



Article

Posture as a Non-Invasive Indicator of Arousal in American Toads (*Anaxyrus americanus*)

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Abstract: Animal welfare has become a priority for modern zoos and aquariums. However, amphibians have not yet been the focus of much welfare research, perhaps in part because they do not tend to display many quantifiable active behaviors. This study focused on nine zoo-housed American toads (*Anaxyrus americanus*), a species that displays long periods of sedentary behavior, to explore whether more subtle cues could serve as welfare indicators. A novel American toad posture index was developed that characterized toad posture based on the angle of their forelimbs, visibility of ventral regions, and body weight distribution. As an indicator of arousal, approximate breathing rates were assessed based on the rate of expansion of the toads' throats. Subsequent analyses revealed that lower body postures were associated with slower rates of throat expansion and raised postures with faster rates of throat expansion, suggesting that posture may be a promising way to quickly and non-invasively assess toad arousal. This work lays important groundwork for assessing welfare of an understudied species, and we are optimistic that, with additional validation, these approaches can be applied in future amphibian welfare research.

Keywords: amphibian; welfare; breathing; zoo-housed; behavior



Citation: Woody, S.M.; Santymire, R.M.; Cronin, K.A. Posture as a Non-Invasive Indicator of Arousal in American Toads (*Anaxyrus americanus*). *J. Zool. Bot. Gard.* **2021**, *2*, 1–9. <https://doi.org/10.3390/jzbg2010001>

Received: 28 October 2020

Accepted: 4 December 2020

Published: 7 January 2021

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1. Introduction

Many modern zoos and aquariums monitor and improve animal welfare using a scientific approach. An emerging unifying framework for doing so is the Five Domains Model which posits that affecting any of the four domains of nutrition, environment, health, or behavior can lead to differential positive, neutral, or negative impacts on an animal's mental state [1]. Within the mental domain, affective states are classically categorized along two main dimensions: arousal, or the level of autonomic activation, and valence, the extent to which an emotion is positive or negative [2,3]. Within the field of animal welfare science, there is a strong taxonomic bias toward research on mammals, with behavior being the predominant method for measuring welfare [4]. Because methodology focusing on quantifying active behaviors is so widespread, there is little precedent for studying species that exhibit less variety in behavior, or that exhibit less overt behavior. One such understudied group is amphibians, a diverse taxon comprised of three orders, Anura (frogs and toads), Caudata (salamanders), and Gymnophiona (caecilians).

Despite less developed methodology, it is not impossible to make welfare insights in the absence of active behaviors. In humans, posture is known to convey valenced emotional states [5], and there is an increasing interest in studying the relationship between posture and emotion in other animals. Literature on posture and emotion has primarily focused on farm animals. For instance, Boissy et al. found that, in sheep, neutral and unpleasant scenarios corresponded with horizontally or backwards pointing ears respectively [6]. Previous research has also shown ear position to be a promising indicator of welfare in goats [7] and cows [8], and tail posture has been linked to welfare in goats [7] and pigs [9]. One amphibian-focused study characterized some elements of postural and behavioral

responses in four species (*Aquiloerycea cafetalera*, *Craugastor rhodopis*, *Lithobates berlandieri*, and *Rheohyla miotympanum*) that differed when housed in optimal versus stressful temperature ranges [10].

In addition to posture, breathing rates may be particularly useful for assessing the arousal component of welfare. Higher breathing rates typically indicate high arousal states as increased intake of oxygen prepares the body for increased activity [11]. In mammals, measuring breathing rate is less common perhaps because this technique usually requires the aid of technology. For instance, Gonzalez-Sanchez et al. explored the use of non-contact sensors to monitor stress in laboratory mice [12]. Breathing rate may be more easily measured in anurans as many species have distinct and visible throat expansions. Breathing in anurans occurs through three mechanisms which vary in how much they contribute to visible throat expansions. The first mechanism, buccal ventilation, occurs continuously [13] and is characterized by slow oscillations of the buccal floor which tidally ventilate the buccal cavity [14]. Interestingly, this process does not involve the lungs [15]. Lung ventilation occurs intermittently [13], and in addition to the raising and lowering of the buccal floor, it involves fine control over the opening and closing of the nostrils and glottis, the opening to the lungs [14]. Finally, some gas exchange occurs through the skin, although in a lesser capacity in terrestrial amphibians than in aquatic amphibians [14]. In toads, the visible throat contractions and expansions are thought to be a product of the raising and lowering of the buccal floor, a process present in both buccal and lung ventilation [16].

