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SHORT COMMUNICATION

Observations on habitat preference of juvenile eastern hellbender salamanders (*Cryptobranchus alleganiensis*)

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Abstract
Laboratory experiments have provided a wealth of knowledge on antipredator responses and habitat preference in both terrestrial and aquatic salamanders, many of which are difficult to study under natural conditions. However, there remains a dearth of carefully designed experiments to elucidate habitat preferences of aquatic salamanders, many of which are of growing conservation concern. This experimental study evaluated shelter selection of nearly threatened larval eastern hellbender salamanders, *Cryptobranchus alleganiensis*, in North Carolina. We performed three experiments: 1) ability to burrow under 50% embedded cobble, 2) preference for cobble (~ 120−150 mm) versus gravel (~ 30−60 mm) in an aquarium (ex situ), and 3) preference for cobble over gravel in a natural stream setting (in situ). We found that salamanders ($N = 11$) showed a preference for cobble (~ 135−137 mm) in both aquarium and in-stream testing environments (87.7% and 88.5% of time, respectively) over gravel and burrowed under cobble in 95% of aquarium and in-stream trials. Moreover, salamanders were unable to burrow under 50% embedded cobble and were actively moving 38.1% of the time in our burrowing experiment, highlighting the potential for larvae to remain exposed to predators if streams contain buried substrate. Our study provides preliminary evidence on the behavior of these cryptic larval salamanders, suggesting that they prefer cobble substrate and are unable to utilize embedded cobble. These findings have management implications for juveniles of this species, particularly in areas where prolonged sedimentation has the potential to make cobble unavailable for larvae.

Keywords Freshwater streams · Animal behavior · Underwater video · Embedded substrate

Introduction

Many animals inhabiting aquatic environments often rely on refugia during some portion of their early life history stages, presumably to facilitate predator avoidance (Wahle and Steneck 1992). Increased sedimentation as a result of land use disturbance can fill interstitial spaces among rocky substrates and thus function as a major threat to the availability of resources that amphibians depend on (Willson and Dorcas 2003; Sepulveda and Lowe 2009), and has the potential to indirectly influence population size and persistence among other demographic parameters. Salamanders often represent a dominant vertebrate and make up exceptionally large biomass in Appalachian streams (Crawford and Peterman 2013). Substrate composition and antipredator responses are among the most important drivers of juvenile salamander survival and behavior (Parker 1991; Storfer and Sih 1998; Tyler et al. 1998). Moreover, embeddedness of substrate (percent to which fine sediments surround substrate on the surface of a streambed) may reduce the availability of interstitial spaces needed by salamanders as refugia from predators (McHugh and Budy 2005; Bank et al. 2006).

Among salamanders, the eastern hellbender, *Cryptobranchus alleganiensis* (hereafter, hellbender), is encountered throughout Appalachian streams of the Southeastern USA (Petranka 2010). This species is experiencing precipitous declines in many watersheds throughout its geographic range (Wheeler et al. 2003; Burgmeier et al. 2011) and is near threatened (Hammerson and Phillips 2004). However, very little is known regarding the behavior of early life stage, gilled, larval hellbenders. Hellbenders rely

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on rock crevices as shelter, and previous research has found that gravel beds are important substrate for larval hellbenders (Nickerson et al. 2003). Moreover, as hellbenders develop into juveniles and adults, they exhibit a shift in microhabitat, with larvae found in interstitial spaces of gravel beds (Hecht et al. 2019) and adults found under larger shelters (Rossell et al. 2013). As many of the habitats hellbenders utilize in streams are increasingly under threat of anthropogenic mortality associated with movement of substrate (Unger et al. 2017), it is imperative to further study their preference for substrate for conservation management.

