes confused with large lymphocytes (Fig. 1m), but the staining of the nucleus and cytoplasm can distinguish this.

Thrombocytes are spindle-shaped with oval nuclei stained dark purple. The cytoplasm is stained purple, stronger at the cell poles (Fig. 1o). Unactivated thrombocytes are easily mistaken for small lymphocytes. As shown in Fig. 1p, the thrombocyte in the center resembles a naked-nucleated small lymphocyte. However, it can be distinguished by the color of the cytoplasm.

The total cell count includes erythrocytes, leukocytes, and thrombocytes. In the Natt-Herrick staining solution, the morphological characteristics of erythrocytes are easily identified, and erythrocytes are stained in lavender. Leukocytes and thrombocytes are stained dark purple. Different types of leukocytes can be seen, but they cannot be distinguished precisely. Small lymphocytes and thrombocytes are easily confused when counted but can be better distinguished after an h of staining. Thrombocytes can be recognized by their U-shaped nuclei with

abundant cytoplasm. The counts of erythrocytes (RBCs), leukocytes (WBCs), and thrombocytes all conformed to the normal distribution, which were 0.85 ± 0.13 10^{12}/L, 6.72 ± 1.89 10^{9}/L, and 31.67 ± 8.79 10^{9}/L, respectively.

The hematocrit (HCT) corresponded to the normal distribution ($P=0.973$), which was 33.01 ± 4.95%. There was no significant gender difference between the sexes ($P=0.113$). Males and females had HCT levels of 35.05 ± 3.74% and 32 ± 5.25%, respectively. Hemoglobin (HGB) was normally distributed ($P=0.054$), 139.55 ± 38.03 g/L in males and 118.9 ± 46.22 g/L in females. There was no significant gender difference ($P=0.120$). Specific hematological data is presented in Table 2.

Plasma biochemical

The VS2 biochemical analyzer analyzed 13 blood indexes, but the total bile acid (TBA) and uric acid (UA) values were Zero. The reason could be that the levels were too low to measure the specific values. To measure these two plasma values, other biochemical analyzers (BS-240VET, Mindray, Shenzhen, China) were used for detection. The biochemical plasma values are listed in Table 3. All data except creatine kinase (CK) ($P=0.033$), creatinine (CREA) ($P=0.018$), TBA ($P=0.001$), and albumin (ALB) ($P=0.022$) conformed to the normal distribution. Across all data, only calcium (CA) ($P<0.001$), phosphorus (PHOS) ($P=0.002$), and TBA ($P=0.003$) showed significant gender differences. The contents of CA and PHOS in females were higher than in males. CA and PHOS were 8.52 ± 1.01 mg/dL and 4.31 ± 0.55 mg/dL in males and 9.90 ± 0.75 mg/dL and 5.00 ± 0.50 mg/dL in females, respectively. CA: PHOS was 1.99 ± 0.18, which showed no significant gender difference ($P=0.972$). Male TBA was higher than female, 6.85 ± 3.89 μmol/L and 3.05 ± 3.15 μmol/L, respectively.

Body composition and bone mineral density

Except for Fat% ($P=0.003$), Fat in tissue ($P=0.003$), and Lean% ($P=0.004$), the other indicators were in line with normal distribution. Only bone area ($P=0.049$) had significant gender-specific differences. Females were higher than males, 29.98 ± 3.53 cm^2 and 27.67 ± 3.13 cm^2, respectively (Table 4). It is believed that this may be due to the larger size of the females. The acquired images are shown in Fig. 2, in which the distribution of the various components can be seen.

Discussion

We established reference intervals for hematology, plasma biochemistry, body composition, and BMD in *C. cranwelli* at 12–14 months of age. *C. cranwelli* erythrocytes are oval with nuclei. Males were observed to be slightly longer than females and slightly shorter in width than females. However, there was no significant gender difference in erythrocyte length and width, which is not true for all species. Female erythrocyte length is longer than males in *P. teraiensis* (Anura: Rhacophoridae) (Das and Mahapatra 2014), *R. rugulos* (Anura: Dicroglossidae) (Chen et al. 2022), and *R. macrocnemis* (Anura: Ranidae) (Arserim and Mermer 2008), indicating that the gender relationship of erythrocytes differs between species. It has also been reported that erythrocytes are related to metabolism (Wojtaszek and Adamowicz 2003). More active species have been shown to have smaller erythrocytes, and species with lower oxygen consumption have larger erythrocytes (Arserim and Mermer 2008; Fathinia et al. 2020).

Different species have different RBCs. As shown in Table 5, the RBCs of *C. cranwelli* were higher than in most collected species but lower than in *L. podicipinus* (Anura: Leptodactylidae), *P. viridis* (Anura: Bufonidae), *R. rugulosa* (Anura: Dicroglossidae) and *X. tropicalis* (Anura: Pipidae). RBCs are seasonally correlated. *B. bombina (L)* had higher RBCs in spring than summer and autumn (Wojtaszek and Adamowicz 2003). Seasonal differences may be related to metabolism, with metabolic activity increasing from the end of hibernation to awakening, leading to the highest RBCs being reached in spring (Arserim and Mermer 2008). A study of *R. catesbeiana* during the hibernation period (January) and the active period (May) found that the RBCs, HGB, and HCT during the hibernation period were higher than those during the active period (Peng et al. 2016). In addition, hematological indicators are related to the living environment of the species. Hence, the RBCs of terrestrial species are higher than those of semi-aquatic species, and the HCT and HGB of terrestrial species are higher than those of semi-aquatic and aquatic species (Gul et al. 2011).

