EFFECTS OF PREDATOR-RELATED CHEMICAL CUES ON THE ACTIVITY

LEVEL OF HOUSTON TOAD (*BUFO* [*ANAXYRUS*] *HOUSTONENSIS*) AND

COASTAL PLAIN TOAD (*BUFO* [*INCILIUS*] *NEBULIFER*) TADPOLES

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Predation is a strong ecological force and plays a role in shaping communities. In
response to predation, many prey species have evolved predator avoidance mechanisms. One
such avoidance mechanism is the detection of chemical cues from predators. This
mechanism of predator detection is especially important in aquatic systems. Many anuran
larvae can detect chemical cues created by the presence of aquatic invertebrate predators and
consumption of conspecific and/or concurrent anurans by these same predators. A

commonly documented larval anuran response to the presence of predator-related chemical cues is the reduction of activity.

Two species that have not been tested for antipredator response in the presence of chemical cues during their larval stage are the coastal plain toad (*Bufo* [*Incilius*] *nebulifer*) and the federally endangered Houston toad (*Bufo* [*Anaxyrus*] *houstonensis*). I conducted two experiments. In the first, I tested if *B. nebulifer* exhibited a reduction of activity (antipredator response) in the presence of chemical cues produced from the presence of an invertebrate predator (kairomone cues) or chemical cues produced from the predation of conspecifics by an invertebrate predator (conspecific diet cues). I also examined whether aggregation status (solo tadpoles vs. groups of tadpoles) mediated the response of *B. nebulifer* to both cues. In the second experiment, I tested if *B. houstonensis* exhibited a reduction of activity (antipredator response) in the presence of kairomone cues, conspecific diet cues, or congeneric diet cues produced from the predation of larval *B. nebulifer*.

I found that individuals and groups of *B. nebulifer* tadpoles reduced their activity level when exposed to conspecific diet cues, but only individuals reduced their activity level when exposed to kairomone cues. I found that *B. houstonensis* larvae lowered their activity in the presence of conspecific diet cues and congeneric diet cues, but not in the presence of kairomone cues. Taken together, these results imply a stronger response by both bufonids to predation than to the presence of a predator alone. I propose some possible hypotheses for the disparity in activity levels between solo and group treatments, diet and kairomone cues, and discuss the ecological implications of these disparities.

CHAPTER I

AGGREGATION STATUS MODIFIES COASTAL PLAIN TOAD *(BUFO [INCILIUS]*

NEBULIFER) TADPOLE RESPONSE TO PREDATOR-RELATED CHEMICAL CUES

Abstract

Many anuran larvae exhibit an antipredator response to chemical cues released by potential predators. The genus *Bufo* is no exception, as many species exhibit an antipredator response (reduction in activity) to the presence (recent and current) of predators. Reduced activity comes at a cost to resource acquisition and growth. Using a mesocosm experiment in a field laboratory setting, I showed that *Bufo (Incilius) nebulifer* tadpole groups and individuals responded to chemical cues produced by 1) the presence of odonate nymphs, and 2) the predation of conspecifics by odonate (dragonfly) nymphs. Data were analyzed using a blocked ANOVA followed by a Tukey's HSD analysis. I found chemical cue to be significant, but did not find the aggregation status (individuals vs groups) to be significant. However, tadpoles appeared to reduce their activity in response to cues produced from predation in both individual and group treatments, but only appeared to reduce their activity in response to cues produced from the presence of predators when they were alone. These results suggest that these tadpoles modulate their response to specific types of chemical cues depending on their aggregation status.

<u>Introduction</u>

Predation is an important ecological factor (Curio 1976). Strong predatory forces can result in reduced prey populations (Macan 1965). This, in turn, can lead to additional effects on prey populations, such as decreased chances of finding a mate or reduced genetic variability (Frankham 1996). Predators can be non-random in their selection of prey, often preferring to prey upon certain size classes (Scott and Murdoch 1983, Quinn and Kinnison 1999) sexes (Quinn and Kinnison 1999), or age classes (Sogard 1997). This selective predation can have potentially negative effects on the population structure of prey populations (Taylor 1983, Banks et al. 2000). Other effects include reduced foraging by prey species (Schmitz 1998), the reduction of prey clutch size and condition (Travers et al. 2010), and the exacerbation of negative effects such as parasitism (Johnson et al. 2006) or increased stress levels (Grafe et al. 2004). In extreme cases, extirpation of the prey species can occur (Fritts and Gordon 1998, Nakata and Goshima 2006). In such situations, the predator-prey system is unstable.

A stable predator-prey system depends on a reproductively viable prey population. One of the ways this is accomplished is through a substantial portion of the prey population avoiding death by predation. To avoid predation, many species have developed predator recognition mechanisms. In aquatic systems, these recognition mechanisms include visual and hydrodynamic mechanisms (Tikkanen et al. 1994). Another mechanism is the recognition of predation-related chemical cues.

There are different types of chemical cues released by predators that appear to be a blend of metabolic wastes and hormones. Chemicals produced via normal metabolic activity can be used for intraspecific communication (pheromones), but can also be detected by prey (Kats and Dill 1998, Turner et al. 1999, Relyea 2001, Mathis et al. 2003). When prey utilize these chemicals for predator avoidance, they are referred to as kairomone cues. Kairomone cues, in predator-prey interactions, can allow earlier detection of the predator by the prey, resulting in a negative effect (missed predation opportunity) for the predator and a positive effect (greater chance of avoiding predation) for the prey, and can be more reliable than visual cues (Hickman et al. 2004). Another type of cue results when syntopic organisms release injured skin cell cues. These release events are generally due to predation or injury, and are referred to as alarm cues. Organisms that produce alarm cues are often (but not necessarily) conspecific or congeneric to the receiver (Chivers and Smith 1998, Chivers and Mirza 2001, Sullivan et al. 2005). The exact chemical makeup of alarm cues are largely unknown, but are thought to be tetrodotoxins in some amphibians (Zimmer et al. 2006). Both kairomone and alarm cues are a component of diet cues, which are a combination of predator metabolic waste products and digested prey, and are capable of inducing learned predator recognition (Mathis and Smith 1993, Chivers et al. 1996). They can be recognized when produced by the consumption of certain conspecific or heterospecific sympatric organisms (Hazlett 1990, Petranka and Hayes 1998, Anholt et al. 2000).

