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Biochemical and Hematologic Reference Intervals for Aged *Xenopus laevis* in a Research Colony

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Xenopus laevis*, the African clawed frog, is commonly used in developmental and toxicology research studies. Little information is available on aged *X. laevis*; however, with the complete mapping of the genome and the availability of transgenic animal models, the number of aged animals in research colonies is increasing. The goals of this study were to obtain biochemical and hematologic parameters to establish reference intervals for aged *X. laevis* and to compare results with those from young adult *X. laevis*. Blood samples were collected from laboratory reared, female frogs (n = 52) between the ages of 10 and 14 y. Reference intervals were generated for 30 biochemistry analytes and full hematologic analysis; these data were compared with prior results for young *X. laevis* from the same vendor. Parameters that were significantly higher in aged compared with young frogs included calcium, calcium:phosphorus ratio, total protein, albumin, HDL, amylase, potassium, CO_2, and uric acid. Parameters found to be significantly lower in aged frogs included glucose, AST, ALT, cholesterol, BUN, BUN:creatinine ratio, phosphorus, triglycerides, LDL, lipase, sodium, chloride, sodium:potassium ratio, and anion gap. Hematology data did not differ between young and old frogs. These findings indicate that chemistry reference intervals for young *X. laevis* may be inappropriate for use with aged frogs.

The biomedical use of the South African clawed frog, *Xenopus laevis*, has increased substantially in recent years. The generation and use of transgenic *X. laevis* has been accelerated by advances in transgenesis methods, further promoting the importance of the species in developmental and cell biology research.[23] The Marine Biology Laboratory estimates that research in *Xenopus* will continue to climb substantially in the next years.[14] Recently, *Xenopus* has been identified as a model for heterotaxy, a congenital heart disease.[4] There is current interest in exploring mechanisms of maturation and aging in *X. laevis* and other nonmammalian vertebrate species.[3,5,10,16] As such, baseline laboratory data for *X. laevis* have become essential in the phenotyping of transgenic animals and the interpretation of clinicopathologic experimental data. Serum biochemical reference intervals for young adult wild-caught and laboratory-reared *X. laevis* were reported recently.[26] Hematology and serum biochemical reference intervals for older adult and geriatric *X. laevis* have not been reported to our knowledge.

Here, we report the clinical chemistry reference intervals for a large population of aged laboratory-reared *X. laevis*. The values are compared with prior clinical chemistry values for young *X. laevis* from the same vendor, and significant differences are highlighted and discussed. The reference intervals and comparisons presented here will enable researchers and clinicians to interpret clinical chemistry data from aged and geriatric *X. laevis*.

Materials and Methods

Animals. All animal procedures were conducted in accordance with a protocol reviewed by Stanford University's Administrative Panel on Laboratory Animal Care, the University's IACUC panel. All frogs were laboratory-reared *X. laevis* purchased from NASCO (Fort Atkinson, WI) and housed in an AAALAC-accredited facility. All animals were mature female frogs with ages ranging from 10 to 14 y according to known purchase dates. Prior to blood collection, laboratory-reared frogs had been housed for at least 8 y under similar conditions of water temperature (16 to 22 °C), room lighting (12:12-h light:dark cycle), and diet (Frog Brittle, NASCO). Frogs were maintained in a timed flow-through water system supplied by municipal water after passage through particulate and reverse-osmosis filtration systems. Water-quality parameters were spot-tested regularly and maintained within institution-specific ranges considered acceptable for housing of aquatic amphibians.[24] A total of 142 frogs were examined by necropsy and histopathology, with 52 of the frogs randomly selected for blood collection for hematology and clinical biochemistry analysis.