Here, in line with the Five Domains Model, we attempt to bridge the knowledge gap that exists in the field of animal welfare science by focusing on the mental domain of American toads (toad; *Anaxyrus americanus*). This species' sedentary nature makes the traditional method of collecting standardized behavioral data challenging and potentially uninformative for inferring welfare. Our aims were to: (1) characterize toad postures and (2) explore the relationship between posture and throat expansion rates. We predicted that more raised postures would be indicative of higher arousal and therefore associated with higher throat expansion rates. Although throat expansion rates may be informative on their own as a measure of arousal, posture is potentially a more practical measure of welfare in the field because it can be assessed instantaneously and even when a toad's throat is out of sight to the observer. Since arousal is an important component of welfare, determining the relationship between posture and arousal is an important first step towards understanding if posture can be used as a practical indicator of amphibian welfare.

2. Materials and Methods

2.1. Ethical Statement

All methods were designed to be minimally disruptive to the toads. This study was approved by the Lincoln Park Zoo Research Committee (approval number 2019-016).

2.2. Study Species

American toads are common throughout their native range in the Northeastern and Midwestern United States and parts of Canada [17] and are commonly housed in zoological institutions. While they have the ability to travel long distances (females can disperse up to 1.5 km. post-breeding) [18], during most of the year they occupy smaller home ranges of over 30 square meters [19] and only emerge from hiding in shallow burrows to feed at night [17]. The species is primarily terrestrial, but males aggregate in shallow waters bodies during the breeding season (March through July in most of their range) to call for females [17]. General husbandry of captive toads calls for loose soil for burrowing, water to soak in, abundant live insects, and minimal handling [20].

2.3. Subjects and Housing

The study was conducted at Lincoln Park Zoo (Chicago, IL, USA) with nine zoo-housed American toads. Preceding this study, two of the toads were located on exhibit

in view of public and the remaining seven toads were housed off exhibit. During the study, all toads were housed in identical 208.2-L tanks set up in a behind-the-scenes space. Data collection took place as part of a larger ongoing study of toad habitat preferences. Therefore, one side of each tank represented a more basic enclosure style, while the other represented a more enriched enclosure style. Both sides contained a shallow water bowl and two small (14 cm × 13 cm × 10 cm) plastic hides (manufactured by Kaytee). The “basic” side contained a single layer of bark chips while the “enriched” side had a thick (approximately 8 cm) layer of soil that allowed toads to burrow. The enriched side included features that the basic side did not, including a ramp made of cork bark and rock leading up to a raised platform that provided an additional source of dark cover with a live plant underneath, and a slowly dripping water source stationed above the tank (manufactured by ZooMed Laboratories, Inc.). Toads were removed from the study enclosure three times a week for feeding of live crickets or mealworms in separate bins.


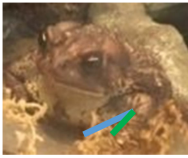

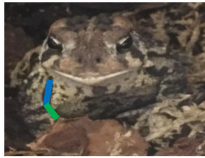



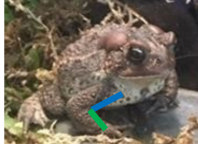


Data for the current study were collected from September 2019 through January 2020. Over a 4-week period, toads were housed in pairs (with the exception of one toad who was singly housed throughout) in either the basic or enriched side of the enclosure, or with access to both. Order of housing type was varied among pairs. An additional week of data collection occurred from February 2020 through March 2020 during which each pair-housed toad was singly housed with access to both sides of the enclosure. Although the role of habitat type and social housing were not the focus of this inquiry, we include both factors in our analyses to rule out any contributing variance (see Section 2.6) as we attempt to isolate the relationship between posture and throat expansion rates. Data collection was completed before the onset of breeding season. Building lights were on daily from 6:00 AM to 5:00 PM although toads may have been exposed to different levels of natural light depending on the time of year.

2.4. Method Development

To determine whether or not the novel method of counting throat expansions was accurate, eight 1-min videos of toads were recorded and the data collector scored the number of throat expansions from the video upon first playback at real speed (Supplementary Materials, Video S1). The video was then played back at a slow speed ($0.1 \times$ speed) on Boris (version 7.9.7, [21]) to determine the true number of expansions.