HCT is related to the size of erythrocytes, and smaller erythrocytes often have higher HCT. However, the relationship between them is not linear but curvilinear (Forzán et al. 2016), which is similar to the results of this study. It has been reported that erythrocytes become progressively smaller with increasing height (Baraquet et al. 2013; Fathinia et al. 2020). In a study of *Bufo gargarizans* (Anura: Bufonidae), elevation differences were found to result in increases in HGB, HCT, and RBCs, increased oxygen affinity of hemoglobin, and smaller erythrocytes (Pu et al. 2021). A similar situation was found when studying *N. parkeri* (Anura: Dicroglossidae) at high and low altitudes (Niu et al. 2022).

After summarizing 19 species, the relationship between RBCs, HCT, and length is shown in Fig. 3, and a slight trend can be seen: with the increase of RBCs, HCT also

Table 2 Hematology variables of *C. cranwelli*

Variable	n	Mean ± SD	Median	Min	Max	RI (90% CI)	*P* value	*P* value (sex)
RBCs (10^{12}/L)	**30**	**0.85 ± 0.13**	**0.85**	**0.60**	**1.04**	**0.81–0.89**	**0.356**	**0.802**
♂	10	0.84 ± 0.12	0.85	0.68	1.04	0.78–0.90	0.851	
♀	20	0.86 ± 0.13	0.85	0.60	1.03	0.81–0.90	0.303	
HCT (%)	**30**	**33.01 ± 4.95**	**33.15**	**23.57**	**44.17**	**31.48–34.55**	**0.973**	**0.113**
♂	10	35.05 ± 3.73	35.47	30.00	40.80	33.22–37.02	0.451	
♀	20	32.00 ± 5.25	31.59	23.57	44.17	30.23–33.78	0.880	
HGB (g/L)	**30**	**125.79 ± 44.12**	**117.91**	**61.16**	**260.94**	**112.10–139.47**	**0.054**	**0.120**
♂	10	139.55 ± 38.03	134.58	81.87	196.72	120.29–158.66	0.886	
♀	20	118.90 ± 46.22	102.28	61.16	260.94	103.13–137.38	0.010	
MCV (fL)	**30**	**395.88 ± 81.20**	**401.37**	**231.05**	**595.10**	**370.69–421.07**	**0.484**	**0.176**
♂	10	424.54 ± 81.57	426.31	288.46	595.10	384.49–466.19	0.326	
♀	20	381.54 ± 79.14	387.62	231.05	570.71	352.86–410.41	0.700	
MCHC (g/dL)	**30**	**378.91 ± 113.46**	**357.48**	**250.66**	**717.54**	**347.32–414.65**	**0.008**	**0.287**
♂	10	395.85 ± 91.25	397.15	262.98	543.42	351.03–440.73	0.925	
♀	20	370.43 ± 124.41	341.12	250.66	717.54	330.60–420.99	0.005	
MCH (pg)	**30**	**1507.69 ± 585.39**	**1406.89**	**621.24**	**3526.28**	**1343.61–1696.62**	**0.008**	**0.075**
♂	10	1683.53 ± 496.94	1749.49	871.01	2522.04	1426.02–1919.38	0.941	
♀	20	1419.77 ± 617.77	1304.45	621.24	3526.28	1219.72–1671.23	0.001	
WBCs (10^9/L)	**30**	**6.72 ± 1.89**	**6.41**	**3.48**	**10.90**	**6.13–7.31**	**0.296**	**0.342**
♂	10	6.25 ± 2.20	5.67	3.48	10.70	5.19–7.34	0.483	
♀	20	6.96 ± 1.73	6.63	4.44	10.90	6.41–7.66	0.389	
Thrombocytes (10^9/L)	**30**	**31.67 ± 8.79**	**30.00**	**16.67**	**53.33**	**28.94–34.39**	**0.367**	**0.145**
♂	10	35.00 ± 10.80	31.67	16.67	53.33	30.00–40.00	0.843	
♀	20	30.00 ± 7.33	30.00	16.67	43.33	27.33–32.50	0.600	
Neutrophil (%)	**30**	**8.62 ± 3.32**	**7.42**	**4.33**	**18.00**	**7.71–9.62**	**0.003**	**0.586**
♂	10	9.10 ± 3.20	8.59	4.83	15.67	7.55–10.85	0.764	
♀	20	8.38 ± 3.44	6.67	4.33	18.00	7.19–9.70	0.002	
Lymphocyte (%)	**30**	**71.33 ± 6.81**	**72.42**	**56.00**	**84.17**	**69.21–73.44**	**0.903**	**0.221**
♂	10	69.15 ± 6.45	68.17	61.50	81.50	66.10–72.55	0.201	
♀	20	72.42 ± 6.88	74.00	56.00	84.17	69.81–74.69	0.195	
Eosinophi (%)	**30**	**8.15 ± 3.42**	**7.42**	**3.00**	**15.67**	**7.17–9.21**	**0.147**	**0.111**
♂	10	9.57 ± 3.87	9.00	3.33	14.17	7.60–11.35	0.301	
♀	20	7.44 ± 3.04	7.17	3.00	15.67	6.43–8.55	0.136	
Basophil (%)	**30**	**9.41 ± 3.24**	**9.00**	**2.67**	**17.17**	**8.40–10.41**	**0.351**	**0.321**
♂	10	10.25 ± 2.87	9.58	7.00	16.83	8.97–11.76	0.151	
♀	20	8.98 ± 3.40	8.67	2.67	17.17	7.81–10.27	0.757	
Monocyte (%)	**30**	**2.46 ± 1.12**	**2.25**	**1.17**	**5.50**	**2.12–2.83**	**0.005**	**0.066**
♂	10	1.93 ± 0.61	2.00	1.17	2.67	1.63–2.25	0.145	
♀	20	2.73 ± 1.23	2.42	1.17	5.50	2.28–3.19	0.089	
N: L	**30**	**0.12 ± 0.06**	**0.10**	**0.05**	**0.26**	**0.11–0.14**	**0.004**	**0.268**
♂	10	0.14 ± 0.05	0.13	0.06	0.23	0.11–0.16	0.799	
♀	20	0.12 ± 0.06	0.09	0.05	0.26	0.10–0.14	0.002	

Abbreviation: *N: L* Neutrophil: lymphocyte

increases, accompanied by a decrease in the length of erythrocytes. However, when RBCs are between 0.45 and 0.55, both the HCT and the length of the erythrocytes are low, suggesting that the relationship between them is non-linear rather than linear.