Previous studies on salamanders under laboratory conditions have added greatly to the understanding of amphibian behavior. Many of these experimental studies conducted in tank or pool enclosures found salamanders' increased use of refugia shelter (Rundio and Olson 2003; Sih et al. 1988) or decreased activity in presence of predator cues (Crane et al. 2012). Subsequently, the availability of substrate and thus refugia habitat is an important consideration for juvenile salamander survival (Rittenhouse et al. 2004), yet information is lacking on burrowing behavior in stream salamanders. Terrestrial salamanders are capable of passive burrowing using their snout and body to enlarge holes, while others may be unable to burrow (Semlitsch 1983). Moreover, few studies have assessed habitat selection or burrowing behavior of aquatic salamanders under embedded cobble substrate simulating increased sediment, a factor which may affect their abundance (Miller et al. 2007). Other aquatic organisms such as crayfish have shown reduced burrowing ability in pebble versus cobble substrate particularly when elevated sediment is present (Dyer et al. 2015). Presently, behavioral preferences of available microhabitat (cobble versus gravel preference) are lacking in the literature for stream dwelling hellbender gilled larvae.

We performed three short-term field experiments designed to elucidate hellbender larval behavior. We experimentally tested larval hellbender preference for cobble versus gravel using a modified aquarium choice chamber and similar design in a stream side channel. We also tested whether salamanders were able to burrow under 50% embedded cobble. We predicted that larval hellbenders would show a preference for larger sized substrate and show limited ability to burrow in embedded cobble.

Methods

We sampled gilled larvae in May and June of 2019 in a tributary of the Hiwassee sub-basin, Nantahala National Forest, Clay County, North Carolina (exact location on file with the North Carolina Wildlife Resources and withheld to preserve site location). This second-order stream has a mixed canopy of *Rhododendron maximum*, *Tsuga canadensis*, and *Pinus strobes* and is at 580-m elevation. Briefly, we searched for free-living gilled larval hellbenders under 100-mm total length. Hellbenders in this size class were 5–8 months in age (unpublished data). We searched for larvae by conducting stream gravel-cobble surveys (lifting rocks ~ 60–200-mm diameter). When a larvae was encountered, we immediately subjected them to experiments. Upon experiment completion (~ 50 min following capture), we recorded total length, mass, and whether or not gills were present for all individual captures ($N = 11$), as well as the size of the shelter rock each larvae occupied in the stream at capture.

Upon capture, we subjected each larvae to a series of three short-term, timed experiments: burrowing, ex situ habitat selection, and in situ habitat selection. We subjected each larvae to all three experiments in a random order, and all larvae were subject to each experiment individually. All experiments took place in temporary enclosures set up along the stream bank. After completing all three experiments and processing, we returned the larvae to their exact point of capture.

Burrowing experiment During the burrowing experiment, we used a small net to place individuals in a 380 mm × 330 mm × 150 mm container filled with stream water (water depth = 10 cm) and 50% embedded small-cobble substrate (~ 145-mm average length) surrounded by a fine layer of very coarse-gravel (~ 40-mm average length) substrate. Individuals were observed for 10 min to assess whether they would exhibit any burrowing behavior (actively retreating under 50% embedded cobble). This level of embeddedness was chosen to standardize trials and simulate sediment loads in streams which would require active burrowing by larvae. We recorded burrowing as a binomial response (success or failure) and recorded movement of salamanders as the total amount of time in seconds when larvae were observed to be actively moving versus sitting still, similar to Davis et al. (2017).

Ex situ habitat selection During our ex situ habitat selection experiment, we placed individuals in the middle of a 50 cm × 30 cm × 25 cm aquarium filled with stream water (depth = 10 cm). We covered the aquarium on three sides to limit potential background disturbance. We provided the larvae a choice between two substrate sizes by covering one-third of the aquarium floor with very coarse gravel (~ 41 average mm length; range 30–56 mm) and the other third on the opposite side of the aquarium floor with small cobble (~ 137.4 average mm length; range 129–150 mm). The middle area was designated as a release area between the two substrate choices and clear of any substrate, similar to Mathis and Unger (2012). We arranged cobble on both substrate sides atop a fine layer of gravel to mimic non-embedded substratum in streams (based on personal observation) (Fig. 1). Substrate size classes were defined according to the Wentworth scale (Wentworth 1922; Hynes 1970). Placement of cobble used in treatments for ex situ experiments was randomized across individual trials according to Ousterhout et al. (2014).