A novel toad posture index (Table 1) was developed in the weeks preceding the study by taking 50 photos of free standing, stationary toads (limb position not impacted by the tank wall or other hardscape habitat features) and manually clustering similar postures together. The five resulting clusters were then described based on the degree to which a toad was raised from the substrate which could be separated into three distinguishing criteria: (1) the angle between the toads’ upper and lower arm as estimated from the inner crook, (2) the visibility of the ventral regions, including the throat (area that expands during ventilation), chest (narrow band extending between the locations where the arms meet the front body), and underbelly (area of trunk posterior to chest), and (3) majority body weight distribution along the toad’s frontal plane. To use the tool, a stationary toad is assigned to the category with the most matched criteria. If a toad equally meets criteria for two or more postures, the posture that is less raised from the substrate (lower posture ID number) is chosen. Sometimes each of the two forelimbs can fall into a different category for criterion 1 (angle between upper and lower arm), in which case criterion 1 is excluded from the decision process.

Table 1. Toad posture index. A tool to characterize stationary American toads (*Anaxyrus americanus*) based on three criteria when toads are visible, not amplexed, and not supported by habitat structures. Postures are numbered from the least to the most amount of the ventral surface that is raised off the substrate. Majority weight distribution refers to how much of the toad's trunk is shifted posteriorly.

	1 "Hunkered Down"	2 "Low Sit"	3 "High Sit"	4 "Tall Legs"	5 "Spider"
Angle between upper and lower arm (x)	0°	0° > x > 55°	55° ≥ x ≥ 95°	x > 95°	x > 95°
Visible ventral regions	Partial or full throat No chest No underbelly	Full throat Partial or full chest No underbelly	Full throat Full chest Up to ½ of underbelly	Full throat Full chest ½ to ¾ of underbelly	Full throat Full chest >¾ of underbelly
Majority weight distribution	Even distribution	Inferior ¾ of body	Inferior ½ of body	Inferior ⅓ of body	Even distribution
Anterior view					
Lateral view					

To determine whether this approach resulted in a reliable posture index (i.e., one that another observer could use independently), the primary data collector shared the index with a naive second observer. The second observer then scored toad posture independently from 45 photos and an inter-observer-reliability test was conducted. Posture 5, characterized by forelimbs and hindlimbs extended away from the body wall, was excluded because it did not occur frequently enough to generate a set of photos for inter-reliability testing.

2.5. Data Collection

Throat expansion and posture data were collected by a single observer at 11:00 AM and 14:30 PM every day, Monday through Friday for the 5 weeks of the study. The data collector positioned herself in front of a tank housing a pair of toads. After a 5-min habituation period intended to allow toads to acclimate to the experimenter's presence, posture was recorded for one of the toads according to the toad posture index and throat expansions were counted for 1 min. A toad was recorded as "not visible" if only one criterion could be determined or if no criteria could be determined based on the toad's location and "amplexed" if engaged in amplexus with another toad. At the 10-min mark, this procedure was repeated for the other toad. Observation order was predefined and an equal number of observations were collected per toad in the morning and afternoon. The habitat type that the toad was currently occupying (basic or enriched) was also documented at the time of data collection. Because some toads were visible more often than others, the total throat expansion rates analyzed per toad ranged from four measurements to 43 measurements with an average of 23 measurements analyzed per toad.

2.6. Statistical Analysis

We used R statistical software (R Studio version 3.6.3, [22]) to perform a linear mixed effects analysis (lme4 package, [23]) to assess whether or not the four commonly seen postures (1, 2, 3, and 4) predicted throat expansion rate. Posture 5 was too rare for analyses (observed two times across two toads during the 5-week study) and therefore excluded. The dependent variable was throat expansion rate (events per minute, as an integer). The main predictor variable of interest was posture (factor with four levels: 1, 2, 3, and 4). Other factors controlled for in the model included whether or not a conspecific was present (factor with two levels: paired, unpaired), habitat type (factor with two levels: basic, enriched), time of day (factor with two levels: AM, PM), and date (factor with 85 levels). One random

effect of individual toad IDs (factor with nine levels) was included and each toad was assigned a random intercept to account for differences in throat expansion rates across individuals. Coefficients from the full model were plotted with the package GGally [24]. In addition to the full model, one reduced model was created to isolate the fixed effect of interest, posture ("Reduced: No Posture" model). We compared Akaike information criterion with correction (AICc) values (AICmodavg package, [25]) to compare model fits. We obtained p-values of specific variables through a likelihood ratio test of the best-fitting full model with the reduced model, using the analysis of variance (ANOVA) function and χ^2 distribution. The ANOVA was followed by a post-hoc Tukey's test to make pairwise comparisons among individual postures using the emmeans package [26]. For all models, visual inspections of residual plots did not reveal any obvious deviations from linearity, normality or homoskedasticity. Tests relying on probability distributions to determine significance were two-tailed and $\alpha = 0.05$.

