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Innate Predator Recognition and the Problem of Introduced Trout

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Abstract

Innate predator recognition typically only occurs when there is an evolutionary history between predator and prey. Predator introductions thus can pose a substantial threat to native fauna that rely heavily on inherent identification of predators. In permanent aquatic habitats prey often encounter a variety of predatory and non-predatory fish species, and the ability to distinguish between the two is essential to avoid wasted time and energy spent in unnecessary antipredatory efforts. Here, we present a study evaluating the ability of lab-reared larvae of an endangered fully aquatic salamander (hellbenders: *Cryptobranchus alleganiensis*) to recognize chemical cues from native and introduced fish predators. We recorded responses of hellbender larvae to chemical stimuli from native and non-native predatory fishes, a non-predatory fish and a blank control. Eastern hellbender larvae (*C. a. alleganiensis*) significantly reduced activity in response to chemical stimuli from native predators (*Micropterus salmoides*, *Micropterus dolomieu*, *Ambloplites rupestris*, *Sander vitreus*, and *Cottus carolinae*), but responses to non-native rainbow (*Oncorhynchus mykiss*) and brown (*Salmo trutta*) trout were not significantly different from responses to the non-predatory control (redhorse sucker, *Moxostoma* spp.). Responses of larval Ozark hellbenders (*C. a. bishopi*) to brown trout were similar to that of the native fishes and different from the blank control, but responses to rainbow trout did not differ from the blank control. The generally weak responses of larval hellbenders to chemical cues from introduced predatory trout could lead to increased predation in the wild, which may have exacerbated the decline of hellbender populations.

Introduction

Innate predator recognition should result in optimal predator avoidance when the youngest age classes experience intense predation or when there is a low probability of survival once prey are detected and captured by a predator (Murray et al. 2004). Because recognition can occur immediately after hatching, a dangerous encounter with a predator is not required and any possibility of erroneous learning is eliminated. However, innate predator recognition should only evolve in stable habitats with constant predatory regimes (Wisenden 2003) and where predator and prey co-exist over evolutionary time (Kats & Ferrer 2003; Ferrari et al. 2007a). Moreover, this type of predator recognition likely limits the extent of predators that can be identified and leaves prey vulnerable to predation by introduced predators (Wisenden 2003).

Fishes are considered the most destructive predators of amphibian larvae (Petranka et al. 1987; Semlitsch & Gavasso 1992), and are common predators of amphibian eggs, larvae, and adults (Duellman & Trueb 1986). Many species of salmonids, such as rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*), have been extensively stocked for recreational fishing, with little consideration of their potential effects on native species (Fausch 1988). Trout are effective predators and have been reported to be a factor in the global decline in amphibian populations. For example, in Spain, introduction of both rainbow trout and brown trout reduced amphibian abundance, and diversity (Braña et al. 1996). In the western United States, an in-depth survey of 1700 mountain lakes found that introduced trout distributions were strongly negatively correlated with the distribution of the mountain yellow-legged frog (Knapp & Matthews 2000), and experimental removal of trout from five lakes resulted in the rapid recovery of frog populations (Vredenburg 2004).

Although many studies have examined the behavioral responses of aquatic amphibians to introduced predatory species (e.g. Kiesecker & Blaustein 1997; Kiesecker et al. 2001; Pearl et al. 2003; Marquis et al. 2004; Bosch et al. 2006), these have primarily focused on (1) the aquatic larvae of a terrestrial amphibian and (2) permanent aquatic habitats historically devoid of predatory fishes (e.g. high elevation lakes). The effect that introduced fishes have on amphibians that have co-evolved with other predatory fishes has not been well studied, and we are aware of only a single study that documents the response of a permanently aquatic amphibian to native and introduced fishes. In that study, Epp & Gabor (2008) demonstrated that captive-reared *Eurycea nana* showed innate recognition of chemical cues from a non-native predator, the redbreast sunfish (*Lepomis auritus*). Because other native sunfish of the same genus co-occur with the prey species, the authors attributed the response to the non-native species to the close degree of relatedness between the native sunfish and the non-native species: closely related species should share a similar chemical signature (Ferrari et al. 2007a, 2008).