A wide variety of organisms detect chemical cues and alter aspects of their life history, morphology, and behavior in response to them (Magurran 1989, Wisenden et al. 1994, Brown and Smith 1996, Chivers et al. 1996, Chivers and Smith 1998, Kats and Dill 1998).

Detection spans multiple kingdoms and phyla, as protists, rotifers, bryozoans, cnidarians, mollusks, arthropods, echinoderms, and chordates can exhibit responses to chemical cues (Chivers and Smith 1998, Kats and Dill 1998). Exposure causes some species to exhibit life-history shifts, such as the gastropod *Physella virgata* (freshwater snail), which delays reproduction (Crowl and Covich 1990), the crustacean *Gammarus pseudolimnaeus* (an amphipod), which reduces the time invested in forming a precopulatory pair (Mathis and Hoback 1997), or the amphibian *Bufo marinus* (cane toad) which metamorphoses at a smaller size and increases energy invested in predator defense (Hagman et al. 2009). Responses can be morphological as well, such as in the gastropod *Thais lamellose* (sea snail), which develops larger apertural teeth in the presence of predatory crabs (Appleton and Palmer 1988), or as in the zooplankton *Daphnia lumholtzi*, which develops rigid head spines when exposed to predatory fish chemicals (Engel and Tollrian 2009). However, behavioral responses are most commonly documented, and include submergence into substrate, migratorial avoidance of a detected cue, adoption of a watchful posture, cessation of feeding, frantic movement, and reduction of activity (Howe and Sheikh 1975, Smith 1979, Sih 1986, Lawrence 1991, Peterson et al. 1992, Rittschof et al. 1992, Marvin and Hutchison 1995, Yamada et al. 1998).

Anurans exhibit a behavioral response, such as spatial avoidance, to diet and kairomone cues (Petranka and Hayes 1998). However, the most commonly documented behavioral response is the reduction of activity and/or swimming speed, and has been demonstrated using bullfrogs (*Rana catesbeiana*), green frogs (*Rana clamitans*), leopard frogs (*Rana pipiens*), wood frogs, (*Rana sylvatica*), and American toads (*Bufo americanus*) (Skelly

and Werner 1990, Petranka and Hayes 1998, Anholt et al. 2000, Eklöv 2000, Relyea 2004, Fraker 2010). Chemical cues are most likely detected by anurans through their skin, which readily absorbs chemicals from the environment (Hayes et al. 2003), or through their nares, as tadpoles detect intraspecific chemicals using this method (Waldman 1985).

Anuran responses to different types of cues can vary within the same predator/prey species interaction. For example, a study by Buskirk and Arioli (2002) found that increasing the number of proximal larval anisopteran predators was associated with increased morphological responses in pool frog (*Rana lessonae*) larvae, whereas an increase in the number of predation events was associated with increased behavioral responses. Predator diet is also a factor in the response of prey to chemical cues, as response strength to diet cues is positively correlated with phylogenetic closeness of the organism consumed to produce the diet cue (Laurila et al. 1997, Fraker 2009b). However, response to a predation event does not always require the consumption of a conspecific. Some aquatic organisms (larval anurans) exhibit a response to consumption of congenerics (Fraker 2009b) and others (gastropods) respond to phylogenetically distant, but syntopic, species (Dalesman et al. 2007).

Larval anurans are often sympatric with invertebrate predators, which can significantly decrease their survival rate (Eklöv 2000). Chemical detection is a common method of avoiding predation from these invertebrates (Kats and Dill 1998, Petranka and Hayes 1998). The reduction in activity level as described above helps protect larval anurans from nymphal odonate predators such as the dragonfly *Anax junius*, which are attracted to active prey (Folsom and Collins 1984). The exact chemical makeup of anisopteran-released

cues is unknown, but tadpoles show antipredator responses to both kairomone and diet cues released from these predators (Petranka and Hayes 1998, Anholt et al. 2000, Eklöv 2000, Buskirk and Arioli 2002).

Bufo nebulifer is a toad of least concern commonly found throughout southern Texas, Louisiana, and Mississippi, as well as northeastern Mexico (Hammerson & Canseco-Márquez 2004). It was distinguished from the more southern tropical *Bufo* [*Incilius*] *valliceps* by Mulcahy and Mendelson (2000) using mtDNA analysis.

Tadpoles of *B. nebulifer* can be sympatric with nymphal anisopteran (dragonfly) predators. Nymphs are predatory generalists, and will eat other aquatic insects, minnows, crustaceans, and tadpoles (Proche and Runyan 1996). I used *Anax junius* (Aeshnidae)*,* the common green darner, which is globally widespread (Paulson 2009), has been previously identified both in nymphal and adult form in central Texas (Lasswell and Mitchell 1997), and occurs locally within the Lost Pines of Bastrop County, Texas. It is attracted to active over inactive prey (Folsom and Collins 1984), making actively moving tadpoles more vulnerable to predation. In an experiment in which bullfrog (*Rana catesbeiana*) tadpoles were exposed to a fish predator (*Lepomis macrochirus*) and an anisopteran nymph predator (*A. junius*) one at a time and in combination, tadpoles experienced the highest mortality in the presence of the larval odonates alone (Eklöv 2000). Because anuran breeding often occurs in fishless ponds, this implies that anisopteran nymphs can have a large impact on larval anuran populations. The nymphal stage constitutes the majority of an anisopteran's lifespan, as they can take up to five years to reach adulthood, whereas the adults typically live from a few

weeks to six months. Since many anurans breed one or more times per year, one generation of nymphal odonates has the potential to influence multiple generations of larval anurans.

There are multiple factors influencing the degree of a tadpole's response to dragonfly chemical cues, not all of which have been well studied. Tadpoles have been shown to exhibit a continuous dosage response when exposed to chemical cues, so responses are generally graded rather than binary (Buskirk and Arioli 2002). In general, tadpoles will show a greater degree of behavioral alteration when: (A) tadpoles are small (Anholt et al. 2000), (B) the observed species has a long developmental period (Laurila et al. 1997, Fraker 2010), (C) cue concentration is high (Buskirk and Arioli 2002), (D) tadpoles are satiated (Anholt et al. 2000, Fraker 2010), and E) phylogenetically proximal organisms are consumed (Laurila et al. 1997, Fraker 2009b). However, there have been few studies on the effects of aggregation status and differing diet cues on a larval anuran's activity level (Table 1), and none I am aware of that examine interaction effects between the two. I assessed if and how these two effects mediated the response of *Bufo* [*Incilius*] *nebulifer* larvae to kairomone and diet cues.