In *C. cranwelli*, also found in other reptiles and amphibians, small, discrete basophilic inclusions in the cytoplasm of erythrocytes are occasionally observed, which is considered a normal physiological phenomenon, and it has been suggested that this structure may

Table 3 Plasma biochemical values of *C. cranwelli*

Analyte	Mean ± SD	Median	RI (90% CI)	*P* value	Mean ± SD ♂	Mean ± SD ♀	*P* value (sex)
ALT(U/L)[a]	7.13 ± 4.51	6.25	5.73–8.53	0.068	7.95 ± 5.02	6.73 ± 4.32	0.493
ALP(U/L)[a]	136.80 ± 42.42	132.75	123.62–149.94	0.570	137.9 ± 44.83	136.23–42.35	0.921
AST (U/L)	76.87 ± 27.36	72.00	68.38–85.35	0.305	86.1 ± 26.55	72.25 ± 27.23	0.196
TBA (μmol/L)[a]	0.89 ± 1.10	0.40	0.57–1.21	< 0.001	1.68 ± 1.40	0.50 ± 0.66	0.009
CK (U/L)	604.70 ± 410.91	582.50	482.60–719.31	0.033	796.70 ± 393.40	508.70–394.09	0.075
UA (mg/dL)[a]	1.24 ± 0.42	1.34	1.11–1.37	0.332	1.39 ± 0.49	1.17 ± 0.37	0.164
GLU (mg/dL)	68.57 ± 11.82	66.00	64.90–72.23	0.455	66.10 ± 6.56	69.80 ± 13.71	0.428
UREA (mmol/L)[a]	19.44 ± 6.74	18.85	17.64–21.56	0.012	22.07 ± 7.30	18.13 ± 6.22	0.102
CREA (μmol/L)[a b]	162.23 ± 17.25	154.50	154.27–171.18	0.018	-	-	-
CA (mg/dL)	9.44 ± 1.06	9.45	9.11–9.77	0.390	8.52–1.01	9.9–0.75	< 0.001
PHOS (mg/dL)	4.77 ± 0.61	4.85	4.59–4.96	0.265	5.03 ± 0.47	4.65 ± 0.64	0.002
CA: PHOS	1.99 ± 0.18	2.02	1.93–2.05	0.398	1.99 ± 0.22	1.99 ± 0.17	0.972
TP (g/dL)	3.61 ± 0.47	3.60	3.47–3.76	0.406	3.53 ± 0.53	3.66 ± 0.44	0.497
ALB (g/dL)	0.78 ± 0.17	0.80	0.73–0.84	0.022	0.76 ± 0.17	0.80 ± 0.17	0.703
GLOB (g/dL)	2.82 ± 0.31	2.80	2.73–2.92	0.656	2.76 ± 0.36	2.86 ± 0.30	0.446
K (mmol/L)	2.66 ± 0.73	2.60	2.44–2.89	0.614	2.79 ± 0.80	2.6 ± 0.71	0.152
Na (mmol/L)	108.73 ± 2.72	109.00	107.89–109.58	0.643	108.50 ± 3.50	108.85 ± 2.32	0.429

The total sample was 30, with 10 males and 20 females

[a] Represents the measurement with Mindray (BS-240VET). The unmarked ones are measured by Abaxis (VS2)

[b] Represents analytes with less than 30 samples. CREA = 11

Table 4 Body composition and BMD of *C. cranwelli*

Contents	Mean ± SD	Median	Min	Max	RI (90% CI)	*P* value	*P* value (sex)
BMC (%)	**3.01 ± 0.31**	**2.94**	**2.41**	**3.83**	**2.92–3.10**	**0.728**	0.779
♂	3.01 ± 0.36	2.92	2.65	3.83	2.85–3.21	0.026	
♀	3.01 ± 0.29	3.07	2.41	3.48	2.91–3.11	0.246	
FAT (%)	**25.40 ± 2.69**	**24.81**	**21.50**	**35.08**	**24.67–26.22**	**0.003**	0.983
♂	25.27 ± 1.82	24.99	22.25	28.23	24.38–26.18	0.508	
♀	25.46 ± 3.08	24.81	21.50	35.08	24.41–26.62	0.014	
LEAN (%)	**71.59 ± 2.80**	**72.05**	**61.72**	**75.66**	**70.73–72.36**	**0.004**	0.812
♂	71.72 ± 1.92	71.82	68.70	75.10	70.75–72.70	0.455	
♀	71.53 ± 3.20	72.24	61.72	75.66	70.25–72.58	0.017	
FAT In Tissue (%)	**26.19 ± 2.80**	**25.53**	**22.13**	**36.24**	**25.43–27.04**	**0.003**	0.914
♂	26.06 ± 1.89	25.75	22.86	29.08	25.13–27.00	0.482	
♀	26.25 ± 3.20	25.53	22.13	36.24	25.18–27.49	0.014	
BMD(g/cm^2)	**0.112 ± 0.008**	**0.112**	**0.100**	**0.130**	**0.109–0.115**	**0.487**	0.623
♂	0.113 ± 0.01	0.115	0.100	0.130	0.108–0.118	0.594	
♀	0.111 ± 0.008	0.110	0.100	0.120	0.109–0.114	0.334	
Bone Volume(cm^3)	**1.98 ± 0.31**	**1.93**	**1.53**	**2.65**	**1.89–2.07**	**0.192**	0.311
♂	1.90 ± 0.33	1.88	1.53	2.53	1.75–2.07	0.426	
♀	2.02 ± 0.29	1.96	1.66	2.65	1.92–2.13	0.059	
Bone Area(cm^2)	**29.21 ± 3.52**	**28.03**	**23.75**	**36.21**	**28.20–30.20**	**0.083**	0.049
♂	27.67 ± 3.13	26.81	24.59	34.98	26.20–29.18	0.830	
♀	29.98 ± 3.52	29.55	23.75	36.21	28.76–31.27	0.966	