Chemically mediated predator detection is widespread among amphibians and enables prey to identify predators that are cryptic or in low-visibility habitats (Kats & Dill 1998). Using chemical information, prey can gauge the current level of predation risk, allowing them to minimize the threat of predation and maximize alternative fitness-increasing activities (Lima & Dill 1990). Although some amphibians learn to recognize predators through experience (Woody & Mathis 1998; Ferrari et al. 2007b; Mathis et al. 2008), many innately recognize chemical stimuli from predators (Kats et al. 1988; Elliott et al. 1993; Sih & Kats 1994; Kiesecker & Blaustein 1997; Griffiths et al. 1998; Petranka & Hayes 1998; Gallie et al. 2001; Epp & Gabor 2008). Innate predator recognition should be especially important in amphibians because small (young) individuals are particularly vulnerable to predation; many individuals would be consumed before they have the opportunity to learn to identify unfamiliar predators.

The hellbender (*Cryptobranchus alleganiensis*) is a fully aquatic salamander whose two subspecies are native to the continental United States. The eastern subspecies (*C. a. alleganiensis*) has a larger geographic distribution, ranging south from New York to northern Georgia and west to Missouri (MO) (Nickerson & Mays 1973). The Ozark hellbender (*C. a. bishopi*) is geographically limited to the White and Black River drainages of southern Missouri and northern Arkansas (Nickerson & Mays 1973). In MO, both subspecies, have declined by an average of 77% between the early 1980's and late 1990's (Wheeler et al. 2003), and declines also have occurred in many other parts of the hellbender's range (Mayasich et al. 2003). In addition to declining numbers, a shift in age structure also was observed for all populations in MO, with significantly fewer young individuals sampled in the late 1990's (Wheeler et al. 2003). The apparent decline in recruitment is likely attributable to either depressed reproduction or low egg/larval survivorship. Larval hellbenders hatch between 23 and 30 mm in total length (TL) (Smith 1912a), metamorphose 1.5–2 yrs after hatching (Grenell 1939) at 100–130 mm TL (Smith 1912a), and attain sexual maturity after 4–6 yrs (Smith 1907). As a result of their small size and slow developmental rate, larval hellbenders are particularly vulnerable to predation. Because hellbenders are unusual among amphibians by occupying permanent streams, they are vulnerable to predation by fishes.

The number of trout stocked and the density of trout/km of stream has risen dramatically in MO over the last 40 yrs [Alsup 2005; Missouri Department of Conservation (MDC) 2006]. The extent of the role that these introduced fishes have played in recent hellbender declines is unknown. Although hellbenders have successfully co-existed with many species of predatory fishes for thousands of years, there are no native members of the family that includes trout (Salmonidae) in MO. The purpose of this study was to determine whether larval hellbenders hatched in captivity can distinguish between (1) native predatory and non-predatory fishes and (2) native fishes and trout.

Methods

Egg Collection and Larval Maintenance

Two clutches (690 eggs) of eastern hellbenders (*C. a. alleganiensis*) were collected from the Big Piney River and two clutches (136 eggs) of Ozark hellbenders (*C. a. bishopi*) were collected from the North Fork River between Sep. and Nov. 2007 at developmental stages ranging from 17 to 23 (Smith 1912b). Clutches were housed at Shepherd of the Hills Fish Hatchery (SHFH) in Branson, MO, where they were maintained in separate fish egg incubation trays. Larval eastern hellbenders were supplied with a constant inflow of well-water (16°C) that continually drained from the trays (a 'flow-through' system). Upon hatching, eastern hellbender larvae (n = 466) were transferred to covered aluminum raceways with well-water flow-through systems and no substrate. Because Ozark hellbender eggs (n = 61) may have been exposed to the chytrid fungus (*Batrachochytrium dendrobatidis*) (Bd), which is known to occur in the North Fork River (Briggler et al. 2008), they were maintained in a 170-l polyethylene tank with a closed filtration system and no substrate. Once the majority of the yolk sac was absorbed (approx. 4 wks), larvae were fed a mixture of isopods and amphipods and black worms 5 d/wk.

