

## ENVIRONMENTAL SUBSTRATES ALTER SURVIVAL AND FORAGING EFFICIENCIES IN TADPOLES

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**Abstract.**—Tadpoles of many anuran species live in bodies of water that contain a variety of environmental substrates to which food can be affixed. Especially for benthic and planktonic feeders, acquiring food from various surfaces may differentially wear tadpole mouthparts and cause alterations in foraging efficiencies and subsequent growth. We conducted an experiment to test the hypothesis that foraging substrate type (leaves, stones, wood, glass slides, and no-substrate control) would affect Southern Leopard Frog (*Lithobates sphenoccephalus*) (syn. *Rana sphenoccephala*) tadpole mouthpart damage, percentage of gut that contained food (gut contents), and subsequent body condition. Substrates differentially impacted tadpole survival with significant mortality observed in leaf treatments compared to no-substrate controls. According to a path analysis, only substrate and gut contents were significantly related. Substrate type did not directly influence mouthpart damage or body condition, nor were significant pathways observed between the dependent variables. We conclude that environmental substrates may alter feeding efficiencies and subsequent survival in amphibian larvae, and future research must account for the effects of these naturally occurring variables.

**Key Words.**—environment; feeding; larvae; life-history traits; path analysis

### INTRODUCTION

Growth and development of any organism are influenced by a number of biotic and abiotic factors such as climate change, contamination, habitat desiccation, predation, and competition (Denver 1998; Tsoularis and Wallace 2002; Groner et al. 2013; Caruso 2014; Hanlon and Parris 2014). In aquatic systems, organisms such as larval amphibians are especially susceptible to stressors attributable to their status as obligate-aquatic animals (Siegfried 1993; Relyea 2005; Cox and Lima 2006). Although factors such as predation and competition may inhibit access to a food source, characteristics of a food source alone may be sufficient to alter a potential consumer's food-acquisition efficacy.

Tadpoles occur in a variety of aquatic habi-

tats that can contain any combination of leaves, wood, stones, sediment, or other substrates (Duellman and Trueb 1986). A primary food source for many tadpole species is periphyton (Altig et al. 2007), a mixture of algae, cyanobacteria, and other microbes that attach to submerged surfaces in aquatic systems (Azim et al. 2005). To consume periphyton, tadpoles must first scrape it off of a surface (Kupferberg, et al. 1994; Venesky et al. 2010b). The released particles are then sucked into the tadpole's mouth via buccal pumping and strained by gill filters and papillae or affixed to mucus in the pharynx (Wassersug 1973; Venesky et al. 2010a, 2010b). Because periphyton can attach to multiple substrates, tadpole mouthparts can potentially be worn differently according to the environment. Previous work has shown that mouthpart damage is a suspected pathway by

which reductions in tadpole growth may occur (Venesky et al. 2013). However, no study has investigated differential environmental substrates as a possible mechanism for reductions in growth due to mouthpart damage.

We tested the hypothesis that environmental substrates would differentially affect mouthpart damage and subsequent growth. We conducted a laboratory study using Southern Leopard Frog (*Lithobates sphenoccephalus*) tadpoles. Previous work has shown that *L. sphenoccephalus* graze on substrates (e.g., glass slides to which food is attached) and ingest the released food in experimental settings (Venesky et al. 2010a). Using path analyses, we tested how environmental substrate, mouthpart damage, and percentage of gut that contained food (hereafter, gut contents) influenced body condition (growth measure). We predicted that substrates would vary in their contribution to mouthpart damage and the subsequent pathways (e.g., effects of mouthpart damage on percent food ingested, which in turn could affect body condition). Specifically, we predicted that damage would be greatest in stone treatments, followed by glass, wood, leaves, and no-substrate controls, attributable to differential malleability of the surfaces (more malleable surfaces would allow mouthparts to penetrate the substrates versus scraping against harder surfaces). Finally, we predicted that substrates would not affect tadpole survival.