The total sample was 30, with 10 males and 20 females

Abbreviations: *BMC* Bone mineral contents, *BMD* Bone mineral density, *fat* fat contents, *lean* lean body contents

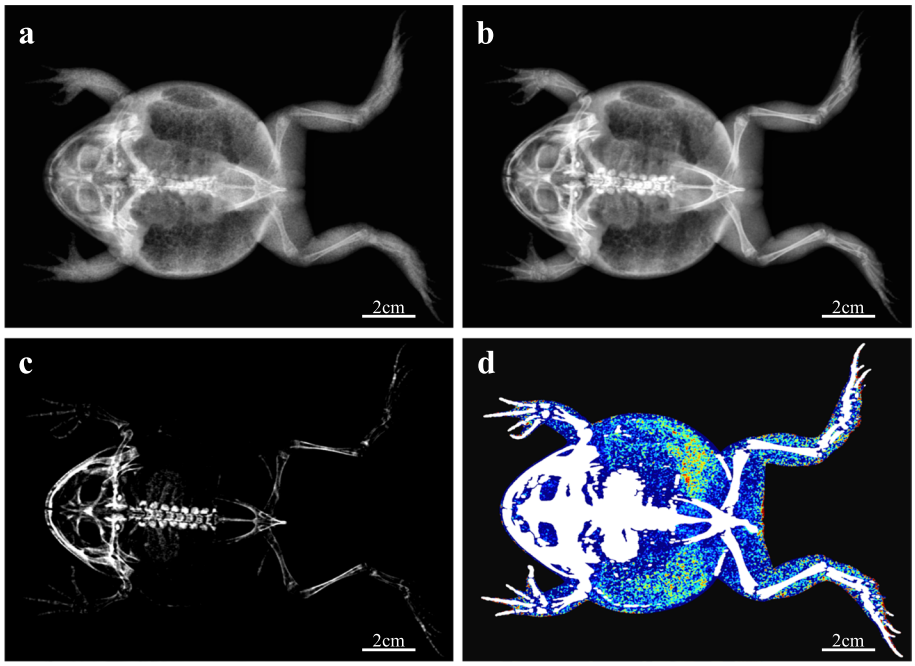

Fig. 2 Images of *C. cranwelli* by dual-energy X-ray absorptiometry. **a** High body image, **b** Low body image, **c** Bone image, **d** Composition image (blue and green areas: mix lean and liquid; red areas: fat contents; white areas: bone)

represent a degenerate organelle (Chung et al. 2009). Some researchers believe this to be the same Heinz body observed in humans, a product of hemoglobin precipitation (Basile et al. 2011). The study of Chrysemys picta found that basophilic inclusion bodies may be related to the maturation process of erythrocytes, and there was a tendency for expression in older erythrocytes (Davis and Holcomb 2008).

The results of the WBC differential count of *C. cranwelli* and other frogs are shown in Fig. 4. Among the taxonomic proportions, *C. cranwelli* had the highest proportion of lymphocytes, similar to that of other frogs, suggesting that lymphocytes were among the most important leukocytes in amphibians. Like *L. podicipinus*, *L. luctator*, *R. clamitans,* and *R. sylvatica*, *C. cranwelli* has a lower proportion of neutrophils, but neutrophils present the highest proportion of leukocytes in *X tropicali*s. In addition, monocytes represented the lowest percentage of leucocytes in this study, while other frogs (Fig. 4) have the lowest proportion of basophils or eosinophils. Furthermore, statistics showed that the observed values of eosinophils and basophils of *L. infrafrenata* were all 0, indicating a significant difference in the WBC differential counts between different species. It should be noted that while there is a difference in blood indices between wild and captive amphibians (Sabrina et al. 2011), most of the data collected in the existing studies are from field-captured frogs, so all of the above differences are also possibly related to animal sources.

The number of leukocytes varies between species and can be influenced by many other factors, including developmental stage, age, sex, season, reproductive stage, wild versus captive, diet, ambient temperature, and humidity (Allender and Fry 2008). RBCs, HCT, and HGB of Australian tree frogs were higher in the wet season than in the dry season. RBCs, PCV, and HGB changes have been hypothesized to be due to increased hematopoietic capacity in warmer environments (Young et al. 2012). Temperature can influence the number and proportion of leukocytes in the body. Temperature significantly impacted lymphocytes and eosinophils, while neutrophils negatively correlated with temperature. Lymphocytes, eosinophils, and neutrophils responded to seasonal changes, and the response of lymphocytes to short-term temperature changes was delayed. Temperature and season did not significantly affect basophils (Maniero and Carey 1997; Raffel et al. 2006). The dimensions can affect the absolute leukocyte count. As such, lymphocytes and monocytes increase towards the north, while eosinophils, neutrophils, and basophils decrease (Sacchi et al. 2020).