3. Results

3.1. Accuracy and Reliability of Methods

The scores from the real-time and slow-motion toad throat expansion videos were strongly correlated, indicating high accuracy for real-time coding of throat expansion rates (Pearson correlation, $r = 0.97$, $p < 0.001$). Posture scores based on still images from the original and naive observers demonstrated good agreement (Cohen's kappa, $k = 0.63$).

3.2. Model Significance

The best fit model for predicting throat expansion rate was the full model that included posture (AICc full model = 2159.59; AICc reduced model = 2169.54). An ANOVA comparing the full and reduced models revealed a significant difference, indicating that including posture significantly improved the model ($\chi^2_{1,2} = 26.41$; $p < 0.001$). Evidence ratios considering the differences in AICc values for each model revealed that the full model was 144.89 times more parsimonious than in the reduced model where posture was omitted, indicating that posture was an important predictor of throat expansion rate.

3.3. Best-Fit Model Output

We continued our analysis with the full model since it was the best fit model. Overall, our predictor of interest, posture, did significantly predict throat expansion rate with higher postures predicting higher throat expansion rates and vice versa (Figure 1). The coefficients in the model reflected the difference in mean throat expansion rates per minute for postures 2 through 4 in reference to posture 1, the posture where the toad was the least raised from the substrate. This estimate accounted for any contributing variance of other effects in the model (i.e., conspecific presence, habitat type, time of day, and toad ID). Increasing posture index numbers indicated that the toad was progressively more raised from the substrate (i.e., posture 3 was more raised than 2 which was more raised than 1). Mean throat expansions per minute did not significantly differ when toads moved from posture 1 to posture 2 (Estimate = 0.13, $p = 0.98$). However, when the degree to which the toad was raised off of the substrate increased, throat expansion rates significantly increased. Specifically, as toads moved from posture 1 to posture 3, mean throat expansion rate increased by 17.49 expansions per minute ($p = 0.001$). As toads moved from posture 1 to 4, there was a significant increase of 40.77 expansions per minute ($p < 0.001$). Three other significant predictors of throat expansion rate emerged from the full model. When toads were housed with a conspecific, mean throat expansion rates were 39.67 expansions per minute higher compared to when toads were singly housed ($p = 0.05$). Mean throat expansion rates were 24.98 per minute lower when toads were on the enriched side of the enclosure ($p < 0.001$) and 7.43 per minute lower in the afternoon ($p = 0.02$).

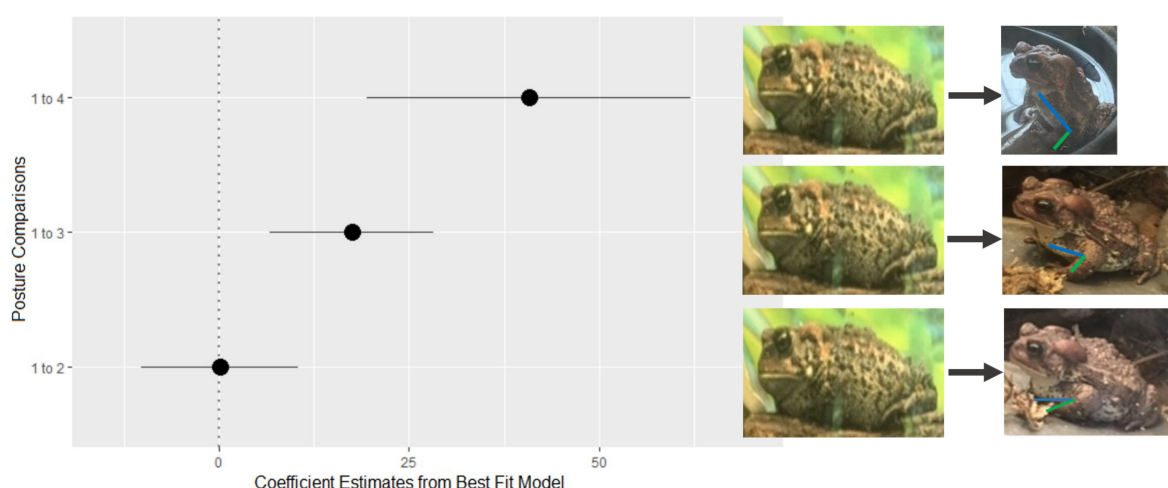


Figure 1. Output of posture coefficients from the Full model demonstrating the mean change in throat expansions per minute as toad postures become progressively more raised off of the substrate. Horizontal lines represent 95% confidence intervals. Coefficients represent significant differences ($p < 0.05$) between postures if the 95% confidence interval bars do not cross the y-axis at 0.