Publication	Cue	Group Size
Skelly & Werner 1990	Conspecific & Heterospecific Diet (uncontrolled)	10
Laurila et al. 1997	Conspecific & Heterospecific Diet	1
Petranka & Hayes 1998	Kairomone & Conspecific Diet	10
Anholt et al. 2000	Conspecific Diet	10
Eklöv 2000	Kairomone	22
Gallie et al. 2001	Kairomone	5
Buskirk & Arioli 2002	Conspecific Diet	2
Relyea 2004	Conspecific Diet	20/40/80/160
Awan & Smith 2007	Kairomone	1/2/4
Fraker 2008	Conspecific Diet	10
Fraker 2009a	Conspecific Diet	10
Fraker 2009b	Conspecific & Heterospecific Diet	10
Fraker 2010	Conspecific Diet	10

Table 1. Publications describing larval anuran response to chemical cues emphasizing the shortage of comparison between differing chemical cues and aggregation status, and lack of publications incorporating both at once.

<u>Materials and Methods</u>

I reared experimental organisms and ran trials from May 5, 2011 to June 15, 2011 on the Welsh tract, a property owned by Bastrop County and managed by the Texas State University's Department of Biology. Amplectant *B. nebulifer* pairs were collected from the adjacent Griffith League Ranch (GLR), a 404.7 ha tract of land owned by the Boy Scouts of America and placed into plastic tubs approximately 2.1 meters in diameter and 0.9 meters deep. I oriented the tubs on a decline so that the downhill half accumulated rainwater, while the uphill half was filled with sand. Standpipes kept the water from overflowing onto the sand in the event of heavy rainfall. The amplectant pairs laid eggs in these tubs, and provided the tadpoles utilized in this study.

I used a three by two factorial design. The factors were: 1) cue, with three levels, and 2) aggregation status, with two levels. Cue levels were control, kairomone cue, and diet cue. Aggregation status levels were solo and group.

Cue Production

Cue production was similar to protocols followed by Fraker (2010). The control consisted of well water drawn onsite. I produced the kairomone cue in the following manner: 15 dragonfly (Anisoptera) nymphs of the genus *Anax* were put into aquaria and fasted for 24 hours, after which they were separated and transferred to individual plastic containers with 1050mL of well water. I left them undisturbed for one hour and then removed them from their containers. The resulting solution provided kairomone cue. I produced the diet cue by placing 13 *Anax* into an aquarium containing 1050 mL of water per individual, then I

deposited three tadpoles per *Anax* into the aquarium and left them alone for 24 hours, after which I removed the *Anax* and surviving tadpoles (if any). Both diet and kairomone cues were stirred with a sterile instrument to standardize them before use. Both cues were used within 12 hours of production.

Cue Exposure

I used both individuals and groups of five *B. nebulifer* larvae to examine the effects of aggregation status, as some tadpoles are known to aggregate as an antipredator response (Watt et al. 1997, Spieler and Linsenmair 1999). I placed these individuals and groups into a 37.8L aquarium containing 8.4L of well water and allowed them to acclimate for 15 minutes. I introduced approximately 0.86 g of rabbit chow to provide a foraging incentive, started a stopwatch, and began recording the activity level. I quantified the activity level by summing Activity Events (AEs) in five-second intervals over a period of 15 minutes. I defined an AE as a tadpole swimming at least one body length (tail included), or a tadpole ingesting a piece of rabbit chow. I used five second observation intervals with a three second recovery period. If an individual performed an AE during an observation interval, I tallied a mark for that interval. Subsequent AEs performed by that individual were not counted for the remainder of the interval. After the interval, I allowed a three second recovery period to pass without recording regardless of whether there was an AE or not. This served to standardize the time it took for the observer to record data after every interval.

I recorded AEs over 15 minutes, at which point I introduced 1050mL of the respective experimental cue by pouring it gently into the center of the aquarium from > 2cm

above the water's surface to minimize disturbance. There was no visible tadpole response to the physical disturbance of cue introduction. I continued observations over 15 more minutes. AEs were summed for each 15 minute period. There were 12 repetitions of each treatment, with the exception of the group diet cue, which had only 11 due to a paucity of cue available.

I directly observed the solo treatments and recorded AEs as they occurred, whereas I filmed group treatments with a Sony DCR-HC62 Handycam, observing and recording AEs from the film. In the group treatments, I divided the total AEs per 15-minute period in each aquarium by five to determine the AEs per tadpole per aquarium.

Statistical Analyses

I calculated Response Strength (RS) as the proportional reduction in AEs between the first (pre exposure) 15-minute period and the second (post exposure) 15-minute period ([pre exposure AEs - post exposure AEs] / pre exposure AEs). A higher RS implied a stronger reduction in activity.

I evaluated normality in the data set using a Shapiro Wilk test and normal Q Q plot, then applied a 3 way blocked nested ANOVA with aggregation status nested within cue. This was followed by a Tukey's HSD test to examine differences between specific treatments. Calculations were performed in R (R Core Team 2012).

<div align="center">

Results

</div>

Twelve of 39 tadpoles remained after production of diet cue, indicating that 27 had

been consumed by *Anax* in the 24-hour feeding period. This indicated that there was a

predation component in this cue.

The data were normally distributed (Shapiro Wilk test: P = 0.117). Figure 1 shows