## MATERIALS AND METHODS

**Animal collection and husbandry.**—We collected 12 *L. sphenoccephalus* clutches from ponds within the University of Memphis Edward J. Meeman Biological Field Station (MBS), Meeman-Shelby State Park, Shelby County, TN (35° 22' N, 90° 01' W) and Shelby Farms Park, Shelby County, Tennessee (35° 9' N / 89° 51' W) between 6 March and 9 March 2013. We transported the eggs to the laboratory at the University of Memphis, Memphis, Tennessee, where, upon hatching, we maintained tadpoles in

8-L aquaria (filled with 4 L of aged tap water). After reaching the free-swimming stage (stage 25; Gosner 1960), we combined the tadpoles from the different clutches into a stock pool from which subjects were selected for the experiment. While in the laboratory, we maintained tadpoles on a 12 h light:12 h dark photoperiod at 19° C.

**Experimental design.**—We used the following substrates in the experiment: a glass microscope slide (positive substrate control), *Quercus rubra* (Red Oak) leaf, *Q. rubra* bark (wood), and a stone (granite river rock collected from a gravel driveway, all with similar textures and composition). We chose these substrates to represent a broad range of possible environmental substrates (Shane M. Hanlon, pers. obs.). Prior to the start of the experiment, we rinsed each substrate with distilled water and placed in an oven at 38° C for 48 h for sterilization. We used this method rather than autoclaving because substrates such as leaves and wood did not withstand the autoclaving process in pilot trials.

We prepared substrates for the experiment by first dissolving Sera Micron® (Sera North America Inc., Montgomeryville, Pennsylvania, USA) fish food in aged tap water at a ratio of 90 mg to 3 mL (as used for husbandry by our lab group). Upon dissolving the food, we pipetted 0.5 mL of the food/water mixture onto one of the four experimental substrates. We then placed substrates in a drying oven at 25° C for 24 h to allow the Sera Micron to dry and affix to the surfaces. To ensure that our substrate-affixing methods were sufficient, via pilot trials, we affixed Sera Micron to each substrate. We then weighed the substrates, placed them into water-filled containers for five days, removed and allowed them to dry in a drying oven at 25° C for 24 h, and then reweighed the substrates. Pre- and post-substrate weights were not significantly different ( $\alpha \leq 0.05$ ) for any substrate. In addition to the substrate treatments, we added 15 mg of food (the approximate amount added to each substrate) directly to the experimental containers as a no-substrate control.

This amount was sufficient to maintain proper tadpole health and has been used previously in our lab for tadpole husbandry. To control for various substrate dimensions, we chose or altered all substrates to reflect dimensions most similar to the microscope slide (75 mm × 25 mm × 1.2 mm).

For the experiment, we randomly selected tadpoles from the stock pool and placed them individually into 1.5 L plastic containers filled with 1.0 L of aged tap water. The five treatments were replicated 12 times for a total of 60 units. We changed the water in each container every five days. Between water changes, we cleaned the containers to prevent accumulation of algae on container walls and fed the tadpoles after each water change. We applied the food/water mixture to each experimental substrate 24 h prior to feeding and allowed it to dry and affix to the substrate(s) in a 25° C drying oven. On each feeding day, we added the substrate (with the affixed food/water mixture, or no-substrate control) to the experimental containers. Substrates remained in each treatment for five days until the next water change. Because of natural degradation, we used new leaves and wood for each feeding. With the slides and stones, we randomly chose substrates for each feeding event from a sterilized stock pool.

We were interested in the effects of substrate on larval measures so we ended the experiment on day 50, prior to initiation of metamorphosis (Gosner 1960). On day 49, we carried out water changes for every individual and introduced food (in each treatment-specific manner) for 24 h. On day 50, we sacrificed tadpoles through lethal exposure to MS-222 according to approved IACUC animal care protocols. We calculated body condition as a function of tadpole mass divided by snout-vent length (SVL). We weighed each tadpole (to the nearest 0.01 g) and measured SVL (to the nearest 0.01 mm).

To measure mouthpart damage, we calculated a deformity index of mouthpart damage adapted from Hanlon et al. (2013). We assessed defor-

mities in 10 zones of the oral disc: labial teeth (anterior tooth rows [3 zones], posterior tooth rows [3 zones]) and jaw sheaths (4 zones). Using a Nikon® SMZ800 dissecting scope with ×10 to ×60 magnification, one observer estimated the proportion of damage per zone for each tadpole on a scale of 0 to 1. We repeated this process for all ten zones for each tadpole. The observer was blind to treatment combinations when performing these measurements.

