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Echocardiographic Assessment of Cardiac Morphology and Function in *Xenopus*

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Advances using *Xenopus* as a model permit valuable inquiries into cardiac development from embryo to adult. Noninvasive methods are needed to study cardiac function longitudinally. The objective of this study was to evaluate the feasibility of echocardiographic studies in *Xenopus* and establish normative data of adult cardiac structure and function. Doppler and 2D echocardiograms and electrocardiograms were acquired from adult *Xenopus laevis* and *X. tropicalis*. Frogs were exposed to either isoflurane or tricaine to discern the effect of sedating agents on cardiac function. Cardiac dimensions, morphology, flow velocities, and electrophysiologic intervals were measured and evaluated by using bivariate and regression analyses. Normal cardiac dimensions relative to body weight and species were established by echocardiography. Normal conduction intervals were determined by electrocardiography and did not vary by body weight or species. Anesthetic agent did not affect ejection fraction or flow velocity but did alter the QRS duration and QT interval. Echocardiographic and electrocardiographic studies in *Xenopus* provide information about cardiac anatomy and physiology and can readily be used for longitudinal analyses of developmental inquiries. Body weight, species, and anesthetic agent are factors that should be considered in experimental design and analyses.

Xenopus laevis, the South African clawed frog, and *Xenopus tropicalis*, the Western clawed frog, are useful animal models for developmental studies. Historically, the ease of experimental embryonic manipulation and the rapid maturation of the free-living embryo attracted scientists interested in vertebrate development to these frogs. More recently, studies have taken advantage of the conservation across phyla of the development of organ systems, especially the development of the heart. Aberrations in cardiac development and function can be characterized through a variety of elegant techniques in the embryo to give insight into the origins of human disease.[3,5,13,15,19] The *Xenopus* heart has 3 chambers, with right and left atria, a common atrioventricular valve, and a single ventricle that gives rise to the truncus arteriosus.[10] *Xenopus* studies traditionally use *X. laevis*; however, work has expanded into *X. tropicalis* as the field has progressed; although smaller than *X. laevis*, *X. tropicalis* matures more quickly and has a fully sequenced diploid genome.

Cardiovascular development studies in *Xenopus* can phenocopy human congenital heart disease closely. Defects in cardiac looping, septation, trabeculation, valve formation, and outflow tract development have been demonstrated in association with altered gene expression and environmental exposures.[1,4,7-9,18] Techniques for investigations into cardiac development, including transgenic methods, continue to expand in the *Xenopus* model.[2,20,29,30] These techniques position the *Xenopus* system for longitudinal analyses of the cardiovascular effects of altered gene expression. As long-term studies expand, noninvasive methods are needed for serial analyses of cardiac anatomy and function through maturation. For example, transgenic lines of *Xenopus* are currently being propagated in which heart tissue expresses either green fluorescent protein or its variants under the control of a cardiac actin promoter.[26] These lines allow easy visualization of myocardial structure in living animals.

Methods to study cardiac physiology have been described for *Xenopus* embryos and, although less well-described, adults.[5,11,25] Echocardiography can add a valuable dimension to cardiac assays. Although echocardiography is used widely in other animal models for noninvasive examination of cardiac anatomy and function,[14,17] data on the utility of this methodology in *X. laevis* and *X. tropicalis* are sparse.

In the present study, we used echocardiography to characterize the anatomy and function of the adult *Xenopus* heart. Cardiac anatomy, chamber dimensions, and flow velocities were characterized by using 2D imaging and pulse-wave Doppler ultrasonography. Parameters were analyzed relative to body weight, species, gender, and anesthetic agent to ascertain differences. Adult *Xenopus* can vary considerably in size within groups and also by gender and species. Male *Xenopus* are smaller than their female counterparts, and *X. tropicalis* are much smaller than *X. laevis*. In addition, previous studies in animal models have shown that anesthetic agents affect hemodynamics.[21,23] To help tease out the effect of the anesthetic agent, isoflurane, commonly used in rodent models, was compared with tricaine, commonly used in frogs.