the mean activity events in the pre and post exposure periods in each treatment.
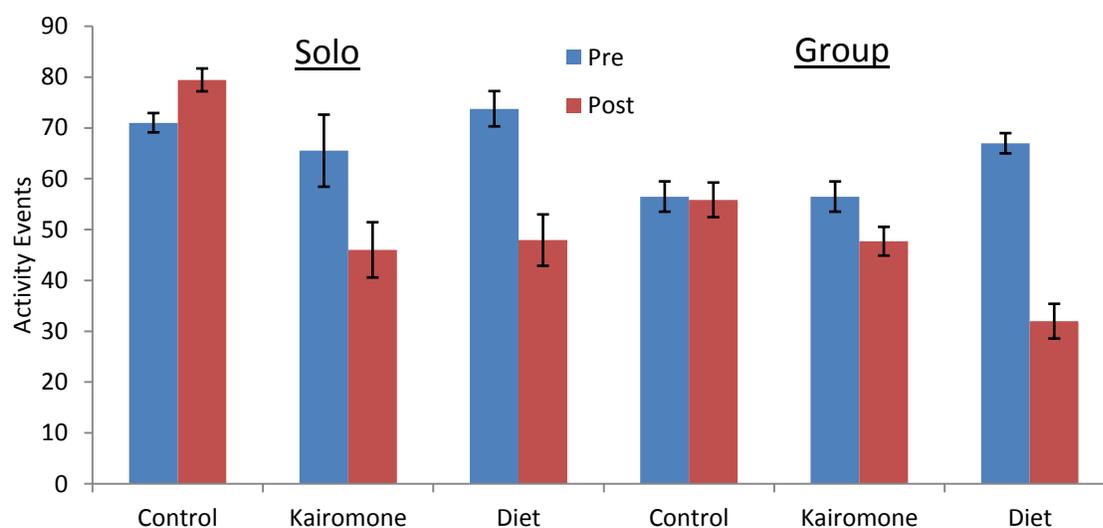
Figure 1. Mean activity events (±SE) of individuals and groups of *Bufo nebulifer* tadpoles
per aquarium per tadpole before (pre) and after (post) exposure to three chemical cues. Both
pre and post exposure periods were 15 minutes long.

When I analyzed the data set with an ANOVA, cue was a significant factor in RS

(ANOVA: $F_{2,64}$=53.81 P < 0.0001), but block ($F_{1,64}$=0.139 P = 0.71) and aggregation status

($F_{1,64}$=2.139, P = 0.15) were not. However, aggregation nested within cue was significant

($F_{2,64}$=7.362, P = 0.0013), indicating that aggregation status had a significant effect in at least

one of the treatments.

In the solo treatments (Fig. 2), the control RS was less than that of the kairomone (Tukey's HSD: p < 0.0001, df = 65) and diet (p < 0.0001, df = 65) cue treatments, and RS of the kairomone and diet cue treatments did not differ from one another (p < 0.98, df = 65).
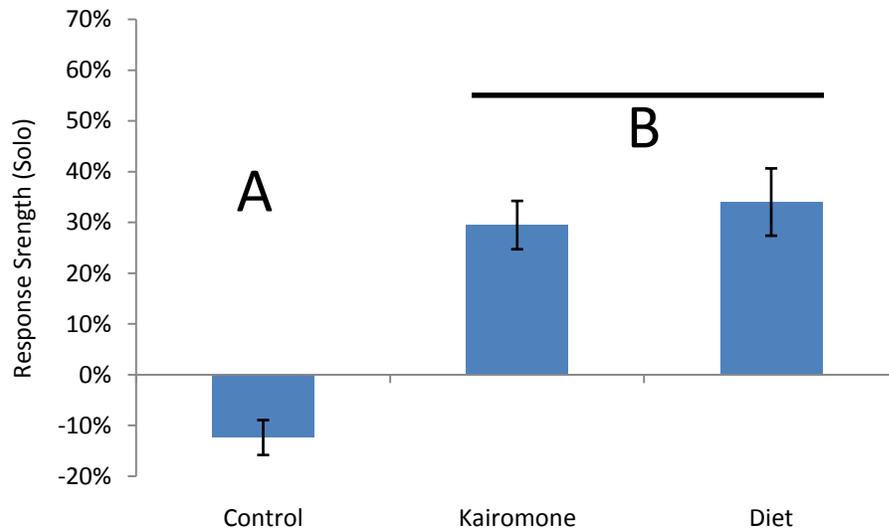
Figure 2. Mean response strength of individual *Bufo nebulifer* tadpoles (±SE) in response to three chemical cues. Response strength was measured in proportional reduction in activity events from the pre exposure period to the post exposure period. Letters in figure indicate groupings from Tukey's HSD means comparisons.

In the group treatments (Fig. 3), the control RS did not differ from the kairomone cue (Tukey's HSD: p = 0.314, df = 65), but it differed from the diet cue (p < 0.0001, df = 65). The kairomone and diet cue differed from one another (p < 0.001, df = 65).
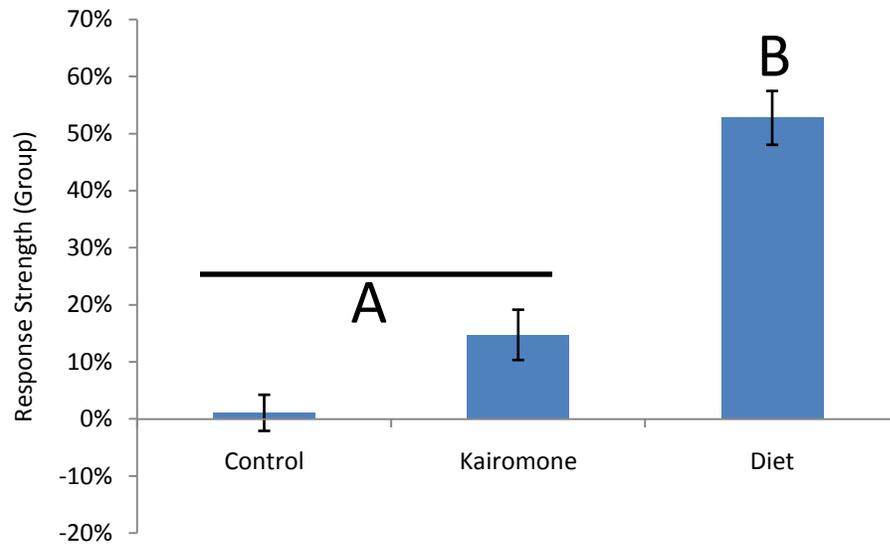
Figure 3. Mean response strength of groups of five *Bufo nebulifer* tadpoles (±SE) in response to three chemical cues. Response strength was measured in proportional reduction in activity events from the pre exposure period to the post exposure period. Letters in figure indicate groupings from Tukey's HSD means comparisons.

Discussion

The significant effect of chemical cue on RS (antipredator response) is consistent with other studies reporting a response by anurans to the presence of predators or predation of conspecifics (Skelly and Werner 1990, Laurila et al. 1997, Petranka and Hayes 1998, Anholt et al. 2000, Eklöv 2000, Chivers and Mirza 2001, Relyea 2001, Relyea 2004, Awan and Smith 2007, Fraker 2009b, Fraker 2010). This activity reduction may slow *B. nebulifer* growth and/or development rates, as has been demonstrated in other anurans (Skelly and Werner 1990, Eklöv 2000, Buskirk and Arioli 2002, Relyea 2004, Hagman et al. 2009), though the duration of this experiment was too short to test this.