The ratio of neutrophils to lymphocytes (N: L) is often considered useful for assessing stress levels in amphibians (Davis et al. 2008). For example, amputations, breeding, continuous lighting, osmotic stress, and other surgeries can all lead to an increase in neutrophils and a

Table 5 Comparison of some hematological parameters in different species

Family	Species	n	RBC	L	W	HCT	Coordinate	Altitude
Ceratophryidae	*C. cranwelli*	30	0.85	18.36	11.09	33.01	30°28′29″N 114°21′24″E	< 100 m
Bombinatoridae	*B. bombina (L)*	10–12♂	0.34	22.08	14.74	20.00	-	< 300 m
		17–19♀	0.29	-	-	19.00	-	
Bufonidae	*R. fernandezae*	23	0.51	16.42	11.43	27.37	31°40′29″S 60°20′13″W 31°38′26″S 60°40′22″W	-
	P. viridis	4♂	0.98			58.5		
		6♀	0.94			43.75		
Dicroglossidae	*N. parkeri*	8	0.51	16.44	12.25	21.07	-	3400 m
		8	0.62	16.54	12.15	28.16	-	4600 m
	R. rugulosa	15♂	1.12	14.61	9.83	-	31°51′43″N 117°15′25″E	< 100 m
		15♀	1.33	15.09	10.60	-		
Hylidae	*H. arborea*	4♂	0.65			45.00	-	-
		11♀	0.73			50.18	-	-
	H. cordobae	10	-	23.42	15.18	-	33°09′S 64°W	808 m
		21	-	23.66	15.06	-	32°S 64°W	930 m
		15	-	22.93	14.62	-	32°48′S 66°05′W	1634 m
		9	-	21.62	15.35	-	32°23′S 64°55′W	2107 m
		6	-	22.41	15.04	-	31°49′S 64°51′W	2150 m
		5	-	21.14	13.85	-	32°00′S 64°56′W	2310 m
	L. caerulea	80	0.74	19.00	12.00	38.00	16°55′32″S 145°46′31″E	< 200 m
	L. infrafreta	66	0.72	18.00	11.00	30.00	19°15′S 146°49′E	< 100 m
Leptodactylidae	*L. luctator*	30	0.73	-	-	21.00	35°08′15.87″S 57°23′35.21″O	-
	L. podicipinus	22	3.31	-	-	13.75	19°34′37″S 57°00′42″W	< 200 m
Pelodytidae	*P. caucasicus*	20	0.78	15.29	9.68	-	40°36′56″N 40°18′07″E	1090 m
Pelobatidae	*P. syriacus*	13	0.75			40.00		
Pipidae	*X. laevis*[c]	10	0.80	17.50	10.50	36.90	-	-
	X. laevis[d]	166	0.76	-	-	36.90	-	-
		109	1.10	-	-	49.40	-	-
		20	0.67	-	-	41.20	-	-
	X. laevis[e]	52	1.22	-	-	47.00	-	-
	X. tropicalis	33–41	1.5	13.10	9.90	40.80	46°12′0″N 63°12′0″W	< 100 m
Ranidae	*P. bedriagae*	25	0.25	23.50	13.63	-	51°34′E 30°13′N	900 m
		43	0.21	21.70	12.55	-	51°34′E 30°40′N	1810 m
	R. Catesbeiana[a]	35♂	0.25	25.70	15.20	20.24	-	-
		35♀	0.24	25.60	15.70	20.32	-	-
	R. catesbeiana[b]	302	0.42	24.20	16.20	30.10	-	-
	R. dalmatina	6♂	0.65	-	-	30.26	-	-
		9♀	0.72	-	-	34.91	-	-
	R. pipiens	20–56	0.32	24.00	17.00	24.65	-	-
	R. macrocnemis	15♂	0.51	22.32	13.65	32.00	40°07′964″N 29°06′753″E	1617 m
		15♀	0.24	23.03	14.59	35.00		-
	P. ridibundus	5♂	0.76	-	-	38.19	-	-
		5♀	0.89	-	-	43.19	-	-
	R. sylvatica	26–40	0.41	25.00	18.00	29.50	46°12′N 63°12′W	< 10 m
Rhacophoridae	*P. teraiensis*	40♂	0.59	16.87	8.95	50.62	20°31′11′′N 85°49′11′′E	< 200 m
		40♀	0.62	19.77	8.62	51.55		
	P. maculatus	40♂	0.48	18.64	12.86	28.65	28°18′N 85°50′E	< 100 m
		40♀	0.57	18.43	11.36	23.80		

A total of 19 species were collected

Source: *B. bombina (L.)* (Wojtaszek and Adamowicz 2003); *H. arborea* (Gul et al. 2011); *H. cordobae* (Baraquet et al. 2013); *L. caerulea* (Young et al. 2012); *L. infrafreta* (Young et al. 2012); *L. luctator* (Brodeur et al. 2020); *L. podicipinus* (Franco-Belussi et al. 2022); *N. parkeri* (Niu et al. 2022); *P. bedriagae* (Fathinia et al. 2020); *P. maculatus* (Mahapatra et al. 2012); *P. ridibundus* (Gul et al. 2011); *P. syriacus* (Gul et al. 2011); *P. teraiensis* (Das and Mahapatra 2014); *P. viridis* (Gul et al. 2011); *R. catesbeiana*[a] (Peng et al. 2016); *R. catesbeiana*[b] (Coppo et al. 2005);*P. caucasicus* (Arikan et al. 2003); *R. dalmatina* (Gul et al. 2011); *R. fernandezae* (Cabagna-Zenklusen et al. 2011); *R. macrocnemis* (Arserim and Mermer 2008); *R. pipiens* (Rouf 1969); *R. rugulosa* (Chen et al. 2022); *R. sylvatica* (Forzán et al. 2016); *X. tropicalis* (Maxham et al. 2016); *X. laevis*[c] (Hadji-Azimi et al. 1987); *X. laevis*[d] (Sabrina et al. 2011); *X. laevis*[e] (Chang et al. 2015)