Materials and Methods

Animals. All protocols were approved by the facility's institutional animal care review committee with compulsory biannual

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reviews. Studies involved 50 adult frogs housed in an AAALAC-accredited animal care and research facility that uses 12:12-h light:dark cycles; an ambient temperature of 18 to 19 °C; dechlorinated, UV-treated, filtered water, and a commercial diet (Frog Brittle, Nasco, Fort Atkinson, WI). *X. laevis* were purchased from *Xenopus* I (Dexter, MI) or Nasco for these experiments. *X. tropicalis* were purchased from Nasco and are outbred Nigerian frogs from University of Virginia stock. The majority (39 of 50 purchased) were female *X. laevis*, because they are the frogs typically used in experimental protocols. To assess differences in cardiac size and function related to gender or species, we analyzed male *X. laevis* ($n = 5$) and *X. tropicalis* ($n = 6$).

Anesthesia. Animals were maintained at room temperature (22 to 23 °C) and anesthetized with either topical isoflurane (Phoenix Pharmaceutical, St Joseph, MO) or tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma, St Louis, MO). Isoflurane was given by placing a 5.7 × 7.6 cm adhesive bandage (Band-Aid, Johnson and Johnson, New Brunswick, NJ) infiltrated with 100% isoflurane on the dorsal surface of the frog as previously described.[28] Care was taken to ensure the adhesive surface remained covered and did not make contact with the frog. The initial dose of isoflurane was 0.02 mL/g. Repeat doses were given if needed, and total dose was recorded. Because *Xenopus* spp. are aquatic animals, buffered tricaine, the anesthetic traditionally used when working with these animals, was administered by transferring the frog to a 500-mL bath of 1% buffered tricaine.[12] Animals were considered sufficiently anesthetized if loss of the righting reflex was achieved.

Echocardiography. Doppler and 2D echocardiograms were performed (Vevo 770TM, Visual Sonics, Toronto, Canada). Transducers with broadband frequencies ranging from 12.5 to 37.5 MHz (model 710B, Visual Sonics) and 15 to 45 MHz (model 707B, Visual Sonics) and focal lengths of 15 or 12.7 mm, respectively, were used for imaging. Animals were placed supine on the glass-topped animal handling platform with embedded limb recording electrodes. Moistened gauze pads were placed underneath and on top of the frogs to prevent desiccation.

Cardiac images were obtained from parasternal long-axis, subcostal 3-chamber, and high-sternal views for effective imaging of the intracardiac structures, outflow tract, and venous return. Normative values were established by obtaining measurements from 2D images, in accordance with recommendations for chamber quantification from the American Society of Echocardiography.[16]

Ventricular systolic length, diastolic length, systolic diameter, and diastolic diameter were measured from the subcostal view. Length was measured from the plane of the atrioventricular valve annulus to the apical endocardial border. The atrioventricular valve annulus was measured in this view also. The transducer then was rotated 90°, and ventricular diameter between endocardial borders was measured approximately 1 mm below the atrioventricular valve annulus. The area of the ventricle was calculated by tracing the endocardial border in the subcostal view that displayed the longest ventricular length. Ventricular volumes then were calculated by using the formula for an ellipsoid model,

$$\text{Volume} = 0.85 \times (\text{area})^2 \,/\, \text{length},$$[27]

because it closely approximates the ventricular volume in *Xenopus* according to optical coherence tomography.[5] Ejection fraction was calculated by using the ventricular volumes:

$$\text{Ejection fraction (\%)} = [(\text{diastolic volume} - \text{systolic volume}) \,/\, \text{diastolic volume}] \times 100\%.$$

In addition, the diameters of the right and left atria, truncal valve annulus, truncus arteriosus, and right and left systemic arches were measured. Right and left atrial diameters were measured in the subcostal view perpendicular to the plane of the atrioventricular valve annulus from the annulus to the posterior wall of the atrium. The diameters of the truncal valve and truncus arteriosus were measured in the same plane from a parasternal view. The diameters of the systemic arches were measured just past the bifurcation of the truncus, perpendicular to the vessel course.

Doppler interrogation of inflow velocity across the atrioventricular valve and the velocity across the outflow tract were obtained from the subcostal and high-parasternal views, respectively. Atrioventricular valve regurgitation velocity was quantified in the parasternal view when present. Measurements were made in accordance with American Society of Echocardiography guidelines for Doppler interrogation.[22]

Electrocardiography. Electrocardiograms were recorded simultaneously during the echocardiograms by using an animal handling platform. Cardiac intervals were measured by using imaging software (Vevo 770TM, Visual Sonics). Measurements included the durations of the P wave and QRS complex and the PR, QT, and RR intervals. The P wave represents atrial depolarization; the QRS complex illustrates ventricular depolarization. The PR interval is the time from the onset of atrial depolarization to the onset of ventricular depolarization. The QT interval represents the time between the onset of ventricular depolarization and the end of ventricular repolarization, which is represented by the T wave. The RR interval is the time between 2 consecutive ventricular complexes.

