Herpetological Review, 2008, 39(1), 56–58.

A Fast, Non-invasive Method of Measuring Growth in Tadpoles Using Image Analysis

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Measuring the performance of anuran tadpoles under a variety of conditions is a common research theme in herpetology. There are a number of variations in how performance is measured including assessing tadpole survival (e.g., Brown et al. 2006; Loman 2004) or time to metamorphosis (e.g., Holbrook and Petranka 2004). The most often-used variable is the growth rate of tadpoles, measured either as the size of the animals at the end of the experiment (e.g., Pahkala et al. 2003) or by subtracting initial mass from final (e.g., Relyea 2004; Relyea and Hoverman 2003; Rosenberg and Pierce 1995). Rarely though, do researchers measure tadpole mass at multiple times throughout the duration of the experiment, likely due to the time required to perform the measurements or the stress induced upon the tadpoles. Indeed, to do this tadpoles must be weighed by removing them from water and blotting them dry, which if not done quickly, can result in mortality.

In this paper, we describe a novel measure for assessing anuran tadpole growth that is simple, inexpensive, requires minimal removal from water, and is nearly directly correlated with tadpole mass. This measure is therefore useful for obtaining repeated measures throughout experiments without harming individuals. Furthermore, this measure allows for bulk assessments of growth on multiple tadpoles at once if time is limited, or if large numbers of individuals are involved.

Our method involves the use of image analysis, whereby digital photographs of specimens are taken and computer software used to measure features of the specimens (Davis et al. 2004; Davis et al. 2007; Davis and Grayson 2007; Davis and Maerz 2007). Variations of this technique are already frequently employed by herpetological researchers interested in tadpole shape (Relyea 2004, 2005; Relyea and Hoverman 2003; Van Buskirk 2002; Wilson et al. 2005), though to our knowledge, this technique has not been used to assess growth rates or directly compared to other measured of size. We therefore set out here to determine if this technique could be used to assess tadpole size and if this measure of size was related to traditional mass measurements. There were two components to this research. First, we examined a set of lab-reared tadpoles that varied in size, using traditional (tadpole mass) and the image analysis size measures. Second, we employed this technique to measure tadpoles that were being raised in an ongoing, unrelated experiment to evaluate its utility in the field.

For our first component, we obtained egg masses of *Rana sylvatica* from Habersham Co., Georgia during February 2006 and divided them into four tubs in the lab where they hatched. Tadpoles were fed ad libitum thereafter with a powder, ground from a 60:40 ratio of rabbit food pellets to Reptomin floating aquatic turtle sticks. Two weeks later there was a natural range of tadpole sizes in each tub. At this point we haphazardly caught single individuals from each tub with dip nets and placed them each in a standard plastic weighing dish filled with water from its tub. The dish was then photographed from above with a Canon Powershot G6 digital camera that was mounted on a mini copy stand at a fixed distance from the specimens (Fig. 1A). Care was taken to ensure that the tadpole was not moving at the time of the photograph (to avoid blurring), and that its dorsal surface was fully in view. After photographing, the tadpole was removed from the dish, blotted dry and weighed on an electronic balance. This was repeated until 58 individuals were processed. Throughout the experiment, we made an effort to capture tadpoles that varied in size. We also photographed a standard metric ruler to provide a calibration image for the image analysis software (see below).

To measure the tadpoles we imported all digital photos into an image analysis program, Fovea Pro (Reindeer Graphics, Inc.), used previously to measure features of herpetofauna (Davis and Grayson 2007; Davis and Maerz 2007). We first calibrated the software using the ruler image so that the pixel-to-millimeter ratio could be retained for the tadpole images. For each tadpole image we digitally selected the entire tadpole (head and tail), which was made easier by the fact that the tadpole was darkly colored on a white background (Fig. 1B). A Fovea Pro measure routine was then initiated, which measured the total surface area of the selection, which in this case was the surface area of the tadpole (43 mm^2 in Fig. 1B). This surface area measure was the unit of 'tadpole size' for our analyses. Once all images were measured we compared the tadpole surface area data (log-transformed) to the mass data (log-transformed) using Pearson Correlation. There was a highly significant positive relationship between individual tadpole mass and our surface area measure ($r = 0.996$, $p < 0.001$; Fig. 2).

Because a tadpole's body is relatively spherical, one might expect a curvilinear (e.g., exponential) relationship between tadpole dorsal area and mass, and therefore be surprised by the strong linear correlation we observed. However, the minimum and maximum tadpole area measurements were 18 and 200 mm^2, and the relationship between doral area and volume [mass] is relatively linear between these values. The relationship would only be significantly curvilinear if extended to the origin and out well beyond 200 mm^2, but a tadpole never has zero area or mass, and is not infinitely large.