Fish Collection

Eastern hellbender larvae were tested in 11 treatments, including a dechlorinated water blank (control), and chemical cues from six species of native fishes, and both wild-caught and hatchery-reared rainbow and brown trout (Table 1). Fishes were collected by electrofishing by MDC fisheries biologists. For tests with Ozark hellbender larvae, all fishes except walleye were collected from the North Fork of the White River; walleye were collected from Bull Shoals Lake. Eastern hellbender larvae were tested with chemical stimuli from fishes collected from the Big Piney River or a tributary of the river. Fishes were collected in Dec. 2007, except for walleye which were collected in Oct. 2007. Hatchery-reared rainbow and brown trout were held in large outdoor raceways at SHFH and were fed exclusively floating trout feed (Silver Cup; Nelson and Sons, Inc., Murray, UT, USA). All stimulus fishes (three individuals/treatment; Table 1) were transferred to water-filled ice chests (95 × 38 × 38 cm) and acclimated to 16°C over 24 h.

Each fish treatment was categorized as predatory/non-predatory and native/non-native based on whether they are known to consume vertebrate prey and their historical distribution in MO (Pflieger 1997; Table 1). Because few Ozark hellbender larvae hatched (n = 61), fewer treatments were used for this subspecies: a blank control, and water from tanks containing wild-caught brown trout, wild-caught rainbow trout, smallmouth bass, and walleye.

Stimulus Collection

After the 24 h acclimation period, individuals of each species (except banded sculpin) were placed in a 19-l container with an aerator and dechlorinated tap water for 24 h; the blank control was treated the same except no fish was included. A ratio of 25 ml of dechlorinated tap water per 1 g of fish mass was used to standardize the concentration of fish stimulus among different-sized fish. Partitions were placed between each container to minimize visual inconsistencies among containers. Because banded sculpin were smaller than the other individuals (Table 1), we used a 1.9-l container for this species; all other methods of stimulus collection were the same.

After 24 h, the donor fish was removed, and the stimulus water was filtered through filter fiber to remove solid particles and transferred to plastic bottles in 60 ml aliquots. All stimuli were taken to SHFH on ice and frozen at −6°C. All donor fish were returned to the river of origin within 48 h after stimulus collection.

Stimulus from each of the three conspecific predators was not pooled, but randomly assigned during testing of a particular treatment. Within each treatment, responses to stimuli from individual donor fish of the same species were generally consistent.

General Testing Protocol

The testing chamber was a plastic 5.7-l container (31 × 17.5 × 10.5 cm) with 1 kg of stone substrate and 1.8 l of well-water. Four lines were drawn width-wise across the bottom and sides of the container at 5.5 cm intervals. The container was surrounded on all sides with black plastic to minimize external visual influences. A clear plastic cylinder (8 cm length × 7.5 cm diameter) open at both ends was used as an acclimation chamber for the test larva. The cylinder was drilled with 24 holes, 0.278 cm in diameter, to allow water (including the test stimulus) to flow into and out of the cylinder.

The acclimation cylinder was placed upright in the center of the test chamber. Immediately prior to testing, a 60 ml aliquot of a randomly selected

Table 1: Treatments tested for eastern hellbender larvae, including assignment to predator/non-predatory and native/non-native categories, and assigned stimulus fish and corresponding length

Treatment	Native/ non-native	Predator/ non-predator	Fish no.	Fish total length (cm)
Blank control	NA	NA	1	NA
			2	NA
			3	NA
Redhorse	Native	Non-predator	1	25.5
Moxostoma sp.			2	24.7
			3	25.8
Rainbow trout (H)	Non-native	Predator	1	30.8
Oncorhynchus mykiss			2	25.4
			3	26.3
Rainbow trout	Non-native	Predator	1	24.7/31.0
Oncorhynchus mykiss			2	28.3/27.3
			3	26.5/30.3
Brown trout (H)	Non-native	Predator	1	23.5
Salmo trutta			2	26.0
			3	26.5
Brown trout	Non-native	Predator	1	27.0/34.1
Salmo trutta			2	23.2/28.8
			3	25.0/29.0
Largemouth bass	Native	Predator	1	31.1
Micropterus salmoides			2	28.2
			3	26.5
Smallmouth bass	Native	Predator	1	21.3/22.8
Micropterus dolomieu			2	26.0/19.8
			3	30.3/21.1
Rock bass	Native	Predator	1	23.0
Ambloplites rupestris			2	20.6
			3	20.4
Walleye	Native	Predator	1	23.3
Sander vitreus			2	22.2
			3	22.5
Banded sculpin	Native	Predator	1	14.0
Cottus carolinae			2	12.8
			3	16.2