To calculate gut contents, we dissected each tadpole and removed and straightened the intestine on a dissecting pan. We measured the length of the intestine (to the nearest 0.01 mm) and length of food in the intestine (Sera Micron is easily recognizable in the gut), and determined the gut contents by dividing the latter by the former (adapted from Venesky et al. 2010b). The observer was blind to treatment combinations when performing these measurements.

**Statistical analysis.**—We used a generalized linear model (GLM) with a binomial error distribution and the ANOVA function using the car package in R (R Core Team 2013) to assess the effects of substrate on survival. We used Tukey's post-hoc analyses to test for differences between treatments in survival using the multcomp package in R. We used path analysis with maximum-likelihood estimation to examine the effect(s) of substrates on mouthpart damage, gut contents, and tadpole body condition. We tested the hypothesis that: (1) mouthpart damage; (2) gut contents; and (3) body condition were directly (e.g., substrate to body condition) or indirectly (e.g., substrate to mouthpart damage to food in gut to body condition) influenced by environmental substrates. We predicted that environmental substrates would differentially affect mouthpart damage and the subsequent paths to body condition. We conducted path analyses using the lavaan package in R and assessed the relative strength of each path by the standardized coefficients (a larger value indicates a more significant path). We arcsin transformed mouthpart damage

and gut contents and log transformed body condition to meet normality assumptions. Significance was assessed at  $\alpha = 0.05$ . When substrates were found to have a significant effect on any pathway, we used Tukey's post-hoc analyses to determine the effect of each specific substrate. Prior to conducting the path analysis, we used multivariate and univariate analysis of variance (MANOVA, ANOVA) to determine if substrate type differentially affected mouthpart regions (jaw sheath or teeth) and affected gut length. We conducted these tests to determine if the above-mentioned parameters were appropriate for inclusion in our model (i.e., averaged total mouthpart damage versus specific structures and gut contents versus gut length).

## RESULTS

Substrate had a significant effect on tadpole survival ( $F_{4,59} = 13.85$ ,  $P = 0.007$ ). Post-hoc analysis indicated that leaves significantly reduced survival compared to no-substrate controls (Fig. 1). MANOVA indicated the absence of a significant effect of substrate on teeth and jaw sheath when considered simultaneously ( $F_{4,56} = 1.364$ ,  $P = 0.233$ ). Accordingly, we did not conduct subsequent ANOVAs. There was no effect of substrate on gut length ( $F_{4,28} = 1.200$ ,  $P = 0.333$ ). Thus, total mouthpart damage and gut contents were included as variables in the path model. Our model indicated that only the path from substrate to gut contents was significant (Fig. 2); however, there were no significant differences in gut contents among treatments (Fig. 3).

## DISCUSSION

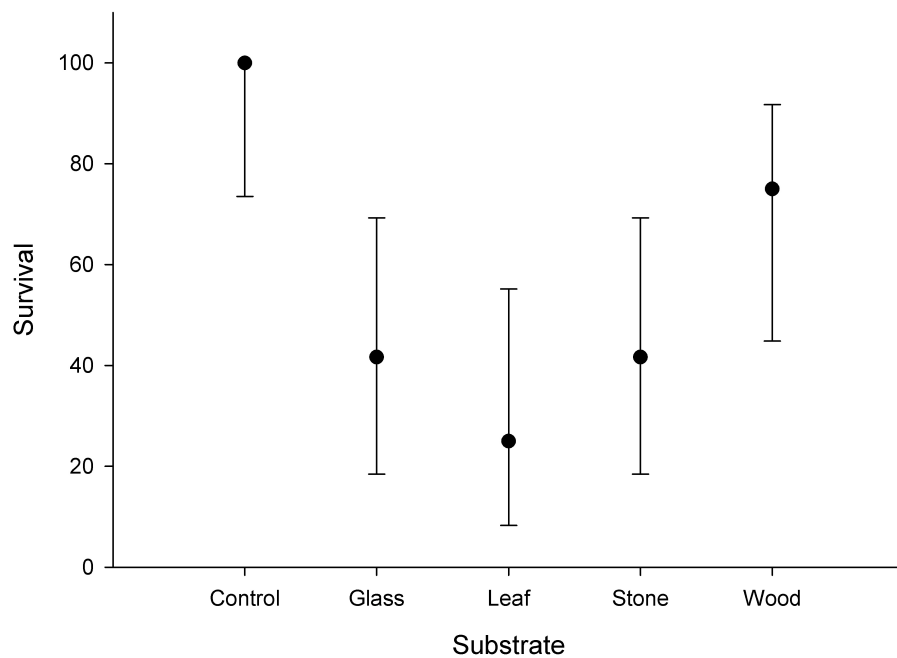
Contrary to our predictions, substrates had varying and significant effects on tadpole survival. In total, 50% of all tadpoles in substrate (excluding no-substrate control) treatments died. The greatest mortality was observed in leaf treatments, which was significantly lower than controls, followed by glass and stone, wood, and control treat-