Abbreviations: *a* Adult, *j* Juvenile

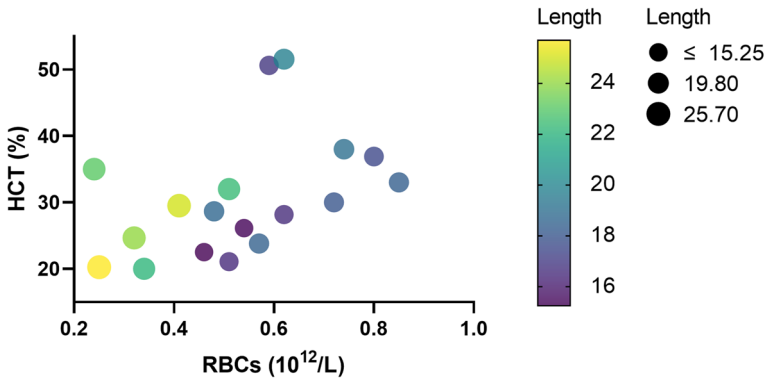

Fig. 3 Relationship between different species' RBCs, HCT, and erythrocyte length. 19 species, each dot represents a species. The size and color of the dots represent erythrocyte length

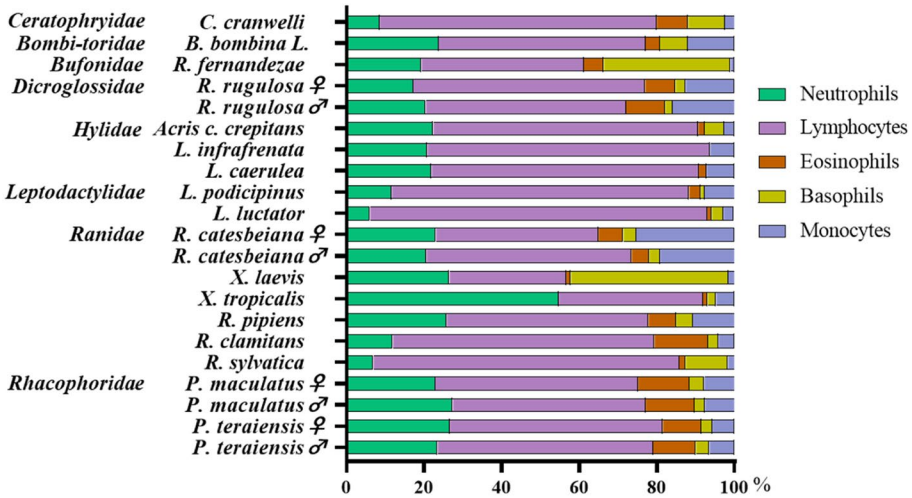

Fig. 4 The proportion of each type of leukocyte in different species. The discrete words on the far-left side of the vertical axis represent families, while the words that lie close to the axis represent specific species

decrease in lymphocytes in *X. laevis*, increasing the N:L ratio (Davis et al. 2008). Studies have shown that salamanders' N: L ratio is higher at high altitudes than at low altitudes, indicating that salamanders at high altitudes may experience greater physiological stress (Xiong et al. 2018). In addition, a study found that the total red blood cell count of wild *L. luctator* (Anura: Leptodactylidae) on the second day after capture was significantly higher than that of the blood immediately after capture and that this was accompanied by an increase in N: L (Brodeur et al. 2020), showing that the post-capture stress response alters the blood index, suggesting that wild amphibians should be bled immediately to obtain more accurate data. The results of our study showed that the N:L of *C. cranwelli* was 0.12 ± 0.06, which falls within the reported N: L range of amphibians (0.1–0.67) (Davis 2009) and on the lower end, indicating that after long-term captive breeding, *C. cranwelli* has adapted to a stable environment free of natural enemies, food competition, and other pressures, resulting in low-stress levels, which is further supported by the lower proportion of neutrophils in *C. cranwelli* compared to other species. Therefore, the N: L value can be used clinically to assess whether pet frogs are exposed to potential stress.

Regarding blood biochemistry results, only BA, CA, and PHOS showed significant differences between males and females, with BA being higher in males than females and CA and PHOS being higher in females than males. Although the levels of CA and PHOS differ between males and females, the CA: PHOS ratios remain the same

(Table 3). The CA: PHOS of *C. cranwelli* was 1.99 ± 0.18: 1, within the reported reference (1–2: 1) (Antwis et al. 2014), suggesting that *C. cranwelli*, which fed on low-calcium, high-phosphorus insects such as crickets, Blaptica dubia, and Tenebrio molitor, required additional calcium supplementation. Long-term consumption of foods low in calcium and high in phosphorus would lead to an imbalance of calcium and phosphate and cause other diseases. Notably, the CA: PHOS of *C. cranwelli* was similar to that of captive *X. laevis* but higher than that of *X. laevis* caught in the field, indicating that the CA: PHOS was different between the captive and wild-caught animals due to different food resources.

In this study, there was no significant difference in protein content between males and females. Previous research has shown that amphibians increase immune cell and protein production during winter, especially in low-temperature environments (Raffel et al. 2006). *R. catesbeiana* had higher immunoglobulin content in the dormant phase than in the active phase (Peng et al. 2016). The measurement methods used for blood protein content may also influence the results. For instance, the refraction method yielded higher total protein (TP) measurements compared to the biuresis method, while the bromocresol green dye binding method yielded lower ALB concentration compared to the electrophoresis method. Lower ALB concentration can lead to pseudo-high-calculated GLOB concentration (Lumeij and De Bruijne 1985; Young et al. 2012). This study used the VS2 automatic blood biochemical analyzer, which uses the biuret method to measure TP, and the bromocresol green dye combination method to measure ALB. However, it is important to note that there may be some errors in the obtained protein content due to the use of this analyzer. Despite this, the VS2 analyzer is commonly used in clinical practice due to its small blood requirement (100 μL), convenience, rapid detection, and lack of specialized amphibian instruments or reagents.