Statistical analyses. Results are presented as mean ± 1SD unless specified otherwise. Unpaired Student *t* tests and Wilcoxon rank sum tests were used for intergroup comparisons. Correlation coefficients were calculated to determine the strength of the relationship between continuous variables prior to use in regression analyses. Multivariate regression analyses were performed to test the association of anesthetic type, species, gender, and weight with the outcomes of cardiac dimensions, flow velocities, and electrocardiographic measurements. Statistical significance was set at a P value of 0.05 or less.

From the results of the multivariate regression, Z-score regression equations were developed for each structure by relating cardiac dimension to body weight. The natural logarithm of the measurement for each cardiac structure was related to the natural logarithm of the body weight to ascertain the slope and y intercept of the relationship. From this correlation, the formula

$$\ln(\text{predicted cardiac dimension}) = \text{slope} \times \ln(\text{body weight}) + \text{y intercept}$$

was solved for each anatomic structure. Nomograms were produced on the basis of the following relationship:[24]

$$z = \frac{[\ln(\text{actual dimension}) - \ln(\text{predicted cardiac dimension})]}{\text{root mean square error}}$$

Data and statistical analyses were performed by using SAS (version 9.1, SAS Institute, Cary, NC) or Prism (GraphPad, La Jolla, CA) software.