For rainbow trout, brown trout, and smallmouth bass, second length is the length of stimulus fish taken from the North Fork River for testing responses of Ozark hellbender larvae. Walleye were collected from Bull Shoals Lake and used in trials for both hellbender subspecies.
H indicates hatchery-reared; NA, not applicable.

stimulus was thawed in a warm water bath. All treatments, except hatchery-reared rainbow and brown trout which were added after the beginning of experimentation, were coded prior to testing so that the observer was blind to treatment selection. Water temperatures were 15.1–17.4°C for eastern hellbender trials and 16.7–19.3°C for Ozark hellbender trials. At the time of testing, eastern hellbender larvae were 6.05 ± 0.04 cm ($\bar{X}$ TL ± SE) and Ozark hellbender larvae were 5.83 ± 0.07 cm ($\bar{X}$ TL ± SE).

Testing Protocol: Eastern Hellbenders

For each trial, a larval hellbender was arbitrarily chosen from a selected clutch and placed inside the acclimation cylinder using a small aquarium net. After 120 s, the test stimulus was gently shaken and poured around the acclimation cylinder. In a previous trial using dye, we determined that 90 s was sufficient for the stimulus to disperse throughout the container and into the acclimation cylinder containing the hellbender. We waited an additional 120 s before slowly lifting the acclimation cylinder and releasing the larva. We recorded the following quantitative response variables during the 10 min observation period: (1) latency to move; (2) latency to cross the first line (recorded when half of the larva's body crossed the line); (3) number of lines crossed; (4) latency to jerk head (rapid, lateral movement of the head, often accompanied with a mouth gape); and (5) number of head jerks. All of these variables are indicators of activity which are frequently used as a measure of antipredator responses in amphibians (Woodward 1983; Feminella & Hawkins 1992; Kats & Dill 1998; Mathis et al. 2003).

Individual larvae were tested only once, with one exception. We initially intended to only test responses to stimuli from wild-caught trout, but decided to add the treatments of hatchery-reared trout to tests of eastern hellbenders once the experiment was in progress. To keep sample sizes equivalent to the other treatments, we re-tested 12 of the 323 eastern larvae; these larvae were exposed to different treatments than in their original tests so that pseudoreplication did not occur. Because most (approx. 3/4) of the tests were completed when we added the additional treatment, we added replicates to the wild-caught trout treatments; the order of testing for the hatchery-reared and wild-caught trout treatments were randomly interspersed with that of the other remaining treatments.

Testing Protocol: Ozark Hellbenders

Because Bd has been found to occur on hellbenders captured from the North Fork of the White River, we used a slightly different testing protocol for Ozark hellbender larvae to prevent potential Bd contamination in uncontaminated areas. Methods for the two subspecies were identical except for the following details. The test chamber, substrate and acclimation cylinder were rinsed with cold tap water prior to rinsing with well-water and the test water (1.8 l) was taken from the Ozark hellbender holding tank. All individual Ozark larvae were tested only once.

Statistical Analyzes

Responses of the hellbenders to the 11 stimuli were compared using Kruskal–Wallis tests (Minitab 15, Minitab Inc., State College, PA, USA) followed by nonparametric multiple comparisons (WINKS SDA 6.0, Texasoft, Cedar Hill, TX, USA). To minimize experiment-wise error, we limited *post hoc* tests to comparisons of each predatory treatment with the non-predatory controls (blank and redhorse). The two subspecies were analyzed separately for latency to move, latency to cross a line, and number of lines crossed.

Head jerks were relatively rare, and so data for both subspecies were combined for analysis of this behavior. We combined treatments *post hoc* into three categories [non-predatory controls (blank and redhorse), trout (hatchery-reared/wild-caught rainbow and brown trout), and native predatory fishes (largemouth bass, smallmouth bass, walleye, rock bass, and banded sculpin] and compared the percentage of trials with head jerks using a chi-squared test.