In the second part of this project, we tested the utility of this technique while conducting an unrelated experiment that involved monitoring the growth of tadpoles within 18 plastic 55-L tubs. Each tub contained 50 tadpoles of *Rana sylvatica,* and our goal was to measure the average size of tadpoles within each tub with minimal mortality. On the day of sampling, the camera and photographing stand were set on a portable table next to the tubs (as in Fig. 1A). Ten tadpoles were dipnetted from the first tub and placed into two large petrie dishes (5 in each) along with water from their tub. When the tadpoles settled, a photograph of each dish was obtained as described above. The tadpoles were then released back to their respective tubs. This process was repeated until all tubs had been sampled. During this process we timed the length of time to sample (i.e., dipnet and photograph 10 tadpoles) each tub,

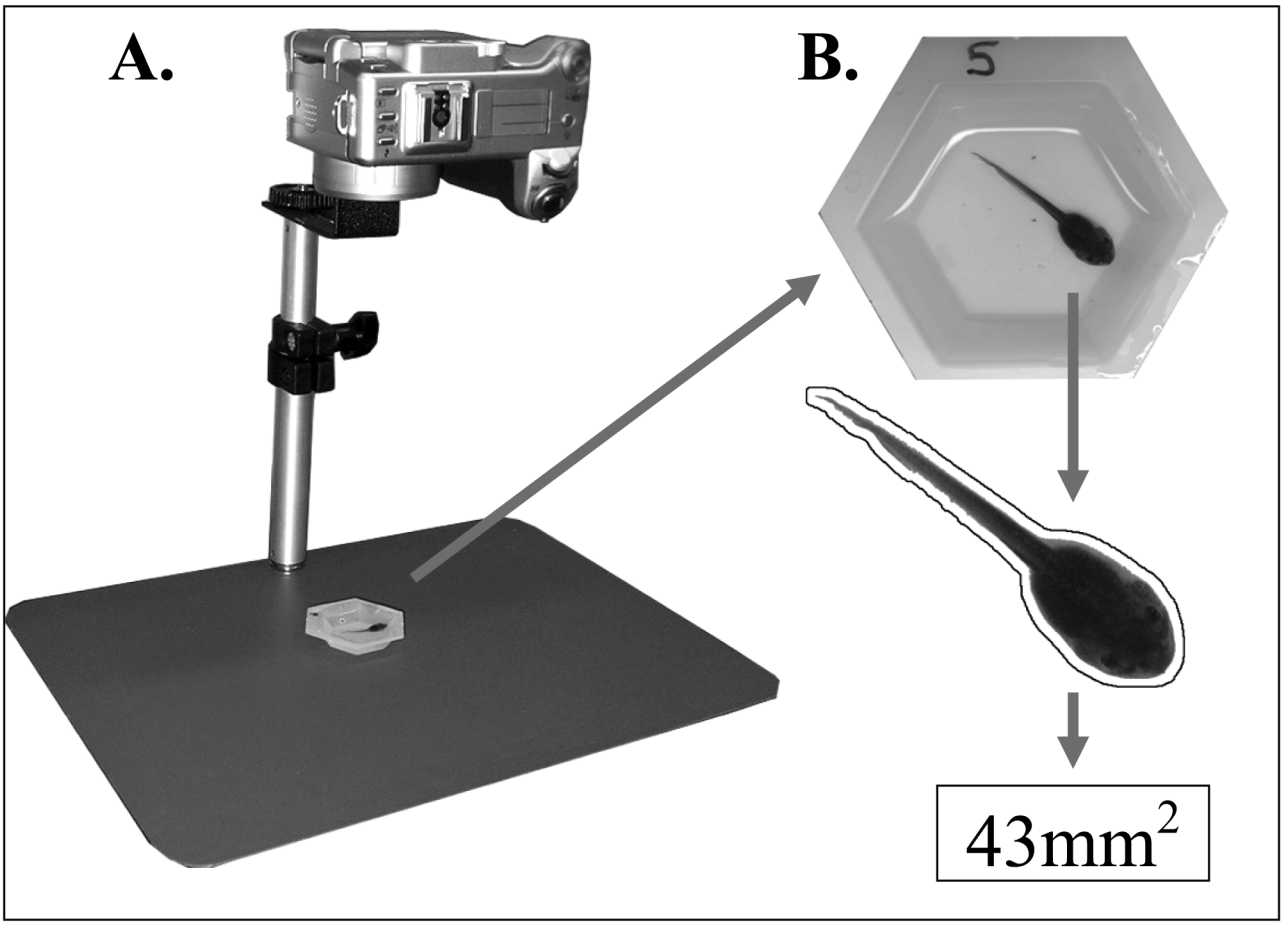

FIG. 1. Camera and photography stand used to photograph tadpoles in the lab and field (A). Tadpoles were held in white plastic weighing boats filled with water during photography. Later, digital images of tadpoles (B) were examined using Fovea Pro software. In the program, the entire tadpole was selected and its dorsal surface area measured in mm^2 based on a previously defined pixel-to-mm ratio obtained from a ruler image.

as well as the start and end times for the entire session. On average it took us approximately 2 min to sample the ten tadpoles per tub, with all 18 tubs finished in 48 min. In the lab, the same procedures were used to obtain size data from all individual tadpoles in the images as described earlier.