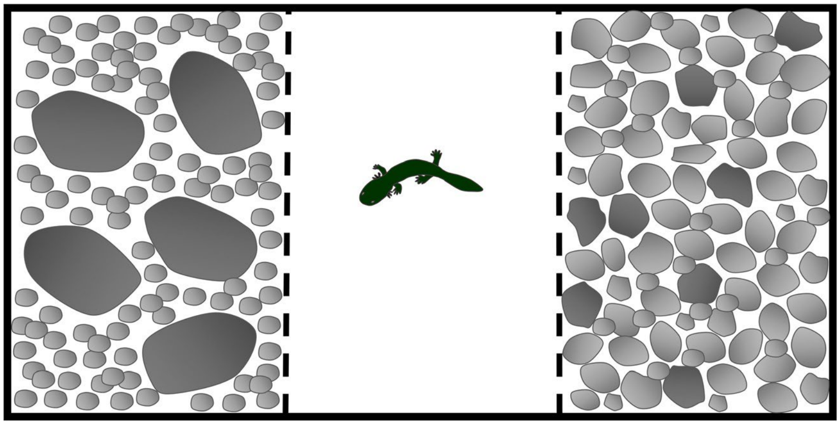

Fig. 1 Experimental design showing individual gilled larval *Cryptobranchus alleganiensis* introduced into ex situ experiment (in-aquarium) showing central testing area where individuals were acclimated and choice of cobble (left) or gravel (right) treatments. Experimental setup for the in situ experiment (in-stream) was similar but performed in a stream bank, while the burrowing experiment was performed in a small plastic container with a different array of 50% embedded cobble placed in gravel

In situ habitat selection During the in situ habitat selection experiment, we attempted to replicate the ex situ experiment in a temporary enclosure within the natural stream. First, we created a shallow depression measuring approximately 500 mm × 300 mm into the natural stream bed in a side channel of the stream that flowed parallel to the main stem where larvae were originally found. Water depth was approximately 10 cm. As in our ex situ experiment, we covered the entire depression with a layer of fine–medium gravel (~ 5–10 mm) and on top of that, covered half of the depression with very coarse gravel (~ 42.2 average mm length; range 30–58 mm), with a similar sized middle area as the ex situ experiment, and the other half with small cobble (~ 135.5 average mm length; range 124–145 mm). Placement of cobble used in treatments for in situ experiments was randomized across individual trials according to Ousterhout et al. (2014).

Experimental procedure For both habitat selection experiments, we introduced larvae into testing enclosures using a small aquarium net. We initially placed them under a clear slotted plastic container (16.2 cm × 7.6 cm × 7.6 cm) for 5 min of acclimation similar to Rittenhouse et al. (2004) and Mathis and Unger (2012). After 5 min, we removed the container and observed larvae for 15 min. We recorded the time spent in either choice chamber (gravel versus cobble habitat), time until salamanders were under cobble shelter (latency to burrow), and choice of shelter (gravel versus cobble). We determined individuals to be fully burrowed under cobble (successful burrowing) if more than 75% of the total body length was under cobble and recorded latency similar to Hickman et al. (2004).

Between trials for burrowing and ex situ experiments, we cleaned the aquarium and container using a 1% vinegar solution, rinsed them with stream water, and fully dried them to maximize independence among trials. Following individual trials for the in situ experiment in the stream area, the depression excavated in the stream was flushed for ~ 5 min to minimize bias between previous individuals or potential chemosensory signals.

We filmed all experiments with a GoPro® action camera (GoPro, Inc., San Mateo, CA, USA) similar to Greene et al. (2016). Observations and video captured during experiments were reviewed by the author (SU) using VLC media player. We compared both time spent moving versus not moving in our burrowing experiment using a Mann-Whitney U test. For in situ and ex situ experiments, we used a Kruskal-Wallis test to assess choice of substrate (gravel versus cobble), accounting for time spent in the middle between treatments. We compared latency to burrow under shelter between in situ and ex situ experiments using a Mann-Whitney U test to examine any potential differences in testing individuals in-stream versus in-aquarium. All statistics were performed in program R version v3.6.2 (R Core team).