ments. Overall, treatments with lower food ingestion (gut contents) tended to also have higher mortality rates; however, this is only a qualitative assessment and future research should investigate possible statistical correlations.

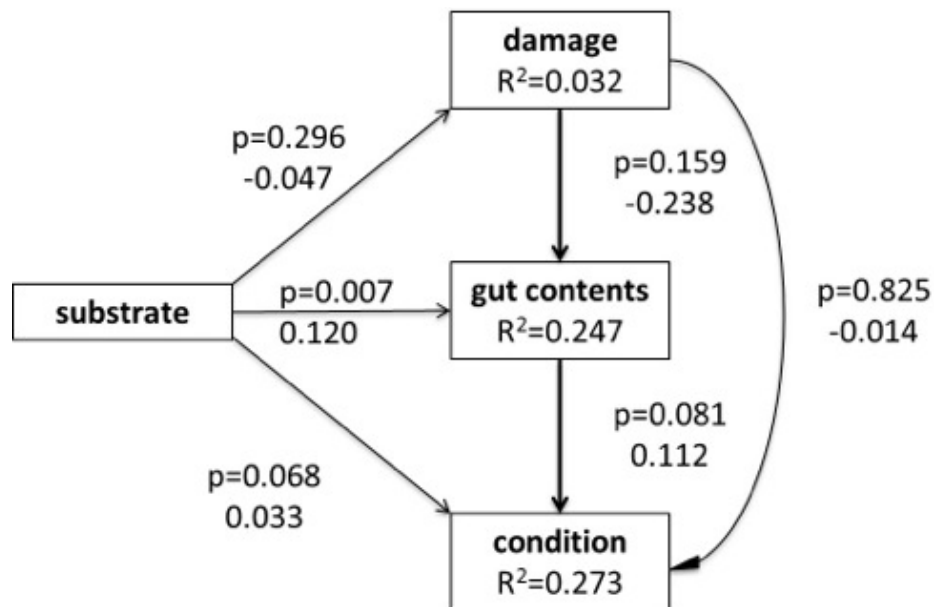
As the significant effect of substrates on survival was unexpected, we are unable to draw any definite conclusions on mechanisms for the occurrence. However, one hypothesis concerning the significant effect of leaves on tadpole mortality concerns the tannins found in the leaves. Earl et al. (2012) showed that leachates from Red Oak (*Quercus rubra*) leaves caused significant mortality in American Toad (*Bufo americanus*) and Gray Treefrog (*Hyla versicolor*) tadpoles. Stoler and Relyea (2011) demonstrated that *H. versicolor* tadpoles in aquatic mesocosms with *Q. rubra* leaves experienced reduced mass compared to those with other leaf treatments. Such findings could help explain our current observation of a significant effect of leaves on tadpole survival through direct toxicity of leaf tannins to tadpoles.

It is important to note the difficulty separating out the effects of each substrate used in the study. Although we controlled for as many variables as possible (e.g., dimensions, organic matter through drying, amount/type of food source), factors such as leaf tannins and other variables were potential confounding factors. We demonstrate here that investigating the role of substrates on amphibian life-history traits and survival can provide valuable insight into environmental impacts on amphibian populations. Moving forward, researchers should continue to investigate this line of research while also finding sufficient ways to control for confounding factors, perhaps through the role of artificial substrates that could serve as a proxy for natural elements.