3.4. Post-Hoc Analysis

Mean throat expansion rates per minute were 80.4 for posture 1, 80.5 for posture 2, 97.9 for posture 3, and 121.2 for posture 4. A post-hoc Tukey's test revealed that mean throat expansion rates were significantly different for four out of six potential posture comparisons. There was a difference of 40.7 breaths per minute between postures 4 and 2 (95% CI: 20.63 to 60.65, $p < 0.001$), 40.8 breaths per minute between postures 4 and 1 (95% CI: 19.51 to 62.04; $p = 0.001$), 17.4 breaths per minute between postures 3 and 2 (95% CI: 8.19 to 26.53; $p = 0.001$), and 17.5 breaths per minute between postures 3 and 1 (95% CI: 6.75 to 28.23; $p = 0.008$). Throat expansion rates in postures 1 and 2 were not significantly different from one another ($p = 1.00$) nor were they different in postures 3 and 4 ($p = 0.11$) with differences of 0.1 and 23.3 breaths per minute respectively.

4. Discussion

Amphibian welfare is understudied partially due to a lack of welfare measures. We successfully created a reproducible American toad posture index that shows promise in serving as a quick and non-invasive measure of welfare in this species. In this novel posture index, postures are distinguished by the degree to which a toad is raised from the substrate. Although this is a continuous gradation, we created distinct threshold points so that postures could be classified in one of five categories that could be reliably scored across observers. We found good agreement between observers coding posture from still images. Agreement may have been further improved if toad postures were scored in person when observers could make judgements after observing the toad from multiple angles rather than from a single dimension provided in photos. The posture index may be adaptable to other anurans, although differing physiology may require some species-specific adaptation of the tool. In addition, we took the first steps towards determining what posture could reveal about toad affective states by ascertaining what relationship, if any, there was between toad posture and arousal, which is one of two components of welfare in addition to valence. Our investigation of throat expansion rates across four of the most common postures revealed that more raised postures predicted higher rates of throat expansion and, by extension, arousal levels.

While there is a logical positive relationship between throat expansion rate and arousal level, the tightness of this relationship requires consideration of some nuances due to the complex nature of anuran breathing patterns. During lung ventilation, toads can exhibit

such fine control over their nares and glottis that a single breath does not always equate to the intake of oxygen [16]. In one breath toads can actually inflate, deflate, or maintain lung volume [14]. Buccal ventilation does play a major role in maintaining high oxygen levels in the buccal cavity [13]. However, the extent to which the oxygenation of the buccal cavity alone relates to overall oxygen uptake in the body remains unclear. Although the exact level at which throat expansions relate to oxygen intake is unknown, a higher rate of throat expansion means that there is a higher chance that more oxygen is being delivered, and therefore that the body is more prepared for increased activity. In this way, anuran breathing is similar to other species where higher breathing rates are indicative of higher arousal [11].

While we found evidence for posture predicting throat expansion rates overall, our results suggest that not every posture comparison corresponds with significantly different throat expansion rates. For instance, the statistical model revealed significant differences in throat expansion rates between toads that were the least raised from the substrate (posture 1) and postures 3 and 4, which were moderately to greatly raised from the substrate. However, posture 2 which was only slightly raised from the substrate did not differ from posture 1. The post-hoc analysis adds support to this conclusion, showing no difference in mean breathing rates in the similarly raised postures 3 and 4. Interestingly, the difference between 2 and 3 was significant despite the similarity of the postures. While it is possible to distinguish toad postures based on small differences, these results demonstrate that not all measurable differences in posture reflect meaningful differences in arousal, a factor that is important to consider when applying this tool to future welfare studies.