In general, aggregation status was not a significant factor on RS. This is consistent with results from previous ethological research on anurans (Awan and Smith 2007) and implies that *B. nebulifer* response to chemical cues is not affected directly by density. However, depending on the cue, aggregation status appeared to mediate the mean RS of tadpoles in a treatment. This result is consistent with a study using *Rana sylvatica* (wood frog) tadpoles, which showed a negative correlation between antipredator response and competitor density (Relyea 2004). Exposure to a kairomone cue, which simulates the presence of predators, but not predation, appeared to induce a significant antipredator response in individual larval *B. nebulifer*, but not in groups. A potential hypothesis for this differential response due to density effects could be an adjustment in activity to increase the likelihood of metamorphosing in a timely manner. Tadpoles developing in ephemeral ponds depend on a steady intake of nutrients in order to metamorphose before the pond dries, and time to metamorphosis in bufonids can increase with increasing densities (Brockelman 1969). Additionally, tadpole deaths by dessication due to relatively high conspecific densities have been observed (Newman 1987). Thus, *B. nebulifer* tadpoles, when in higher densities, may be taking a greater risk of being preyed upon for the tradeoff of more rapid resource acquisition under high densities, which may pose a greater risk of death by dessication. Another possible factor driving the lack of response to kairomone cues when in a group could be a decrease in the perceived degree of predation risk while aggregated. *Bufo* tadpoles can aggregate as a predator avoidance function, (Watt et al. 1997, Spieler and Lisenmair 1999) and *B. nebulifer* tadpoles, when observed on the collection site, were found in groups of >5 individuals without exception (personal observation), suggesting that *B. nebulifer* may

use aggregation to lower the risk of predation. Thus, tadpoles in the group treatments may have already been incidentally displaying a degree of antipredator behavior (aggregation), resulting indirectly in less investment of time by tadpoles in another antipredator behavior (reduction in activity). A final hypothesis for the lack of response is weak cue concentration. Tadpoles have been shown to respond more strongly to increasing concentrations of chemical cues (Buskirk and Arioli 2002), thus the kairomone cue concentration may have elicited a response in the group treatments had it been stronger.

Acknowledgments

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CHAPTER II

HOUSTON TOAD (*BUFO [ANAXYRUS] HOUSTONENSIS*) TADPOLES DECREASE THEIR ACTIVITY IN RESPONSE TO PREDATION OF CONSPECIFIC AND CONGENERIC TADPOLES

Abstract

Anurans may reduce their activity in the presence of predation-related chemical cues. I exposed tadpoles of the federally endangered Houston toad, *Bufo* (*Anaxyrus*) *houstonensis*, to three chemical cues: 1) A kairomone cue produced by the presence of predatory anisopteran nymphs (*Anax junius*); 2) A diet cue produced from the consumption of conspecific tadpoles by *A. junius* nymphs, and 3) A diet cue produced from the consumption of coastal plain toad (*Bufo* [*Incilius] nebulifer*) tadpoles by *A. junius* nymphs. I measured *B. houstonensis* activity levels before and after cue exposure. Tadpole activity was not influenced by the kairomone cue, but activity decreased significantly during exposure to either diet cue. This suggests that predation of both conspecific and congeneric syntopic anurans can lower *B. houstonensis* activity levels. In turn, this reduction in activity may affect *B. houstonensis* growth and development.

Introduction

Amphibian populations are in global decline due to habitat destruction, infectious diseases, and various local factors (Wake and Vredenburg 2008), the current extinction rate possibly exceeding the background extinction rate by a factor of 200 or more (McCallum 2007). Endemic species are particularly at risk, having experienced increased extinction frequency due to human influence (Biber 2002). There are a variety of endemic amphibians occurring in central Texas, including *Eurycea nana* (San Marcos salamander), *Eurycea rathbuni* (Texas blind salamander), and *Bufo* (*Anaxyrus*) *houstonensis* (Houston toad) (Dixon 1987).

The federally endangered (Hammerson and Shepard 2004) Houston toad, first described in the 1950s (Sanders 1953), was once abundant throughout the central plains region of Texas, but has been relegated to an increasingly small area due to urban expansion and drought. There have been no detections in the vast majority of its former home range since 1990 (Hammerson and Shepard 2004), and it has been observed only to inhabit areas with sandy soils (Kennedy 1962, Brown 1971), which may contribute to its endemism.

Anurans known to be sympatric with *B. houstonensis* include *Acris crepitans, Bufo (Incilius) nebulifer, Bufo (Anaxyrus) woodhousii, Gastrophryne olivacea, Gastrophryne carolinensis, Hyla squirella, Hyla cinerea, Hyla versicolor, Pseudacris nigrita, Pseudacris clarki, Rana pipiens, Rana sphenocephala,* and *Scaphiopus hurteri* (Kennedy 1962, Hillis et al. 1984, Brown et al. In Press). Of these, *B. nebulifer,* an abundant toad of least concern throughout the southeastern United States and northeastern Mexico (Hammerson and

Canseco-Márquez 2004), has the potential to impact the remaining *B. houstonensis* population fragments, as their breeding seasons can overlap and there have been multiple recorded instances of hybridization, both in a laboratory setting and in nature (Kennedy 1962, Brown 1971, Hillis et al. 1984).

Understanding the life history and behavior of an organism is important when managing for its persistence (Pearson and Healey 2003, Pecl et al. 2004, Mangel et al. 2006), yet the larval ethology of *B. houstonensis* is largely unknown. Tadpoles develop in chemically complex aquatic environments, often with low visibility. In such environments, aquatic amphibians utilize chemical, rather than visual or tactile, cues to detect predators (Stauffer and Semlitsch 1993, Kiesecker et al. 1996, Hickman et al. 2004) and respond to predation-related chemicals both in laboratory and natural settings (Petranka and Hayes 1998).