Results

Larvae used in our experiments ranged from 2.0–2.4 g and 62.5–75.0-mm total length, and all possessed external gills. All larvae were initially found under cobble ranging in length from 14 to 34 cm (mean $\pm$ 1 SE 20 $\pm$ 1.96). Water temperature for all experiments (stream water) was similar at the time experiments were conducted and ranged from 18.4 to 18.5 °C.

Burrowing experiment We noted a significant difference for time spent moving versus not moving in our burrowing experiment ($U = 0$, $p < 0.001$). On average, individuals spent more time not moving versus moving, mean $\pm$ 1 SE 371.6 s $\pm$ 9.1 and 228.4 s $\pm$ 9.1, respectively. None of the larvae were successful at borrowing under embedded cobble.

Ex situ habitat selection We found a significant difference between time spent in cobble, gravel, and the middle area ($H = 21.0501$, df = 2, $p < 0.0001$, $N = 11$), (Fig. 2). Post hoc tests using the Dunn method revealed significant difference in time spent between gravel and cobble as well as cobble and middle area (both comparisons, $p < 0.001$) but not in time spent between gravel and middle area. Latency to burrow under shelter was mean ± 1 SE 119 s ± 37.9, and all larvae burrowed under cobble. On average, salamanders spent 786.8 s under cobble. We noted that larvae moved along the edge of aquarium during most trials, a behavior described by Greene et al. (2016) as pacing, before selecting cobble.

In situ habitat selection When in the natural stream, we also found a significant difference between time spent in cobble, gravel, and the middle area, ($H = 17.5265$, df = 2, $p < 0.0001$, $N = 11$). Post hoc tests using the Dunn method revealed significant difference between time spent in gravel and cobble ($p < 0.0001$), as well as time spent between cobble and middle area ($p = 0.01$), but not in time spent between gravel and middle area. Latency to burrow varied slightly, mean ± 1 SE 64.5 s ± 24.6.

Comparison of ex situ and in situ habitat selections While we did see a variation in latency to burrow between the in situ and ex situ habitat selection trials, the difference was not statistically significant ($U = 50.5$, $p = 0.535$). Most (10 of 11) larvae selected to burrow under cobble, though one larva burrowed under gravel and spent no time in cobble. Combining percent of time spent in cobble for ex situ and in situ experiments (aquarium and in-stream), salamanders spent 87.7–88.5% of time in cobble, versus 11.3–9.1% of time in gravel (Fig. 2). On average, salamanders spent 796.2 s under cobble substrate.

Discussion

Our short-term observations on the shelter preference of larval eastern hellbenders demonstrate a preference for cobble versus gravel in available cover objects and inability to burrow under embedded cobble. These behavioral preferences are important, as increased sedimentation is often cited as a threat to hellbender populations; however, no empirical research has investigated either the direct or indirect effect of increased sedimentation on this species' behavior or ability to burrow and thus seek shelter from potential stream predators. While both in situ and ex situ experiments are important for obtaining information on larval salamander behavior, we recommend further in situ behavioral observation research, perhaps with underwater action cameras; feeding trials, or to test nocturnal burrowing or anti-predatory behavior, as salamanders may be more active at night or alter behavior patterns. Alternatively, behavior research could be easily modified and performed in a more controlled ex situ captive rearing setting with a longer time frame (e.g., 4–12 h), to allow individuals possibly more time to acclimate or select a wider variety of shelter size and % embeddedness. We did observe a single individual salamander capable of burrowing under gravel and not showing a preference for larger cobble, indicating that natural stream conditions may represent a mosaic of interstitial refugia available to gilled larvae. We point out that our experiments conducted in the field were randomly assigned to salamanders, as we used the same individuals for three treatments, which may have altered behavioral responses based on potential previous experience with any one experiment. However, these results and our findings still have implications for the management of other larger stream salamander larvae