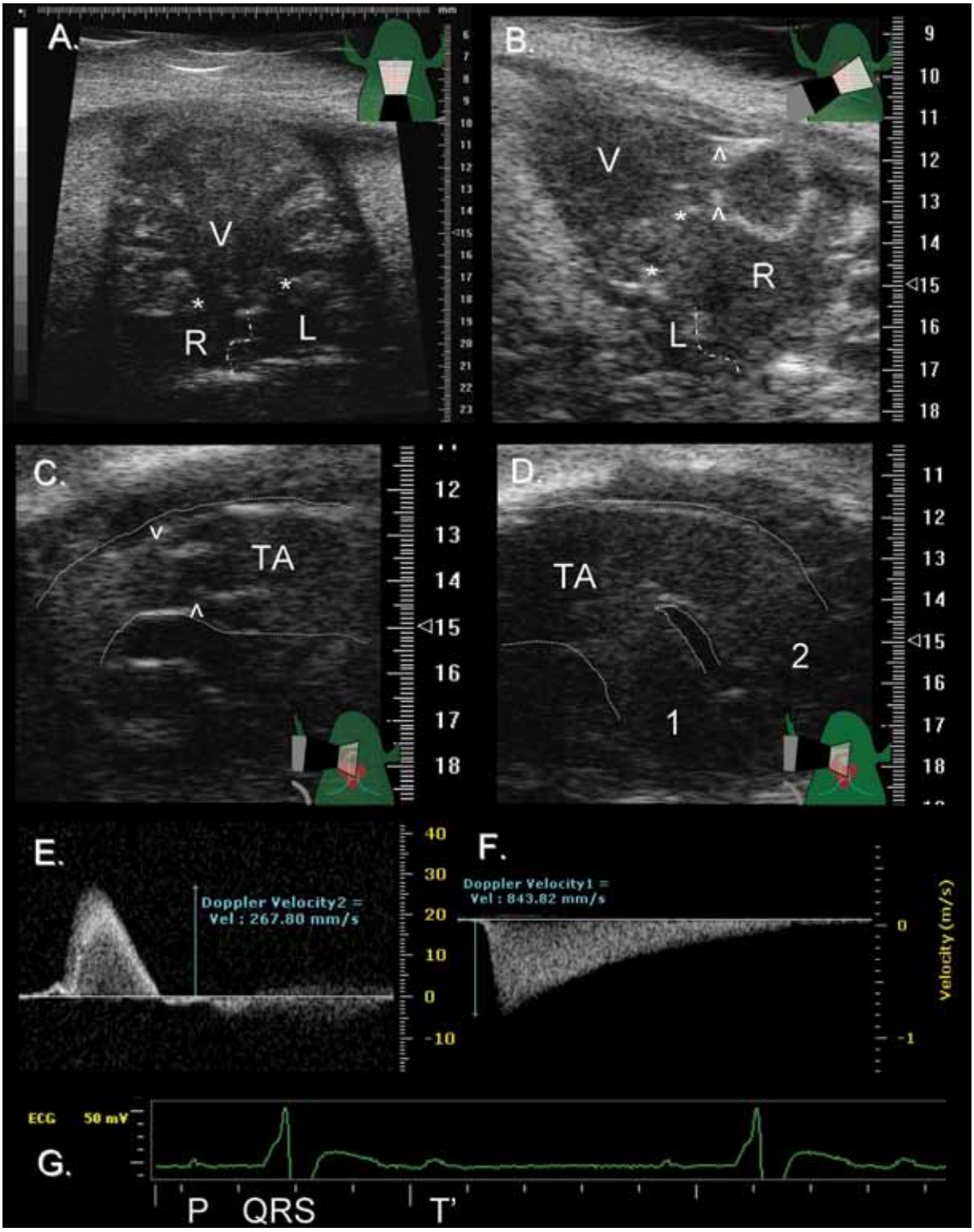

Figure 1. Still-frame images from echocardiograms of *X. laevis* and *X. tropicalis*. The 2D images include insets to illustrate transducer position and image plane relative to the supine frog. The cardiac structure closest to the transducer is displayed at the top of the image. (A) Three-chamber subcostal view of a *X. laevis* female demonstrating the trabeculations of the single ventricle (V), right (R) and left (L) atria, and atrial septum (dashed line). *, Hinge points of the atrioventricular valve leaflets. (B) Parasternal long-axis view of a *X. tropicalis* female gives an additional view of the cardiac anatomy. ^, Hinge points of the truncal valve leaflets. (C and D) High-parasternal views of a *X. laevis* female showing the truncal valve (^), truncus arteriosus (TA), and right (1) and left (2) systemic arches. (E) Doppler interrogation of the inflow velocity across the atrioventricular valve. (F) Doppler interrogation of the velocity across the outflow tract. (G) Electrocardiogram acquired during the echocardiogram showing the P wave (P), QRS complex (QRS), and T wave (T'). For panels A through D, depth (mm) is noted on the right margin. For panels E and F, the scale for velocity (mm/s) is noted on the right margin. For panel G, the vertical lines below the electrocardiogram denote 200-ms increments, the voltage (mV) scale is recorded in the left margin.

Results

Anesthesia. Both topical isoflurane and tricaine were effective anesthetic agents in *X. laevis* and *X. tropicalis*. The initial dose of isoflurane used was 0.02 mL/g body weight. Repeated dosing of isoflurane was often necessary, particularly in small frogs, which required as much as 0.06 mL/g. In addition, 3 frogs received an initial dose greater than 2.5 mL owing to their higher body weight and did not recover from anesthesia. Ultimately, an absolute isoflurane dose of 2.5 mL was found to be effective independent of body weight. The average time to achieving adequate anesthesia with 2.5 mL isoflurane was 22.5 ± 15.2 min, and the time to recover from anesthesia, defined as ability to swim to the tank surface, was 62.5 ± 33 min with a range of 28 to 180 min. Submersion in 1% tricaine for 30 min was uniformly effective to achieve adequate anesthesia. No difficulties were encountered for recovery from anesthesia with tricaine.

Echocardiograms. Echocardiographic analysis of adult *Xenopus* yielded clear images of the intracardiac anatomy, truncus arteriosus, and proximal arches (Figure 1). Imaging was easier in smaller animals, particularly for the systemic arches. Systemic venous return could easily be imaged and interrogated. In smaller frogs, pulmonary venous return could be studied.