If a single exposure to chemical cues induces a behavioral alteration in *B. houstonensis* tadpoles, repeated exposures over a long period of time may induce morphological alterations as well. Anurans repeatedly exposed to predation-related chemical cues exhibit slower growth (Skelly and Werner 1990, Eklöv 2000), smaller size at metamorphosis (Skelly and Werner 1990, Hagman et al. 2009), more energy invested in predator defense (Hagman et al. 2009), and changes in overall body structure (deeper tails and shorter bodies) (Relyea 2004, Maher et al. 2013). In ephemeral water bodies, slower growth may result in a failure to metamorphose before mortality via dessication (Newman 1987) . Slower growth would also result in more time spent in a predation-vulnerable stage, as arthropod predator capture success against tadpoles increases with decreasing tadpole size

(Formanowicz 1986). In addition, tadpoles metamorphosing at a smaller body size may not be able to consume as diverse an array of prey items, as a predator's prey size and diversity typically increase with body weight (Gittleman 1985).

I exposed *B. houstonensis* tadpoles to chemical cues produced from the presence of a dragonfly nymph predator, from the consumption of conspecifics, and from the consumption of *B. nebulifer* tadpoles and observed the effects on activity level in order to test an antipredator response of *B. houstonensis* to these chemical cues.

Materials and Methods

I reared experimental organisms and ran trials from March 18, 2012 to April 12, 2012 on the Welsh tract, described in the previous chapter. I caught nymphs of the dragonfly *Anax junius* (Anisoptera) on the GLR and maintained them in a 66.3L plastic container with aquatic vegetation.

I obtained *B. houstonensis* larvae from the Houston Zoo's headstarting program at Gosner stage 25-36 (Gosner 1960) and released them into plastic tubs approximately 2.1 meters in diameter and 0.9 meters deep, described in the previous chapter. The captive rearing of these *B. houstonensis* individuals likely does not affect their response to experimental stimulus, as captive-reared anurans can have similar stress responses to wild specimens as determined by hematological assay if the behavior is innate (Davis and Maerz 2011).

I obtained *B. nebulifer* larvae from ponds on the GLR. Three amplectant pairs of *B. nebulifer* were caught at night and maintained in the same manner as the *B. houstonensis*

larvae. Of the three amplectant pairs, one had produced eggs the following morning. I then

returned all of adults to their ponds of origin. Tadpoles fed off of naturally occurring detritus

in brooding tanks. Individuals were kept until the time of cue production, 15-19 days later.

At time of cue production, *B. nebulifer* individuals were between Gosner stages 23 and 25

(Gosner 1960). Afterwards, I released all remaining individuals into the pond from which I

had captured their parents. *B. houstonensis* and *B. nebulifer* individuals were kept in

separate brooding tanks throughout the time they were housed at the facility.

Cue Production

I used four treatments: 1) a control, 2) a kairomone cue, 3) a conspecific diet cue,

and 4) a congeneric diet cue. The control consisted of well water drawn onsite. I produced

the kairomone cue by withholding food from *Anax* nymphs for 24 hours, then leaving them

for 12 hours in a proportional (230 times the quantity of water displaced by nymphs)

volume of well water in multiple plastic containers (2 nymphs per container). After cue

production, I poured the kairomone cues from individual containers into a common plastic

bag, mixed them, and froze them at -18˚ C until day of use. Previous experiments have

utilized frozen cues with no apparent effects resulting from freezing (Marvin and Hutchison

1995, Wisenden et al. 1997, Ślusarczyk and Rygielska 2004). I produced the conspecific diet

cue in the same manner, but a consistent mass of *B. houstonensis* (10mL worth of water

displacement) larvae was introduced into the containers to allow *Anax* to feed upon them.

Only containers in which 80% or more of the offered tadpoles had been consumed were

utilized for chemical cue. Remaining tadpoles were removed and put into an empty brooding

tank. These individuals were not sampled for activity observation. I produced the congeneric

diet cue in the same manner, but I offered *B. nebulifer* larvae instead of *B. houstonensis* larvae. Prior to cue production, the plastic containers were cleaned with hydrogen peroxide and triple rinsed with well water.

Cue Exposure

I partitioned two 37.45L aquaria into four compartments each with opaque plastic shielding. Water and chemical cue were not allowed to pass through these compartments. The aquaria were placed adjacent to each other lengthwise. I placed additional plastic shielding between the two aquaria so each group of tadpoles were only able to see each other, not tadpoles in other blocks. I conducted experiments in a two by four randomized block design. I placed five *B. houstonensis* tadpoles into each partition with 2000mL of 22.3° C well water and allowed them to acclimate for 10 minutes. After the acclimation period, I introduced approximately one gram of pre-weighed rabbit chow into each partition to provide a foraging incentive. I then filmed the eight partitions simultaneously with a Sony DCR-HC62 Handycam mounted above the partitions. After an eight minute pre-stimulus period, I introduced 22mL (one part cue per 90 parts water, similar to protocols used in Brown et al. 2011) of respective cue into each partition with a dual injection system to minimize disturbance. After cue introduction, I continued filming for eight minutes more to observe the possibility of a post stimulus response. The dual injection system consisted of two syringes per partition, one of which contained the cue for the treatment in that partition, the other of which contained 22.3° well water. These syringes were connected to the partition with plastic aquarium tubing. During cue introduction, I injected the cue aliquot then immediately injected an equal amount of well water in order to assure that all of

the cue in the tubing had been delivered. I ran 24 repetitions of each treatment using a different group of tadpoles for each repetition. After each filming session, I placed the filmed tadpoles into a separate brooding tank to avoid resampling them during a subsequent trial run.

The observer was blind to the treatment being observed. Activity Events (AEs) in three-second intervals were recorded in the pre and post exposure periods. I defined an AE as a tadpole swimming at least one body length (tail included), or a tadpole ingesting a piece of rabbit chow. If an individual performed an AE during an observation interval, I tallied a mark for that interval. Subsequent AEs performed by that individual were not counted for the remainder of the interval. The reduction in interval time and the omission of the recovery period of the previous experiment allowed for a higher sampling resolution.

Statistical Analyses

All statistical analyses were performed in R (R Core Team 2012). The parameter of interest was Response Strength (RS) mediated by chemical cue. I calculated RS as the proportional reduction in AEs between the first (pre exposure) 8-minute period and the second (post exposure) 8-minute period ([pre exposure AEs - post exposure AEs] / pre exposure AEs). A higher RS implied a stronger reduction in activity. I created a Q-Q plot to test for normality, then performed a two-tailed t-test to determine whether each treatment differed from zero in order to ensure that the control treatment was a reliable baseline. I ran a one-way randomized block ANOVA with response strength as the response variable and treatment (chemical cue) as a categorical factor with four levels (control, kairomone,

conspecific diet cue, and congeneric diet cue). I ran a Tukey's honestly significant difference (HSD) test to determine which treatments differed significantly from one another (α = 0.05).