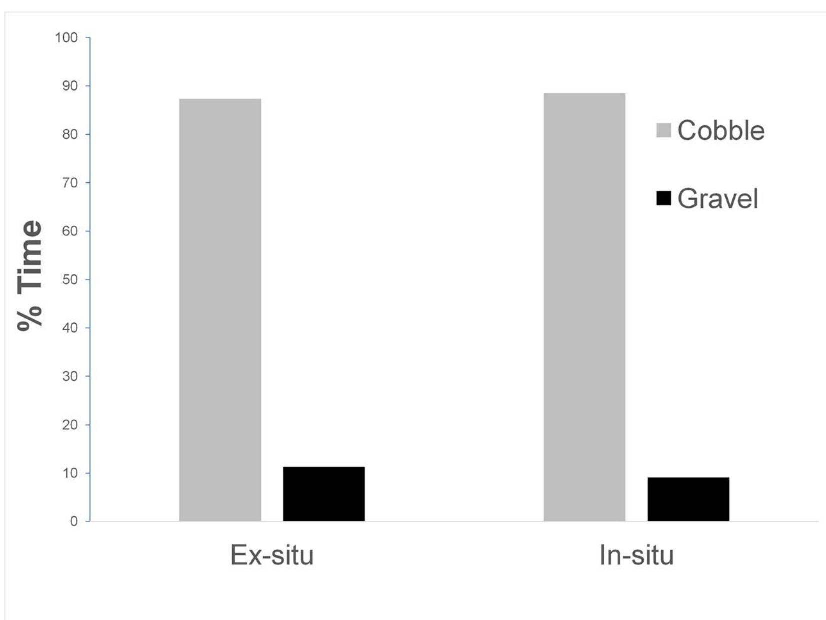

Fig. 2 Percent of time larval *Cryptobranchus alleganiensis* individuals ($n = 11$) spent in habitat (cobble versus gravel) for ex situ experiment (aquarium) and in situ experiment (stream). Note that time spent in central release area was not shown as was less than 5% of time

where cobble habitat may be embedded and unavailable as adequate shelter for the critical larval life stage.

Theory predicts that salamanders should show a preference for habitats that increase their survival or provide shelter from predators (Roberts and Liebgold 2008), but these decisions may often involve tradeoffs between foraging success and predator avoidance (Whitham and Mathis 2000). Moreover, salamanders may alter their microhabitat preference when other salamander species are present (Brodman and Jaskula 2002), indicating that shelter selection in aquatic environments may involve complex ecological and behavioral interactions. Interstitial space and microhabitat are recognized as important potential habitats for larval hellbenders (Nickerson et al. 2003); however, there may be differences across watersheds if increased sediment is altering level of embeddedness, potentially making these shelter habitats unavailable to juveniles. Our findings are similar to Martin et al. (2012), which found that aquatic *Eurycea tynerensis* salamanders always preferred the largest substrate size in pairwise choice experiments. The presence of other salamander species, such as larval *Eurycea* and *Desmognathus*, often encountered in this same stream (S. Unger personal observation), or the presence of conspecifics or even predators may further affect larval hellbender habitat selection. Moreover, larval hellbender refugia choice may be a factor of associated predation risk as noted for other salamanders (Southerland 1986). Native Appalachian potential prey in this stream include trout (Salmonidae) or sculpin (Cottidae) or possibly crayfish, as both crayfish and trout have been observed to feed on salamanders (Tebo and Hassler 1963; Vollmer and Gall 2014). Further ecological and behavioral studies in natural field settings could help determine larval hellbender habitat preferences using more varied substrate type, embedded levels, or combinations of shelter sizes in addition to the effects of prey and predators present in streams with varying sediment loads or embedded substrate.

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Compliance with ethical standards

The experiment followed animal care and use guidelines and was approved by members of the Wingate University Research and Review Board.

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