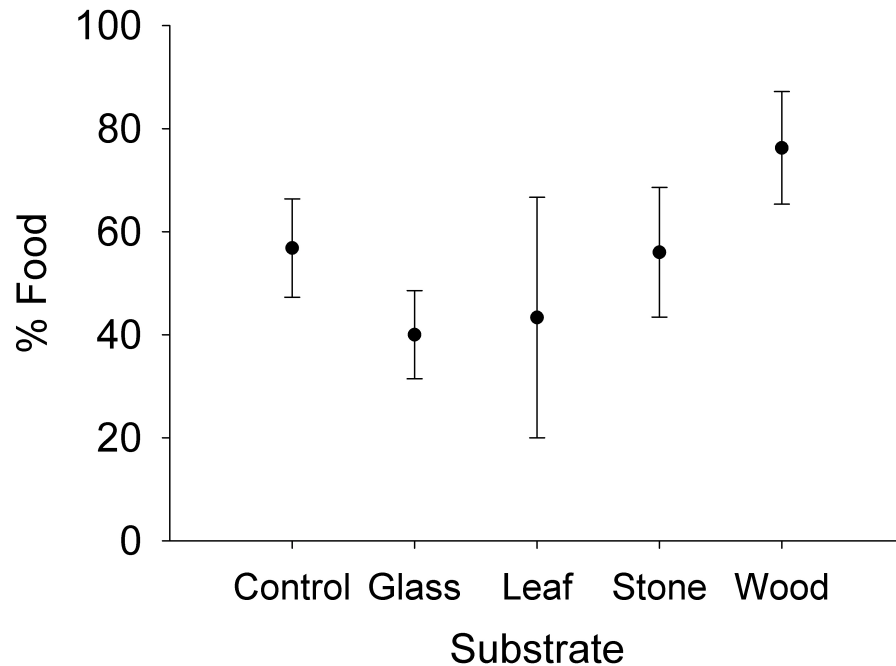
We also found a significant overall effect of substrate on the percent of food ingested; however, we did not detect any significance among-substrate differences in gut contents. Because there was not a significant effect of substrate on mouthpart damage, the exact explanation(s)



**FIGURE 1.** Effects of different environmental substrates on mean survival of *Lithobates sphenoccephalus* tadpoles in different substrates. Survival is displayed as percentage surviving per treatment ( $n = 12$ ). Values plotted are least-square means  $\pm$  95% confidence intervals. Different letters indicate significant differences between treatments ( $\alpha = 0.05$ ).



**FIGURE 2.** Results of the path analysis examining the relationships between environmental substrate, mouthpart damage, gut contents, and body condition in *Lithobates sphenoccephalus* tadpoles. P-values ( $\alpha = 0.05$ ) and standardized coefficients (indicates strength and direction of each relationship) are provided for each pathway.



**FIGURE 3.** Effects of different substrates on gut contents (mean percentage food) of *Lithobates sphenoccephalus* tadpoles. Values plotted are least-square means  $\pm$  1 SE.

for our observations are unknown. Moreover, we must emphasize that any hypotheses on potential mechanisms are speculation. First, the least amount of food ingested was detected in leaf treatments. This was possibly a result of the potentially toxic effects of the tannins in the leaves and may have reduced the ability of tadpoles to successfully obtain and ingest food. In regard to other substrates, one hypothesis is that the composition of the substrate may play a role in foraging efficiencies. For example, the wood used in our study is more malleable than the glass slides. When feeding, tadpoles scrape food off of surfaces to which it is affixed (Venesky et al. 2010b). It is plausible that tadpoles may have been able to consume the food and, inadvertently, the wood to which the food was attached, compared to being able to consume only food attached to less malleable surfaces such as stones or slides. We also propose that the observation of increased food in wood versus no-substrate control treatments could be a function of the location of the

food source. Although tadpoles in no-substrate controls did not have to scrape substrates for food, subjects were forced to forage within the entire experimental container, compared to obtaining food from a single point as in the substrate treatments. Such time and energy spent by tadpoles in no-substrate control treatments could have negated the potential positive effects of not scraping food off of substrates. These potential scenarios may account for the increase in material in the guts of tadpoles in wood treatments compared to other treatments.

Much attention has been given to the effects of disease, pollution, and habitat desiccation on amphibian growth, development, and survival (Denver et al. 1998, Hanlon and Parris 2014). However, the role of factors such as environmental substrates has remained largely unexplored. Given the significant effect of substrate on percent food ingested and survival in our current study, our work highlights the importance of investigating the role of environmental substrates

on amphibian health, life history, and performance.

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