In *C. cranwelli*, there were no significant differences in body composition and BMD between males and females. The BMD of *C. cranwelli* (0.11) was the same as that of *P. nigromaculatus* (Anura: Ranidae) (0.11), higher than *H. japonica* (Anura: Hylidae) (0.03) and *G. rugose* (Anura: Ranidae) (0.09). and lower than *R. catesbeiana* (0.24). *C. cranwelli* had a higher BMC (3.01 ± 0.31) than *H. japonica* (1.9 ± 0.2) but lower than *G. rugose* (4.8 ± 0.01), *P. nigromaculatus* (3.8 ± 0.2) and *R. catesbeiana* (3.4 ± 0.3), as reported in previous studies (Park and Do 2019). DXA can analyze the body's composition, including animal muscle, fat, and bones, and then determine the body's nutritional status. It can also be used to study the relationship between frog movement patterns and habitat and BMD (Vera et al. 2020) and the relationship between BMC and BMD and food resources (Park and Do 2019). In veterinary practice, plasma calcium concentrations alone may not accurately reflect the level of mineralization in the body (Gramanzini et al. 2013). As shown in Fig. 5, we also found that there was no significant linear positive correlation between CA levels and BMD, BMC and BMC% ($P > 0.05$), indicating that they cannot be calculated using the CA content in the blood could be assessed. Our result is similar to Gramanzini's conclusion, which shows the importance of diagnosing metabolic bone disease (MBD) with DXA. MBD is one of the most common diseases in reptiles and amphibians (Klaphake 2010). The most typical symptom is bone deformity and fragility, which can lead to death in severe cases. As a non-invasive screening method, DXA is expected to be an important diagnostic tool for diagnosing and grading MBD in amphibians and reptiles.

Data on hematology, plasma biochemistry, and BMD of diseased *C. cranwelli* were unavailable; therefore, changes in these data under disease conditions were not included in the discussion. Using this data from healthy C. cranwelli will improve the accuracy of diagnosing frog diseases. More clinical cases are expected to be reported, and studies on frogs will continue.

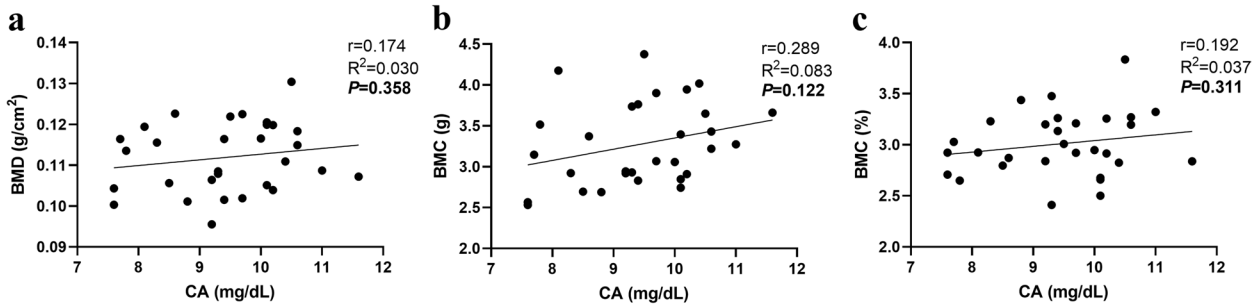

Fig. 5 The correlation between CA and some bone data. There was no significant linear positive correlation between CA and BMD, BMC and BMC % ($P > 0.05$)

Conclusions

This study reports data on the hematology, plasma biochemistry, and bone mineral density of *C. cranwelli*. The established reference interval can allow for hematological control of diseased *C. cranwelli*, which can be helpful in the diagnosis and treatment of the disease. The data obtained can also be useful in the health assessment of the wild population of *C. cranwelli* and even in monitoring changes in the habitat environment.

Materials and methods

Animal

A total of 50 *C. cranwelli* aged 2–4 months old were obtained from local farmers. We artificially reared them for 10 months at Huazhong Agricultural University Veterinary Teaching Hospital (30°28′29″ N/114°21′24″ E, altitude < 50 m). Each frog was reared individually in an amphibious breeding box (19 cm × 12.5 cm), and the ambient temperature and humidity were controlled (temperature 26–29 °C, humidity 60–80%, light and dark for 12 h each). The frogs received a commercial diet fortified with vitamins and calcium carbonate powder every other day, and the goldfish received diets once a week. The water in the rearing box was changed every two days or when feces were visible, and the health status of the frogs was monitored daily.

Randomly, 30 frogs were selected with good spirit and appetite for sampling and were fasted for 48 h prior to sampling. Isoflurane was used for respiratory anesthesia, and all surgical procedures were performed under general anesthesia. Before anesthesia, the frogs and the rearing box were transferred to the anesthesia box and injected with isoflurane gas directly. The frog typically takes 20 min to reach the anesthetized state and, after anesthesia, an operation lasting approximately 30 min. Snout-vent length (SVL) measurements and body weight were taken immediately after anesthesia.

Blood collection

Blood was collected following practice guidelines (Heatley and Johnson 2009). A cardiac puncture was performed using a 29-gauge insulin syringe. The amount of blood collected did not exceed 1% of body weight. The collected blood was immediately transferred into EDTA tubes and lithium heparin tubes. Thorough mixing was carried out to prevent blood clotting. The blood was then stored at 4 °C, and all tests were completed within 6 h.

WBC differential counts, hematocrit, and hemoglobin

Three blood smears were immediately prepared from the blood in the EDTA tube and were air-dried naturally. Subsequently, the blood smears were stained with Wright-Giemsa in the following h. Using a microscope and a cell counting device (ex20, SOPTOP, Hangzhou, China), 200 white blood cells were counted in each blood smear to determine the ratio of each leukocyte.