Blood sample collection. Cardiocentesis for blood collection was performed on 52 frogs anesthetized according to current AVMA guidelines and recently published refinement techniques.[2,25] Briefly, frogs were immersed in approximately 5 gm/L MS222 (Finquel, Argent Chemical Laboratories, Redmond, WA) buffered to a neutral pH with sodium bicarbonate (Sigma Aldrich, St Louis, MO) until animals were fully anesthetized (determined by loss of the righting reflex and a lack of response to toe pinch). Weight and snout–vent lengths were collected; anesthetized frogs were incised from pubis to sternum, and the coelomic and thoracic cavity opened to allow direct viewing of the heart. Whole blood (1 to 3 mL) was collected from the ventricle by using a 3-mL syringe (Kendall Monoject Syringe, Covidien, Mansfield, MA) and a 22- or 23-gauge needle (Becton Dickinson, Franklin Lakes, NJ). Blood was collected into tubes containing EDTA anticoagulant (Covidien) for hematologic analysis and into empty collection tubes (Covidien) for biochemical analysis. After blood collection, the heart was removed per current AVMA guidelines.[2]

Hematologic analysis. Hematologic analysis included total RBC and WBC counts obtained by hemocytometer methodology using Natt and Herrick stain, as previously described for

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Analyte	Method
Albumin (g/dL)	Bromcresol green dye binding; colorimetric detection
ALP (IU/L)	Enzymatic assay detecting transphosphorylation activity of ALP on *p*-nitrophenylphosphate; bichromatic rate detection
ALT (IU/L)	Two-step enzymatic assay detecting transamination activity of ALT on L-alanine and subsequent oxidation of NADH; bichromatic rate detection
Amylase (IU/L)	Enzymatic assay detecting activity of amylase on chromogenic substrate linked with maltotriose bichromatic rate detection
Anion gap	Calculated as (Na^+ concentration + K^+ concentration) – (Cl^- concentration + CO_2 concentration)
AST (IU/L)	Two-step enzymatic assay detecting transamination activity of AST on L-aspartate and subsequent oxidation of NADH; bichromatic rate detection
BUN (mg/dL)	Two-step enzymatic assay detecting urea via urease generation of ammonia and subsequent oxidation of NADH; bichromatic rate detection
Ca^{2+} (mg/dL)	Colorimetric assay detecting calcium complexed with *o*-cresolphthalein complexone; bichromatic endpoint detection
CO_2 (mmol/L)	Two-step enzymatic assay detecting bicarbonate anion via reaction with phosphoenolpyruvate and subsequent oxidation of NADH analog; bichromatic detection
Cl^- (mmol/L)	Ion selective electrode (contained in integrated multisensor)
Cholesterol (mg/dL)	Cholesterol esterase hydrolysis, generation of hydrogen peroxide by oxidation of free cholesterol; polychromatic endpoint detection
Creatine kinase (IU/L)	Coupled enzymatic assay detecting creatine kinase transphosphorylation activity and subsequent reduction of NADP; bichromatic rate detection
Creatinine (mg/dL)	Modified kinetic Jaffe reaction utilizing picrate chromophoric substrate in presence of strong base; bichromatic rate detection
GGT (IU/L)	Enzymatic assay detecting glutamyl transferase activity of GGT on □-glutamyl-3-carboxy-4-nitranilide; bichromatic rate detection
Globulins (g/dL)	Calculated by subtraction of albumin value from total protein value
Glucose (mg/dL)	Modified hexokinase method; glucose phosphorylated then glucose-6-phosphate oxidized and NAD reduced; bichromatic endpoint detection
HDL (mg/dL)	Dextran complexing step, enzymatic detection of hydrogen peroxide generated by oxidation of HDL-cholesterol by cholesterol esterase and cholesterol oxidase; bichromatic endpoint detection
LDH (IU/L)	Enzymatic assay detecting lactate dehydrogenase activity on L-lactate by reduction of NAD; bichromatic rate detection
LDL (mg/dL)	Two-step solubilization, enzymatic detection of hydrogen peroxide generated by oxidation of LDL-cholesterol by cholesterol esterase and cholesterol oxidase; bichromatic endpoint detection
Lipase (IU/L)	Enzymatic assay detecting lipase hydrolysis of ester substrate and subsequent generation of chromogenic free methylresorufin; bichromatic rate detection
Phosphorus (mg/dL)	Detection of phosphate complexed to molybdate, subsequent reduction of complex by *p*-methylaminophenol sulfate and bisulfite; bichromatic endpoint detection
K^+ (mmol/L)	Ion-selective electrode (contained in integrated multisensor)
Na^+ (mmol/L)	Ion-selective electrode (contained in integrated multisensor)
Total protein (g/dL)	Modified biuret method using cupric ion complexed with peptide linkages in basic solution with tartrate as complexing agent; bichromatic endpoint detection
Triglycerides (mg/dL)	Enzymatic assay using lipoprotein lipase generation of glycerol, phosphorylation of glycerol, and oxidation of glycerol-3-phosphate to generate hydrogen peroxide then generation of quinoneimine; bichromatic endpoint detection
Uric acid (mg/dL)	Enzymatic assay detecting uric acid conversion to allantoin by uricase; bichromatic endpoint detection