Cardiac measurements (Table 1) were analyzed to assess the effect of body weight, gender, species, and anesthetic agent on cardiac parameters. Regression analysis showed that cardiac dimensions were a function of body weight and species. Animals with lower weights segregated themselves according to species and gender. The group containing the smallest frogs was comprised entirely by *X. tropicalis*, and the group of second-smallest frogs was comprised solely of male *X. laevis*. All large frogs were female *X. laevis*. Given the wide range of body weight for adult frogs, cardiac dimensions were analyzed as function of body weight by using logarithmic transformations to generate Z scores. Z scores are a well-established mechanism to normalize dimensions relative to body size and indicate the number of standard deviations a measurement is from the population mean. Therefore, valve dimensions with a Z score between +2 and –2 fall within 2 standard deviations of the mean and represent the normal range. Nomograms were generated for the cardiac dimensions listed in Table 1. Z score nomograms for the atrioventricular and truncal valve annular dimensions are presented (Figure 2).

Functional analyses included ejection fraction and flow velocities (Table 2). The ventricular ejection fraction, a marker of systolic function, was 49.3% ± 12.5% for all frogs. The inflow velocity across the atrioventricular valve was 375 ± 146 mm/s. The serpentine course of the truncus arteriosus made parallel interrogation of flow difficult. Peak flow velocities could be obtained in 37 of the 50 frogs analyzed and measured 587 ± 327 mm/s. Evaluation of anesthetic agents, species, weight, and sex was not associated with a statistical difference in the ejection fraction, ventricular inflow, or outflow velocities. Atrioventricular valve regurgitation could be detected in 6 frogs, with a flow velocity of 1197 ± 266 mm/s. Doppler interrogation did not reveal significant truncal valve regurgitation, indicating competence of the bileaflet truncal valve.

Electrocardiograms. Electrocardiographic tracings were adequate for interpretation with minimal baseline artifact in 41 of the 50 frogs studied. The average heart rate was 37 beats per minute, with an RR interval of 1618 ± 281 ms. The PR interval was 322 ± 75 ms and QT interval was 747 ± 197 ms. The P and QRS durations were 65 ± 22 ms and 132 ± 45 ms, respectively.

Although conduction intervals were not associated with gender, species, or weight, there was difference between anesthetic agents (Table 3). Animals sedated with tricaine had significantly ($P < 0.0001$) longer duration of the QRS complex: 181 ± 25 ms compared with 114 ± 37 ms seen with isoflurane. Despite the longer QRS duration, the QT interval was shorter in frogs sedated with tricaine at 634 ± 157 ms compared to those sedated with isoflurane at 788 ± 196 ms ($P < 0.02$).

Discussion

Analysis of cardiac anatomy and function in the *Xenopus* system with echocardiography and electrocardiography is feasible and informative. Here we have presented novel normative data. Baseline information was provided to enable scientists to follow late-onset changes in cardiac structure and function due to

Table 1. Cardiac measurements (mean ± 1SD [*n*]) from 2D echocardiograms

		X. laevis			
		Male	Female		
	X. tropicalis (<25 g)	25–50 g	50–100 g	101–150 g	>150 g
Body weight (g)	18 ± 5 (6)	39 ± 7 (5)	80 ± 15 (12)	123 ± 14 (18)	162 ± 12 (9)
Ventricular length, diastole (mm)	4.1 ± 1.0 (5)[a]	6.3 ± 0.5 (5)	8.5 ± 1.6 (12)	10.1 ± 0.8 (16)	9.8 ± 1.4 (8)
Ventricular area, diastole (mm^2)	15.7 ± 4.1 (5)[a]	40.0 ± 7.7 (5)	67.2 ± 21.7 (12)	89.6 ± 13.4 (16)	83.5 ± 14.1 (8)
Ventricular length, systole (mm)	3.5 ± 0.6 (5)[a]	4.5 ± 0.5 (5)	6.5 ± 1.4 (12)	7.5 ± 1.0 (16)	7.1 ± 1.6 (8)
Ventricular area, systole (mm^2)	10.4 ± 3.0 (5)[a]	23.1 ± 6.1 (5)	41.0 ± 16.2 (12)	55.5 ± 11.4 (16)	48.8 ± 11.6 (8)
Right atrium (mm)	1.42 ± 0.33 (5)[a]	2.42 ± 0.36 (5)	3.0 ± 0.73 (11)	3.17 ± 0.69 (15)	3.52 ± 0.75 (6)
Left atrium (mm)	1.36 ± 0.55 (5)[a]	2.44 ± 0.36 (5)	2.99 ± 0.60 (11)	3.19 ± 0.78 (15)	3.68 ± 0.74 (6)
Atrioventricular valve (mm)	1.92 ± 0.26 (6)[a]	2.94 ± 0.36 (5)	4.12 ± 1.30 (11)	4.79 ± 0.92 (14)	5.2 ± 1.3 (6)
Truncal valve (mm)	1.08 ± 0.16 (6)	1.42 ± 0.15 (5)	1.82 ± 0.51 (10)	1.94 ± 0.45 (13)	2.32 ± 0.49 (5)
Truncus arteriosus (mm)	1.28 ± 0.25 (5)	1.86 ± 0.18 (5)	2.58 ± 0.60 (8)	2.53 ± 0.43 (11)	2.98 ± 0.73 (6)
Right arch (mm)	0.93 ± 0.26 (4)	1.18 ± 0.13 (4)	1.91 ± 0.56 (7)	1.81 ± 0.27 (7)	1.90 ± 0.35 (3)
Left arch (mm)	1.03 ± 0.26 (4)	1.10 ± 0.08 (4)	1.83 ± 0.62 (7)	1.61 ± 0.27 (7)	1.87 ± 0.64 (3)