While our work focuses on the arousal dimension of welfare, future work should also assess valence, a somewhat more evasive measure of emotion that varies on a continuum from negative to positive. Previous work with mammal species has determined emotional valence by assessing posture in animals exposed to situations likely to evoke differing emotional responses. Oliveira and Keeling, for example, integrated multiple postural elements (position of head, neck, and tail) using a PCA analysis, finding that different co-occurrences of postural elements were present in cows when feeding (high arousal and positive valence scenario), waiting to be milked (high arousal, negative valence), or brushed (low arousal, positive valence) [27]. Another method for determining emotional valence is comparing animals' postures in environments that are known to be favorable and unfavorable. Meagher et al. took such an approach, comparing mink (*Neovison vison*) lying postures in enriched versus barren environments [28]. However, no consistent differences in the frequencies of lying postures were found across the three subject groups [28]. Future work with amphibians could employ similar techniques to determine if posture is indicative not only of arousal, but also valenced affective states.

Interestingly, while posture was the main focus of this inquiry, throat expansion rates were not independent of a number of other factors including the presence or absence of conspecifics, enriched versus basic housing, and time of day. Throat expansion rates were lower when toads were housed singly versus in pairs. Toads are solitary by nature and in the wild only interact for breeding purposes [18]. Therefore, higher arousal rates may have been related to sociality either sexual arousal or territoriality. Captive management of anurans often involves housing multiple frogs or toads in one enclosure, making social housing an important area of future research. Throat expansion rates were also lower when toads were on the enriched side of the enclosure which suggests low arousal and may have been indicative of a negatively valenced low arousal state (such as boredom or depression), or, perhaps more likely a positively valenced low arousal state (such as calmness or relaxation). Previous studies have suggested welfare benefits of an enriched environment in other amphibian species, the clearest example being reductions in bite wounds and cannibalism in the common laboratory frog, *Xenopus laevis*, when provided PVC tubes for refuge [29]. It is also possible that the effect of habitat type on throat expansion rates was driven primarily by physiology rather than psychology. The live plant and soil substrate with a high capacity for water retention on the enriched side

could have created cooler microclimates which led to lower throat expansion rates. This effect was demonstrated in the Rococo toad (*Bufo paracnemis*) which showed decreases in ventilatory period as temperatures decreased [30]. While typical husbandry for toads and other anurans centers around the fulfillment of basic needs, our finding suggests that future research be conducted to determine why toads may react differently to different enclosure types and if certain features can promote positive welfare. Finally, in this study toads had lower throat expansion rates in the afternoon than in the morning. This may have been due to increased routine animal keeper activity in the mornings compared to the afternoons. Previous studies have shown deleterious effects of loud, unpredictable noise on large mammals in a zoo environment [31]. How sensitive toads are to ambient noise and whether or not it impacts their welfare may have important implications for captive management. Importantly, the current study identifies posture as a potential welfare indicator that can be applied to these important questions.

Our results add to a limited body of research regarding amphibian welfare and established a novel relationship between toad posture and arousal levels. This work lays important groundwork for a better understanding of how arousal levels relate to valenced affective states in this and related species. Moving forward, posture may provide a promising measurement when investigating how factors influence the welfare of anurans in human care, such as the effects of habitat type, ambient noise, handling, or the presence of conspecifics.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2673-5636/2/1/1/s1>. Video S1: American Toad Throat Expansion.

Author Contributions: Conceptualization, S.M.W., R.M.S. and K.A.C.; methodology, S.M.W., R.M.S. and K.A.C.; formal analysis, S.M.W.; writing—original draft preparation, S.M.W.; writing—review and editing, S.M.W., R.M.S. and K.A.C.; visualization, S.M.W.; supervision, R.M.S. and K.A.C. All authors have read and agreed to the published version of the manuscript.

Funding: S.W. was supported by the Lawrence University Fellowship at Lincoln Park Zoo.

Institutional Review Board Statement: This study was approved by the Lincoln Park Zoo Research Committee (approval number 2019-016).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: We wish to thank Dan Boehm, Lou Keeley, Amanda Barnes, Lauren Carlson, Penny Reidy, and Bryan Summerford for facilitating the study as well as all other keepers who assisted and cared for the toads without whom this research would not have been possible. Thank you to Rick Cortez and the facilities team for building the tanks and to Katie Fowler and Ivy Yen for technical support. We are grateful to Jason Wark, Natasha Wierzal, and Margaret Ramont for their support and contributions. Finally, thanks to Maureen Leahy and Dave Bernier for their input and support throughout.

Conflicts of Interest: The authors declare no conflict of interest.

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