Figure 1. Methods used in automated serum biochemical analysis.

amphibian species.[1,6,27] All hematologic analysis was performed on the day of sample collection. The PCV was determined in duplicate by centrifuging filled microhematocrit tubes for 3.5 min at 1247 × *g* (Autocrit Ultra3 Centrifuge, Becton Dickinson) before reading the PCV. The Hgb concentration was determined by running samples on an automated hematology analyzer

(Cell-Dyn 3500, Abbott, Chicago, IL) and then applying a correction factor provided by the manufacturer. The formula for the calculation is: Hgb concentration = (2.3 × RBC count) – 0.51. The values for MCV, MCH, and MCHC were calculated from the RBC count, hemoglobin concentration, and PCV.[6]

Biochemical analysis. Collected blood was allowed to clot at room temperature for approximately 1 h and was centrifuged for 6.5 min at 18,187 × *g* (Eppendorf Centrifuge 5415R, Hamburg, Germany). Serum was pipetted to a fresh tube and centrifuged again for 3 min at 18,187 × *g*. After centrifugation, serum was pipetted into a 1.5-mL microfuge tube. Although lipemia and icterus can interfere with serum biochemical analysis, no sample collected in this study appeared grossly icteric,discolored or lipemic. Serum analyte values were determined on a laboratory chemistry analyzer (Dimension Xpand Plus Integrated Chemistry System, Siemens, New York, NY); the methods are listed in Figure 1. All biochemical analysis was performed within 2 d of sample collection, with storage of serum at 4 °C prior to analysis. Full biochemical analysis was performed whenever possible. When testing was limited by sample volume, analytes were tested in order of our prioritization list, with sample dilutions performed as needed while remaining within the limits of each analyte's linearity range. The prioritization list was based on our clinical testing prioritization list for amphibian species, with the lowest priority analytes being LDH and uric acid. As such, only LDH and uric acid were not analyzed for all samples.

Necropsy and histopathology. A total of 142 frogs was submitted for gross necropsy and histopathology. After 48 to 72 h of formalin fixation, major organs were trimmed, processed routinely for histology, and slides were stained with hematoxylin and eosin. Special stains (for example, Masson trichrome) were used when deemed necessary.

Statistical analysis. The Reference Value Advisor add-in for Excel (Microsoft, Redmond, WA) was used to calculate descriptive statistics and reference intervals[12] according to guidelines for veterinary species.[11] All data were transformed by using Box–Cox transformation. Outliers were identified and removed as indicated according to the results of both Tukey and Dixon–Reed outlier tests. Robust reference intervals were reported whenever possible, as indicated by results of Anderson–Darling testing for robustness on transformed data. When the *P* value for symmetry testing for an analyte was less than 0.05, reference intervals generated from the standard method were reported instead. Analyte data that failed to demonstrate either symmetry or normality in transformed data were reassessed as described for the nontransformed data, and appropriate reference intervals were reported. Nontransformed data that again failed these tests were reported as nonparametric reference intervals (all nonparametric reference intervals included at least 40 data points). For analytes that yielded values of 0 or less (for example, anion gap), linear transformation was performed to allow inclusion of all data points.