According to regression analysis, weight was a significant ($P < 0.03$) predictor of size for all cardiac dimensions listed in the table.
[a]Dimensions were significantly ($P < 0.005$) smaller in the regression model for species and weight.

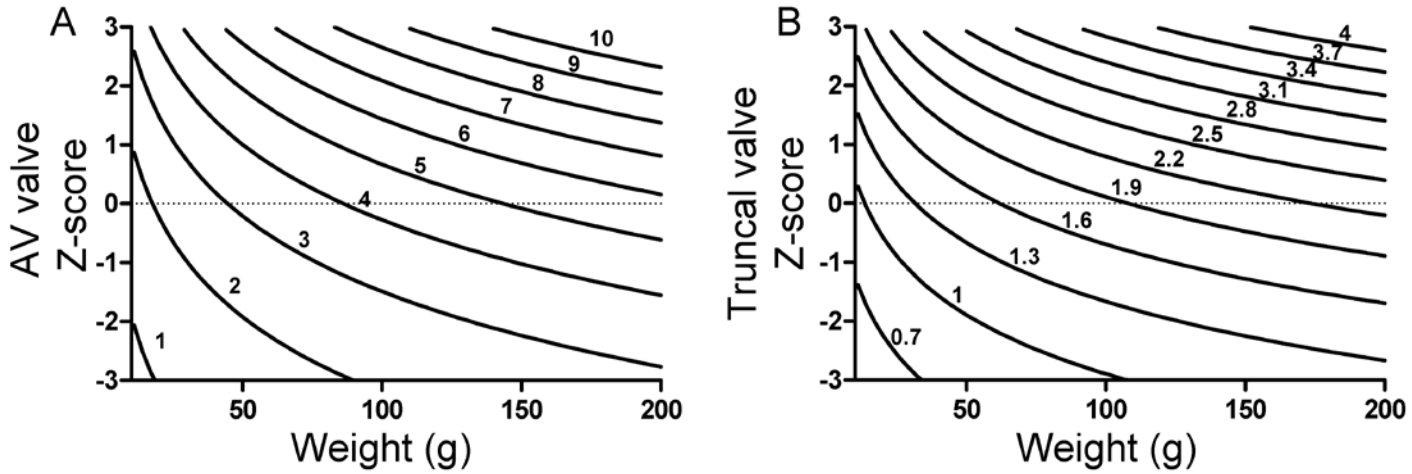

Figure 2. Echocardiographic analysis of *Xenopus* cardiac valve dimension as a function of body weight. Annular dimension z-score nomograms were calculated for the (A) common atrioventricular and (B) truncal valves for annulus size relative to body weight. Annular dimensions are depicted by the curvilinear lines, with the dimension (mm) listed adjacent to the line. Z scores ranging from +2 to –2 represent the normal range for body weight.

genetic, environmental, or nutritional influences by using *Xenopus* as a model organism. These studies improve our understanding of *Xenopus* cardiac anatomy in the context of normal development and function. The atrioventricular valve has dominant anterior and posterior leaflets and small right and left lateral leaflet, similar to the morphology to a common atrioventricular valve seen in human congenital heart disease. The relationship of the bulbus cordis to the ventricle and truncal valve can be easily imaged in *Xenopus*, and depolarization of the bulbus cordis can be recorded from the limb leads. The truncal valve is bicuspid and functions in a competent manner with no significant insufficiency.