In the procedure described here, tadpoles are dipnetted and immediately placed in a dish of water for photography, they remain in this dish for no more than 2 min, and then are released back into their original water. Thus, tadpoles are subjected to minimal stress because they are never long out of water during the procedure. This technique can be used in the lab or field, and in both situations, large numbers of tadpoles can be quickly and non-invasively sampled, and sampling can be done multiple times over the course of experiments. Further, the measure of size we obtained for each tadpole (its surface area) was nearly perfectly correlated with tadpole mass (Fig. 2). Moreover, although not explored here, it is also possible to generate measures of body and tail length from the tadpole images (or the ratio of both). We also note that while the software used here costs ~ $ US800, there is a freely available image analysis program (ImageJ — http://rsb.info.nih.gov/ij/) that can perform the same tasks as outlined here. Thus, the method we describe is arguably faster and safer than mass measurements, and

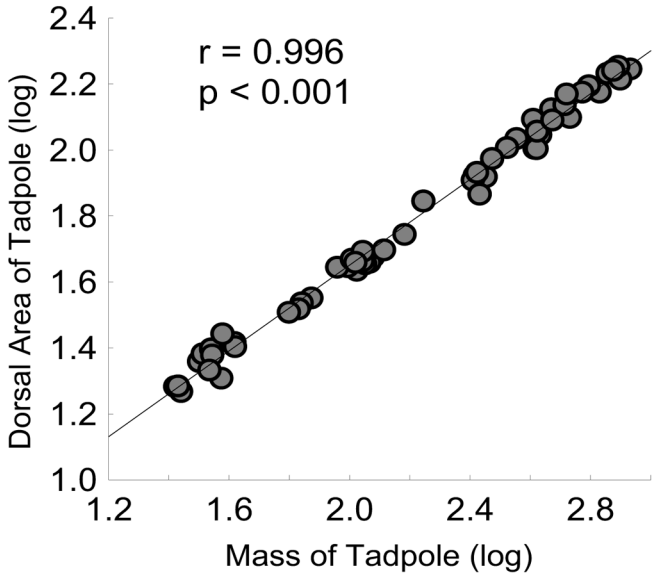

FIG. 2. Comparison of dorsal surface area measurement (log-transformed), obtained from image analysis procedure, and log-transformed mass of 58 lab-reared *R. sylvatica* tadpoles.

allows for a wider range of data to be measured during experiments.

Acknowledgments.—We thank Daniel van Dijk, Alina Ruiz, and members of the University of Georgia 2006 Herpetology class for field and lab assistance. Tadpoles were captured and raised with approval of the Institutional Animal Care and Use Committee of the University of Georgia.

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Herpetological Review, 2008, 39(1), 58–63.

Laboratory Protocols for Husbandry and Embryo Collection of *Anolis* Lizards

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Anolis lizards, or anoles, are a model system for evolutionary biology (e.g., Emerson 2002; Losos 1994), behavioral and physiological ecology (e.g ., Huey et al. 2003; Irschick and Garland 2001; Lovern et al. 2004; Stamps 1983), community ecology (e.g., Pacala and Roughgarden 1985; Schoener 1968), toxicology (e.g., Burger et al. 2004), and physiology and neuroendocrinology (e.g., Greenberg 2003; Wade 2005). Because the genome of *Anolis carolinensis* is currently being sequenced (http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/GreenAnoleLizardAmericanAlligatorSeq.pdf), work on this genus is expected to expand as it becomes more accessible to new disciplines such as developmental and statistical genetics, comparative genomics, and the biomedical sciences. For this reason it is imperative that methods for working with *Anolis* be developed to facilitate comparisons between studies and assure the ethical treatment of these animals as they are used by researchers not accustomed to working with reptiles.

The goal of this manuscript is to describe methods for the maintenance of captive breeding colonies of *Anolis* species. The general characteristics of a species that must be considered to maintain healthy breeding populations include its availability (in the wild or the pet trade), physiological needs (e.g., temperature, humidity, nutrition), and sociality (density of housed individuals) (Greenberg 1992). These factors also must be independently considered for the care of juveniles to assure their proper growth and development. In addition to species-specific factors, practical limitations such as available space, number of species to be housed together, and available resources (incubators, cage washers, climate control systems, etc.) must be considered when developing protocols for the maintenance of animals in captivity. These likely vary greatly from institution to institution. A broad perspective on the general use of reptiles for research can be found in Greenberg (1992) and Pough (1991) and on reptilian egg incubation in Deming (2004).

Here we describe the detailed methods we have found to be successful for the care and maintenance of 13 *Anolis* species from southern Florida and four Caribbean islands that inhabit a wide