The data set we generated from aged frogs was compared with prior hematology and clinical chemistry data from young *X. laevis*. Data from wild-caught frogs were removed from the raw data used in the prior study.[26] The raw data from the current study was used to represent aged *X. laevis*. A 2-tailed *t* test was used to compare the 2 groups (young compared with aged *X. laevis*) for each analyte. A *P* value of less than 0.05 was used to indicate a significant difference.

Results

Clinical pathology results. The mean, 1 SD, and reference interval were calculated for the hematology analytes (Table 1) and most of the chemistry analytes tested (Table 2); reference intervals could not be calculated for uric acid and LDH because too few samples had sufficient volume to permit testing of these analytes. The values for several chemistry analytes differed between aged and young frogs from the same vendor, whereas other analytes did not (Figure 2). Clinical chemistry results from aged *X. laevis* were compared with published values for young adult frogs (Table 3).

Table 1. Reference values for hematology analytes in aged *X. laevis*

Analyte	Mean	1 SD	Reference interval
RBC (x 106/µL)	1.22	0.243	0.84–1.84
PCV (%)	47.0	6.92	32.8–61.6
Hgb (g/dL)	13.18	3.082	6.25–19.10
WBC (x 10^3/µL)	9.20	3.198	3.13–15.93
MCV (fL)	38.56	8.256	24.78–52.96
MCH (pg)	10.60	3.333	5.80–16.91
MCHC (g/dL)	27.92	4.111	21.29–38.12

Necropsy and histopathology results. Gross examination revealed no significant lesions, although approximately half of the frogs evaluated had opaque, bright-yellow gall bladders. However, hematoxylin–eosin and trichrome staining of these gall bladders did not reveal any significant pathology, such that the finding was considered incidental. All frogs had large ovaries filled with mature eggs that did not differ notably in size, shape, or density to those of the younger frogs.

Discussion

Aging-associated changes in clinical pathology analytes occur in numerous mammalian species, for example, domestic dogs.[13,17,18] However, little has been published regarding age-related changes in clinical pathology values in nonmammalian species, particularly amphibians. The summary data and reference intervals determined in the current study therefore provide the groundwork for interpreting clinical pathology data in aged laboratory *X. laevis*.

The comparison of hematology and clinical chemistry data obtained from aged *X. laevis* with data previously obtained from young *X. laevis*[26] serves as a useful baseline from which to understand the potential effects of aging on clinical pathology parameters in frogs. Comparisons with changes in other vertebrate species potentially have limited value. For example, the increased potassium level in aged frogs is similar to that in aged dogs,[18] but other changes seen in aged frogs (for example, increased albumin and decreased ALT) are not associated with aging in dogs.[13,18] Similarly, the increased albumin in aged frogs is opposite to the situation reported for healthy elderly humans, in whom albumin decreases approximately 0.8 g/L per decade in humans older than 60 y.[20] In humans, glomerular filtration rate decreases with aging but creatinine production decreases concurrently, resulting in unchanged serum creatinine levels (but decreased urine creatinine).[8] In addition, our aged frogs did not have increased plasma creatinine values, and elderly humans have normal serum urea nitrogen, calcium, and phosphorus concentrations relative to those in younger adults.[19] Our aged frogs had higher uric acid and calcium levels and lower BUN and phosphorus levels than did the younger frogs in the prior study.[26]

Comparing laboratory data between animal studies can be impeded by several variables, such as differences in husbandry and sampling practices. In our comparisons with prior published data on young adult frogs,[26] the variables were minimal: in