This study presents the range of normal dimensions for 11 cardiac structures by using transthoracic echocardiography. The independent variable that provided the highest correlation coefficient to cardiac dimensions was body weight. Z scores are used to quantify dimensions relative to the normal range and readily allow statistical comparison between experimental subgroups to assess the effect of the intervention.[14,24] Normative data on cardiac dimensions and function are advantageous for studies modeling human heart disease in the frog.

X. tropicalis are markedly smaller than *X. laevis*, with no overlap between the 2 species in the weight of representative animals we evaluated. Regression analyses supported species as a variable, but certainty in this conclusion awaits studies on a larger number of frogs. However, our data indicate that animal weight must be considered when assessing cardiac dimensions and that species-matched controls are important for longitudinal studies. Of note, male *X. laevis* are fully grown by 2 y of age and maintain their weight at approximately 60 g but females of this species continue to grow through age. Therefore, for female *X. laevis*, weight is a surrogate for age. Based on our data, neither weight (nor by association, age) was seen as a modifier of cardiac function.[6]

Anesthesia has been shown to affect cardiac function in other animals, and we have encountered dysrhythmias in *Xenopus laevis* embryos sedated with tricaine.[3,21,23] In this study, we found no difference between the 2 anesthetic agents in adult frogs for parameters other than conduction physiology. Comparative studies on unsedated animals were not practical; however, many of the electrocardiographic values we obtained are comparable to those reported previously in pithed frogs.[11] Topical isoflurane was an effective alternate anesthetic agent to tricaine in adult frogs. In our hands, an absolute dose of 2.5 mL 100% isoflurane topically was more effective and safer than adjusting the dose by frog weight, as discussed elsewhere.[28] However, we observed that isoflurane caused prolonged recovery from anesthesia and transient disruption of the protective mucous that coats the adult frog. In general,

Table 2. Indices of ventricular function determined by 2D echocardiograms

		X. laevis			
		Male	Female		
	X. tropicalis (<25 g)	25 – 50g	50–100 g	101–150 g	>150 g
Inflow velocity (mm/s)	208 ± 142 (5)	297 ± 44 (5)	374 ± 133 (11)	428 ± 156 (15)	431 ± 90 (8)
Outflow velocity (mm/s)	469 ± 362 (2)	619 ± 384 (5)	568 ± 381 (8)	578 ± 273 (16)	651 ± 431 (6)
Ventircular volume, diastole (mm^3)	53 ± 18 (5)	197 ± 71 (5)	460 ± 213 (12)	687 ± 172 (16)	609 ± 133 (8)
Ventricular volume, systole (mm^3)	29 ± 14 (5)	103 ± 48 (5)	233 ± 140 (12)	359 ± 118 (16)	291 ± 96 (8)
Ejection fraction (%)	48 ± 15 (5)	48 ± 11 (5)	51 ± 14 (12)	48 ± 11 (16)	52 ± 14 (8)

Ventricular volumes were calculated using the formula for an ellipsoid model: $V = 0.85 \times (\text{area})^2 / \text{length}$.[27]
Ejection fraction was calculated as: (Ventricular volume during diastole – ventricular volume during systole) / ventricular volume during diastole × 100%.

Table 3. Electrocardiographic Intervals (ms; mean ± 1SD) by anesthetic agent

	P duration	QRS duration	PR interval	RR interval	QT interval
Isoflurane (n = 30)	62 ± 17	115 ± 37[a]	310 ± 73	1636 ± 294	788 ± 196[b]
Tricaine (n = 11)	73 ± 31	181 ± 25	354 ± 74	1557 ± 238	634 ± 157

[a] QRS duration was significantly ($P < 0.0001$) shorter for isoflurane.
[b]QT interval was significantly ($P < 0.03$) longer for isoflurane.

we recommend tricaine for sedation of frogs in most adult studies, owing to the ease of use and safety for the animals.

In summary, echocardiography is a practical, noninvasive method to assess cardiac morphology and function in *X. laevis* and *X. tropicalis*. Cardiac ultrasonography can be performed serially in the same animal and yield valuable data. Morphologic and hemodynamic information can be obtained readily from frogs of various sizes, allowing for sequential monitoring through development and maturation.

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