Results

Eastern Hellbender Activity

In terms of short-term responses to stimuli (latencies), larvae did not statistically differentiate between the blank stimulus and stimuli from the non-predatory control (redhorse), including both latency to move (Fig. 1a) and latency to cross a line (Fig. 1b; Table 2). In contrast, larvae exposed to stimuli from the native predatory species (largemouth bass, small-

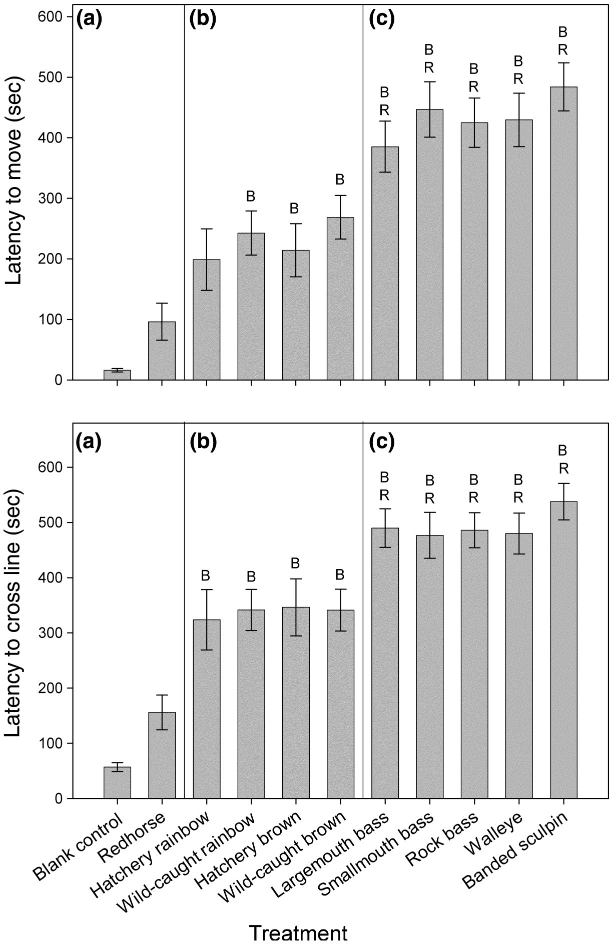

Fig. 1: Mean ± SE latency to move (top) and latency to cross the first line (bottom) for eastern hellbender larvae exposed to (a) controls of dechlorinated tap water (blank) and to chemical stimuli from a non-predatory fish (redhorse), (b) hatchery-reared and wild-caught introduced fish (rainbow and brown trout), and (c) five species of native predatory fish. Different letters indicate significant differences ($p < 0.05$) from blank (B) and non-predator control (R).

Table 2: (a) One-way Kruskal–Wallis (KW) anova results for three response variables for eastern hellbender larvae exposed to predatory and non-predatory stimuli; (b) nonparametric multiple comparisons (test statistic: *Q*) of predatory treatments vs. the blank control and the non-predatory control for three response variables

(a)

Response variable	KW test statistic (*H*)	df	p-Value	Figure
Latency to move	128.00	10	<0.001	Fig. 1
Latency to cross line	122.99	10	<0.001	Fig. 1
Number lines crossed	113.81	10	<0.001	Fig. 2

(b)

Treatment	n	Response variable	$\bar{X} \pm$ SE	Comparison with blank control p-value	Comparison with non-predator p-value
Blank Control	28	Latency to move	16 ± 3		
		Latency to cross line	57 ± 8		
		Number lines crossed	40 ± 4		
Redhorse	28	Latency to move	96 ± 30	>0.5	
Moxostoma sp.		Latency to cross line	156 ± 31	>0.5	
		Number lines crossed	27 ± 4	>0.5	
Rainbow trout (H)	20	Latency to move	199 ± 51	>0.1	>0.5
Oncorhynchus mykiss		Latency to cross line	324 ± 55	<0.02	>0.5
		Number lines crossed	15 ± 4	<0.05	>0.5
Rainbow trout	45	Latency to move	243 ± 37	<0.001	>0.5
Oncorhynchus mykiss		Latency to cross line	342 ± 37	<0.001	>0.2
		Number lines crossed	16 ± 3	<0.002	>0.5
Brown trout (H)	20	Latency to move	214 ± 44	<0.01	>0.5
Salmo trutta		Latency to cross line	346 ± 52	<0.005	>0.5
		Number lines crossed	12 ± 3	<0.01	>0.5
Brown trout	45	Latency to move	269 ± 36	<0.001	>0.2
Salmo trutta		Latency to cross line	341 ± 38	<0.001	>0.1
		Number lines crossed	15 ± 3	<0.001	>0.1
Largemouth bass	29	Latency to move	385 ± 42	<0.001	<0.001
Micropterus salmoides		Latency to cross line	490 ± 35	<0.001	<0.001
		Number lines crossed	6 ± 2	<0.001	<0.001
Smallmouth bass	27	Latency to move	447 ± 46	<0.001	<0.001
Micropterus dolomieu		Latency to cross line	477 ± 42	<0.001	<0.001
		Number lines crossed	6 ± 3	<0.001	<0.001
Rock bass	28	Latency to move	425 ± 41	<0.001	<0.001
Ambloplites rupestris		Latency to cross line	486 ± 32	<0.001	<0.001
		Number lines crossed	7 ± 3	<0.001	<0.002
Walleye	26	Latency to move	430 ± 44	<0.001	<0.001
Sander vitreus		Latency to cross line	480 ± 37	<0.001	<0.001
		Number lines crossed	5 ± 2	<0.001	<0.001
Banded sculpin	27	Latency to move	484 ± 40	<0.001	<0.001
Cottus carolinae		Latency to cross line	538 ± 33	<0.001	<0.001
		Number lines crossed	4 ± 2	<0.001	<0.001