Table 2. Reference values for clinical chemistry analytes in aged *X. laevis*

Analyte	Mean	1 SD	Reference interval
Albumin (g/dL)	1.88	0.29	1.3–2.5
ALP (IU/L)	157.7	49.7	78–279
ALT (IU/L)	11.1	5.7	2–25
Amylase (IU/L)	518.1	342.8	71–1498
Anion gap	16.81	6.09	4.1–29.3
AST (IU/L)	199.3	108.8	49–480
BUN (mg/dL)	6.6	4.0	2–17
BUN:creatinine	17.19	10.14	4.2–47.5
Calcium (mg/dL)	10.13	0.97	7.9–11.9
Ca^{2+}:Phosphorus	1.99	0.72	0.9–3.9
CO_2 (mmol/L)	30.50	5.76	14.2–37.1
Chloride (mmol/L)	80.5	5.6	75–86
Cholesterol (mg/dL)	163.0	72.1	29–325
Creatine Kinase (IU/L)	1753.4	1352.2	431–5716
Creatinine (mg/dL)	0.34	0.09	0.2–0.5
GGT (IU/L)	2.7	2.7	54–206
Globulins (g/dL)	2.1	0.5	1.2–3.1
Glucose (mg/dL)	32.5	11.9	13–61
HDL (mg/dL)	76.9	23.5	29–125
LDH (IU/L)	1532.4	634.4	NA
LDL (mg/dL)	50.4	33.4	2–136
Lipase (IU/L)	53.4	16.8	30–97
Phosphorus (mg/dL)	5.22	2.11	1.0–9.7
K^+ (mmol/L)	3.97	0.86	2.6–6.1
Na^+ (mmol/L)	119.0	3.5	112–126
Na^+:K^+	80.50	2.66	17.6–41.4
Total protein (g/dL)	3.91	0.69	2.3–5.2
Triglycerides (mg/dL)	45.0	20.1	18–104
Uric acid (mg/dL)	0.20	0.00	NA

NA, not available; too few samples had sufficient volume for the test to be run.

both studies, frogs were all laboratory-bred, maintained under similar housing conditions and fed an identical diet; the same anesthetic protocol was used for all frogs; sampling was performed randomly throughout the day, to normalize postprandial and circadian effects; and the same laboratory instrumentation, methodology, and technical personnel were used in both studies. The comparison with other published studies (Table 3) does not take into account these potential variables and should be used for reference only.

Hematology of nonmammalian species is particularly prone to variability between studies. Of the hematology parameters routinely tested, only the PCV can be considered a standard 'automated' method. Automated hematology analyzers cannot discriminate the different populations of nucleated blood cells in nonmammalian blood samples. Automated counts can be used to generate total nucleated cell counts and estimates of MCV, by subtraction of WBC and thrombocyte counts after hemocytometer counting.[6] Alternately, the non-RBC cells can be assumed to minimally affect the automated results and therefore that the results are a reasonable estimate of RBC parameters. In addition, staining methods for hematology can vary between laboratories. Hgb concentrations can be measured via a modified or corrected automated analyzer method or the modified cyanmethemoglobin spectrophotometric method.[1,6] Sources of inaccuracy in Hgb measurement include potential variability in spectrophotometric results due to plasma color variability.[1] Hemocytometers are used to count WBC, thrombocytes, and sometimes RBC in nonmammalian blood samples; the inaccuracy of hemocytometer counting is well documented and is potentially compounded by interobserver variation.[22] The RBC parameters calculated from the RBC count, Hgb and PCV, are accordingly influenced by these sources of variability. In summary,

Aged frog values higher:	Aged frog values lower:	No difference:
Albumin	ALT	ALP
Amylase	Anion Gap	Creatine Kinase
Calcium	AST	Creatinine
Calcium:Phosphorus	BUN	GGT
CO2	BUN:Creatinine	Globulin
HDL	Chloride	LDH
Potassium	Cholesterol	PCV
Red blood cell count	Glucose	Total bilirubin
Total protein	Hemoglobin	
Uric acid	LDL	
White blood cell count	Lipase	
	MCH	
	MCHC	
	MCV	
	Phosphorus	
	Sodium	
	Sodium:Potassium	
	Triglyceride	

Figure 2. Significant differences in hematology and clinical chemistry results between aged *X. laevis* and published results from young *X. laevis*.[26]

Table 3. Table of clinical chemistry values in *X. laevis* and other amphibian species.