For hematocrit (HCT) measurement, 20 µl of blood was drawn from the EDTA tube with a 40 µl capillary and then centrifuged (10,000 g, 5 min) (SmartSpin-12, Topson, Ningbo, China). The HCT was read using a capillary reader.

Hemoglobin (HGB) levels were determined using the colorimetric cyanide methemoglobin (HICN) method. A volume of 10 µl of EDTA anticoagulated blood was collected from 2.5 ml of HGB diluent. Thorough mixing was performed, allowing the mixture to stand for 5 min. The absorbance was measured at 540 nm using a UV spectrophotometer (L6, YOKE, Shanghai, China), and three measurements were averaged.

Blood cell size

Each blood cell's morphology and staining properties were observed under an oil microscope, and images were taken with a camera (E3ISPM, Jingtong, Suzhou, China). Blood smears from each frog were taken at 10 fields of view. The measurements were performed in the Iamgeview software. From 30 frogs, 5 frogs of each sex are selected, and the specific data of 50 erythrocytes for each frog are measured and calculated, including cell length (L) and width (W), nucleus length (NL), and width (NW), cell size (S), nucleus size (NS).

The calculation uses the following formula: $S(\mu m^2) = \frac{LW}{4}\Pi$; nucleocytoplasmic ratio $= \frac{NS}{S}$.

Total cell counts

Ten (10) µl of EDTA anticoagulated blood was added to 1.99 ml of Natt-Herrick staining solution and was mixed well. The mixed solution was aspirated and filled into the counting chambers on both sides of the modified Neubauer counting plate and then placed in a wet gauze Petri dish and incubated for 5 min. The standard method was used for counting. Erythrocytes and thrombocytes were counted from five small squares (4 × 4) in the large square in the center of the counting board, the four corners, and the small square in the center, respectively. Leukocytes were counted from the 9 large squares at the four corners of the counting board. The process was repeated three times to find the average value. The following formula was used for calculation:

$$\text{RBCs}(10^{12}/\text{L}) = \text{Cell Counted} \times 0.01;$$

$$\text{WBCs}\left(10^{9}/\text{L}\right) = \frac{\text{Cell Counted} \times 200}{0.9};$$

$$\text{Thrombocytes}\left(10^{9}/\text{L}\right) = \text{Cell Counted} \times 2.$$

MCV, MCHC, and MCH are obtained by calculation according to the following formula:

$$\mathrm{MCV(fL)} = \frac{\mathrm{HCT(\%)}\times 10}{\mathrm{RBCs}(10^{12}/\mathrm{L})};$$
$$\mathrm{MCHC(g/dL)} = \frac{\mathrm{HGB(g/L)}\times 100}{\mathrm{HCT(\%)}};$$
$$\mathrm{MCH(pg)} = \frac{\mathrm{HGB(g/L)}\times 10}{\mathrm{RBCs}(10^{12}/\mathrm{L})}.$$

Plasma biochemical indicators

Blood anticoagulated with lithium heparin was immediately centrifuged (5000 g, 3 min) (2-16R, Hengnuo, Changsha, China), and 100 µl of plasma was collected for detection using an avian/ reptile biochemical disc (VS2, Abaxis, USA). Measured items included AST, TBA, CK, UA, GLU, CA, PHOS, TP, ALB, GLOB, K, NA. The remaining plasma was used to measure indicators not included in Abaxis, including ALT (ultraviolet-lactate dehydrogenase method), ALP (AMP buffer method), CREA (sarcosine oxidase method), UREA (oxidase method). The instrument used was an automated biochemical analyzer (BS-240VET, Mindray, Shenzhen, China).

Body composition and bone mineral density

Under anesthesia, body composition and BMD were measured using dual-energy X-ray absorptiometry (DXA) (Medikors, InAlyzer, Seongnam, Korea). Before measuring, absorb excess water from the skin's surface with gauze. Stretch the frog's limbs and shoot from the dorsal-ventral position. DXA emits high-energy and low-energy beams, dividing them into bone, fat, and lean areas according to the absorbed dose. The lean area is the part other than the fat area, which is a mixture of water and muscle. The following data were obtained: BMC (g), BMC (%), Fat (%), Fat in Tissue (%), Lean (%), BMD (g/cm^2), Bone Area (cm^2) and Bone Volume (cm^3).

Statistical analysis

Data analysis was performed with SPSS 27, and calculations followed the ASVCP reference manual (Friedrichs et al. 2012). According to the guidelines, a 90% CI should be used for a sample size 30 instead of a 95% CI. This study was based on a small sample, and the reference interval was not verified. All data was analyzed via Shapiro–Wilk normality tests. When data were normally distributed, parametric tests were used to determine reference intervals. When data were unavailable, a robust algorithm was used. The independent sample t-test was used to assess whether there was a difference between the two data sets. If $P < 0.05$, the result was considered significant.

Abbreviations

ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ALB	Albumin
AST	Aspartate aminotransferase
BA	Bile acid
BMC	Bone mineral content
BMD	Bone mineral density
CA: PHOS	Calcium and phosphate ratio
CK	Creatine kinase
CREA	Creatinine
DXA	Dual-energy X-ray absorptiometry
EDTA	Ethylene diamine tetraacetic acid
GLOB	Globulin
GLU	Glucose
HCT	Hematocrit
HGB	Hemoglobin
MBD	Metabolic bone disease
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
N: L	Neutrophils and lymphocytes ratio
RBC	Red blood cell
TP	Total protein
UA	Urine acid
UREA	Carbamide
WBC	White blood cell

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Authors' contributions

SL and YS mainly designed the experiment; SL, YQ, and SZ completed and collected the data; SL processed the data and wrote the manuscript; YS reviewed and revised it. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Experimental Animal Committee of Huazhong Agricultural University (ID number: 202210190004).

Consent for publication

Not applicable.

Competing interests

Yaoqin Shen was not involved in the journal's review or decisions related to this manuscript.

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