Results

The data were normally distributed and homoscedastic, thus it met the requirements of parametric analysis. The mean difference between the pre exposure period and the post exposure period was negligible in the control, slight in the kairomone treatment, and high in both diet cue treatments (Fig. 4).

There was no significant difference between zero and the control treatment (two-sided t-test: t_{23}=0.80, p=0.44) or the kairomone treatment (two-sided t-test: t_{23}=1.35, p=0.19). There was a significant difference between zero and the conspecific diet cue treatment (two-sided t-test: t_{23} = 6.52, p < 0.0001) as well as between zero and the congeneric diet cue treatment (two-sided t-test: t_{23} = 9.51, p < 0.0001).

When analyzed with an ANOVA, cue was a significant factor (ANOVA: $F_{3,69}$= 15.39, p < 0.0001), but block was not (ANOVA: $F_{23,69}$ = 0.89, p = 0.62). There was no significant difference between the control and kairomone treatments (Tukey's HSD: p = 0.85, df = 92; Fig. 5), but there was a significant difference between the control and the conspecific diet cue treatment (Tukey's HSD: p = 0.0007, df = 92; Fig. 5). There was also a significant difference between the control and the heterospecific diet cue treatment (Tukey's HSD: p < 0.0001, df = 92; Fig. 5). There was no significant difference between the conspecific and heterospecific diet cue treatments (Tukey's HSD: p = 0.26, df = 92; Fig. 5).
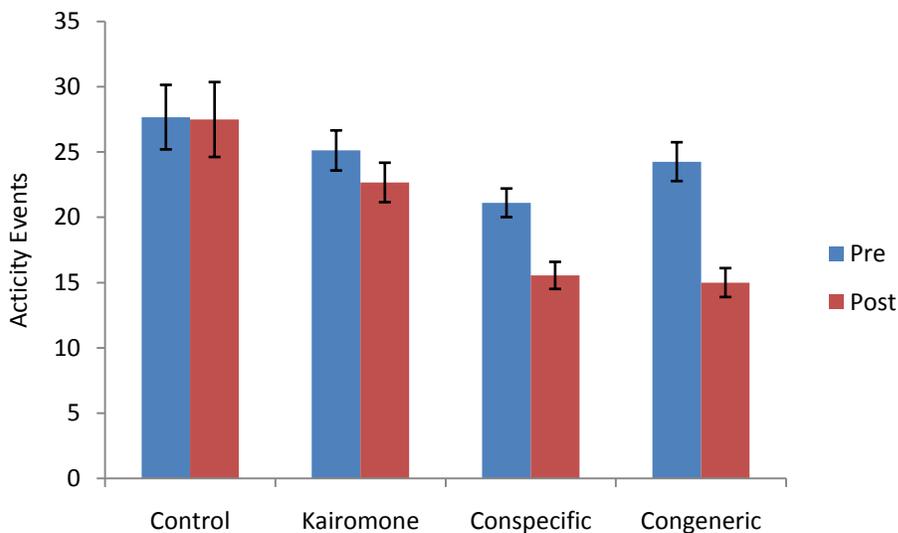
Figure 4. Mean activity events (±SE) of *Bufo houstonensis* tadpoles per aquarium per tadpole before (pre) and after (post) exposure to three chemical cues. Both pre and post exposure periods were 8 minutes long.
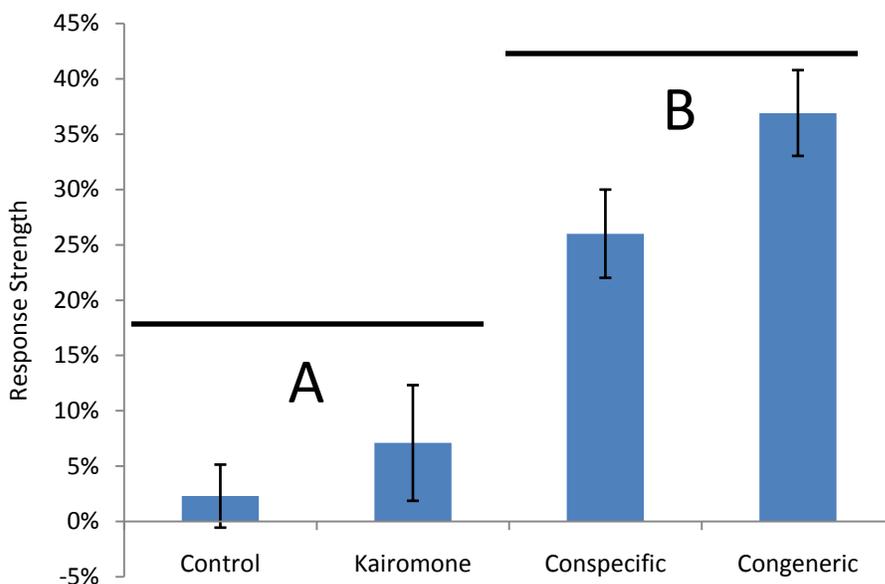
Figure 5. Mean response strength of groups of five *Bufo houstonensis* tadpoles (±SE) in response to four chemical cues. Response strength was measured in proportional reduction in activity events from the pre exposure period to the post exposure period. Letters in figure indicate groupings from Tukey's HSD means comparisons.

Discussion

Bufo houstonensis tadpoles significantly reduced their activity levels when exposed to chemical cues from predation of both conspecifics and individuals of *B. nebulifer*. However, they did not respond to chemical cues produced from the presence of predators alone. Predator avoidance is a trade-off with foraging activity, and an organism must balance predator defense with foraging activities in order to achieve maximum resource acquisition with minimal predation risk (Lima & Dill 1990). The differential response of *B. houstonensis* may indicate that it evolved in conditions in which it was disadvantageous to decrease activity whenever predators were encountered, but it was advantageous to decrease activity when predation of a phylogenetically proximal organism was detected.