		Xenopus laevis (n = 52 current study)		*Xenopus laevis*[26] (n = 166)		*Rana catesbiana*[7] (n = 14)		*Rana argentina*[9] (n = 302)	
	Units	Mean	Reference interval	Mean	Reference interval	Mean	Reference interval	Mean	Reference interval
Anion gap		16.81	4.1–29.3	23.6	13.1–36.1	9.9	1.3–24.2	NA	NA
Albumin	g/dL	1.88	1.3–2.5	1.0	0.1–2.3	1.6	1.0–2.1	1.58	1.02–2.67
ALP	IU/L	157.7	78–279	148	59–282	NA	NA	157	73–248
AST	IU/L	199.3	49–480	453	27–1774	45	22–91	48.1	23–80
BUN	mg/dL	6.6	2–17	5	2–10	3	3–6	8.42	3.01–18.0
Calcium	mg/dL	10.13	7.9–11.9	8.9	5.2–12.3	8.05	6.50–9.60	8.31	6.0–11.2
Carbon dioxide	mmol/L	30.5	14.2–37.1	20.7	8.4–34.3	25	15–32	NA	NA
Chloride	mmol/L	80.5	75–86	82.5	72.7–92.7	77	65–86	108.6	103–116
Cholesterol	mg/dL	163	29–325	232	26–563	NA	NA	62	30–118
Creatine kinase	IU/L	1753.4	431–5716	1658	10–5400	NA	NA	432	156–919
Creatinine	mg/dL	0.34	0.2–0.5	0.4	0.1–1.1	0.99	0.70–3.00	4.83	1.07–12.3
GGT	IU/L	2.7	54–206	4	1–19	NA	NA	9.2	5–20
Globulins	g/dL	2.1	1.2–3.1	2.3	1.1–4.1	NA	NA	NA	NA
Glucose	mg/dL	32.5	13–61	53	18–111	NA	NA	50	10–98
Phosphorus	mg/dL	5.22	1.0–9.7	7.4	3.5–11.6	3.3	2.5–5.2	8.83	4.1–13.7
Potassium	mmol/L	3.97	2.6–6.1	4.0	2.3–7.3	2.7	2.0–3.2	3.62	1.92–5.84
Sodium	mmol/L	119	112–126	123	111–134	108	100–115	118.6	99–144
Total protein	g/dL	3.91	2.3–5.2	3.3	2.0–4.6	NA	NA	4.34	3.05–5.65
Triglycerides	mg/dL	45	18–104	117	57–555	NA	NA	43	20–126
Uric acid	mg/dL	0.2	NA	0.2	0.1–0.4	0.06	0–0.10	1.34	0.13–3.02

NA, not available

the comparison of hematology data for nonmammalian species between different studies is complex and can lead to mistaken conclusions if the data is not directly comparable.

Many of the factors described earlier (for example, husbandry, laboratory methodology) can cause variability in clinical chemistry data and hinder the direct comparison of the data we obtained here with the information obtained in most other studies. The data presented in Table 3 include studies in other amphibian species.[7,15,21] Given that many of the variables in these previous studies differ between studies and from those we accounted for when we compared our current study with that involving young adult frogs,[26] the values should not be compared directly among studies. However, trends in amphibian clinical chemistry are apparent, and a knowledge base can be built through the continued laboratory evaluation of diverse amphibian species.

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