Bufo houstonensis tadpoles did not respond to the kairomone cue. A possible hypothesis for this lack of a response is its life history. *Bufo houstonensis* breeding typically occurs in ephemeral water bodies (Kennedy 1962, Brown 1971), and anurans which have historically utilized temporary water bodies have been shown to respond less strongly to chemical cues (Horat and Semlitsch 1994, Anholt et al. 2000, Fraker 2010). An alternative hypothesis could be that there was a relatively weak response because the individual tadpoles used were in late Gosner stages. Growth-stage and size-moderated responses to chemical cues have been documented, with later growth stages and larger size negatively correlated with cue response (Anholt et al. 1996, Anholt et al. 2000). This would confer greater antipredator resistance, given that larger tadpoles are in less danger of predation by aquatic invertebrates (Formanowicz 1986). Another hypothesis for the lack of response to a kairomone cue is insufficiently high cue concentration. Tadpoles have been shown to respond more strongly

to increasing concentrations of chemical cues (Buskirk and Arioli 2002), thus there may have been a response to the kairomone cue had its concentration been higher.

A final hypothesis for the lack of response to the kairomone cue may be the focal individuals' lack of experience with predator cues. It has been demonstrated that predator-experienced larval anurans can respond more strongly to predator-related chemical cues than non predator-experienced tadpoles (Fraker 2009a). An innate response (as was induced with the diet cue) may indicate a long evolutionary history between the organisms involved in the production and sensing of the cue, as responses in focal organisms are typically stronger when phylogenetically proximal organisms are consumed (Laurila et al. 1997, Fraker 2009b). Innate responses can be advantageous because a previous encounter with a predator is not necessary to induce the response in an individual (Laurila 2000). However, innate responses have the disadvantage of limited predator recognition; they do not confer any resistance against novel predators (Wisenden 2003). The differential response between diet cues and kairomone cues may indicate a substantial period of syntopy between *B. houstonensis* and *B. nebulifer*, as the predation component of either of these species in a cue elicited a response in *B. houstonensis*.

The behavioral response of *B. houstonensis* to a conspecific diet cue is consistent with other studies done with *Bufo* species using alarm cues or diet cues (Petranka 1989, Laurila et al. 1997, Petranka and Hayes 1998). In general, *Bufo* species have been shown to respond more strongly to diet cues produced by the consumption of conspecifics by *Anax* than by kairomones produced from *Anax* predators (Anholt et al. 1996, Laurila et al. 1997, Petranka and Hayes 1998), suggesting that overall, *Bufo* species will respond more strongly

to predation than to the presence of predators. This suggests that *B. houstonensis* tends to favor foraging behavior over predator avoidance behavior, resulting in a minimal fitness reduction due to inducible defenses (Buskirk 2000).

The significant antipredator response of *B. houstonensis* tadpoles to congeneric diet cues is not unique among anurans (Fraker 2009b), but has considerable management implications. The breeding activities of *B. nebulifer* overlap both geographically and temporally with the breeding activities of *B. houstonensis*, to the point which hybrids are produced (Brown 1971, Hillis et al. 1984). Since dragonflies oviposit based on the needs of their nymphs (Buskirk and Sherman 1985), *Anax* may factor prey availability into potential deposition sites. If *B. nebulifer* breeds contemporaneously with *B. houstonensis* there will be ample prey for *Anax* nymphs, which may encourage increased dragonfly oviposition in breeding ponds. In addition, there may be increased predation events overall, as increased prey can lead to increased predation (Estabrook and Dunham 1976). In addition, the contribution of *B. nebulifer* the increase in number of prey may lead to increased abundance and decrease in cannibalism of *Anax* predators, as has been shown with other carnivorous arthropods (Wise 1979, Rypstra 1986).

The life history of *B. houstonensis* may also be impacted by long term exposure to conspecific and *B. nebulifer* diet cues. Repeated exposure to diet cues induces slower growth (Skelly and Werner 1990, Eklöv 2000, Relyea 2004). Because smaller tadpoles are more at risk to predation (Formanowicz 1986, Eklöv 2000), this could increase the time spent in a vulnerable stage and therefore larval mortality. Theoretical models show that anuran larvae should metamorphose out of a dangerous aquatic environment at a cost in body mass

(Wilbur and Collins 1973, Relyea 2007). Experimental data bear this out, as anurans exposed to chemical cues exhibit smaller sizes at metamorphosis (Skelly and Werner 1990, Hagman et al. 2009). This may lead to increased predation of *B. houstonensis* metamorphs by gape-limited predators (Urban 2007), contributing to mortality past the tadpole stage.

Exposure to alarm cues can increase the energy invested by an anuran metamorph in predator defense, leading to higher toxicity (Hagman et al. 2009). This may be the case with *B. houstonensis*, but has not been tested. If *B. houstonensis* develops higher toxicity when repeatedly exposed to *Anax* chemical cues, this may counteract some of the vulnerability of reduced size at metamorphosis. However, the increased antipredator defense may not be enough to offset the negative effects of slower growth rate and smaller metamorph size, as some predators have exhibited both behavioral and morphological defenses against parotoid toxins (Licht and Low 1968, Phillips and Shine 2006), such as the ability to selectively reject individual amphibian prey too toxic to consume (Williams et al. 2003) and the ability to avoid the parotoid glands altogether during consumption (Schaaf and Garton 1970).

From a management perspective, spatial and temporal breeding overlap between *B. nebulifer* and *B. houstonensis* may act antagonistically on the recovery of *B. houstonensis*. Currently, only ponds without fish are recommended for Houston toad breeding. However, predation on tadpoles can be higher in ponds containing only nymphal odonates than in ponds containing both fish and nymphal odonates in combination (Eklöv 2000). If *B. houstonensis* and *B. nebulifer* breed concurrently in these ponds, *B. houstonensis* will most likely be developing in a more diet cue rich environment, as the likelihood of a tadpole encountering an *Anax* or another invertebrate predator will go up. Observationally, larval *B.*

nebulifer on the GLR appear to have a larger foraging range than *B. houstonensis* (Michael Forstner, personal comm.), which may further increase the chance of a tadpole encountering a predator. This indicates that currently recommended breeding sites for *B. houstonensis* may, if their behavioral response leads to a life-history response as in the examples above, contribute to increased mortality via growth and metamorph size retardation.

In summary, the presence of conspecific and a congeneric diet cue cause a behavioral response in *B. houstonensis*. This response, if induced repeatedly during the larval developmental period, may have negative effects on the life history of *B. houstonensis* at larval and post-larval stages. Long-term studies would be useful in order to determine how exposure to predation-related chemical cues over an extended period would affect *B. houstonensis* growth, morphology, and post-metamorph predation vulnerability.

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VITA

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