H, hatchery-reared.

mouth bass, rock bass, walleye, and banded sculpin), had significantly longer latencies to move (Fig. 1a) and to cross a line (Fig. 1b) compared with the non-predatory redhorse and the blank control (Table 2). Qualitatively, responses of larval hellbenders to stimuli from rainbow and brown trout (hatchery-reared and wild-caught) appeared intermediate to responses to the control and native predator stimuli (Fig. 1). Statistically, responses to the trout stimuli did not differ from the non-predator control (redhorse) in either latency behavior (Table 2), and only the response to the wild-caught rainbow trout was not significantly different from the blank control for latency to move.

For more prolonged activity levels (line crosses), larvae were most active following exposure to the

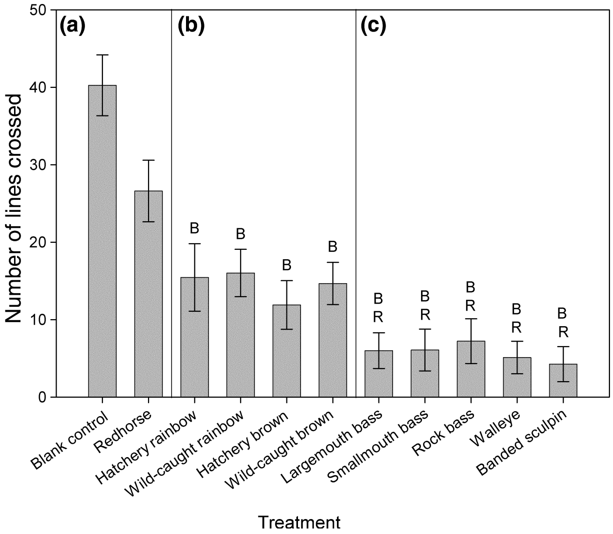

Fig. 2: Mean (±SE) number of lines crossed for eastern hellbender larvae exposed to (a) controls of dechlorinated tap water (blank) and to chemical stimuli from a non-predatory fish (redhorse), (b) hatchery-reared and wild-caught introduced fish (rainbow and brown trout), and (c) five species of native predatory fish. Different letters indicate significant differences ($p < 0.05$) from blank (B) and non-predator control (R).

blank control, but the number of lines crossed by larvae in the blank control was not statistically different compared with the non-predatory redhorse (Fig. 2; Table 2). However, larvae in all treatments for native predatory species crossed significantly fewer lines compared with the non-predatory redhorse treatment and to the blank control (Fig. 2; Table 2). Again, larvae exposed to rainbow and brown trout stimuli (hatchery-reared and wild-caught) appeared to be intermediate in their activity levels (Fig. 2). Statistically, their responses did not differ from larvae in the non-predatory redhorse treatment, but were significantly different from the blank control (Fig. 2; Table 2).

Ozark Hellbender Activity

Larvae exhibited significantly stronger responses to stimuli from wild-caught brown trout, walleye and smallmouth bass than to the blank control in at least one of the response variables (Fig. 3; Table 3). In contrast, larval responses to stimuli from rainbow trout did not differ significantly from the blank control for any response variable (Fig. 3; Table 3).

Head Jerks

Head jerks were never performed in the presence of chemical stimuli from native predatory fishes, but were present in at least one trial for all other treatments (Table 4). Because head jerks were relatively rare, we combined treatments for statistical analysis and compared the percentage of trials with head

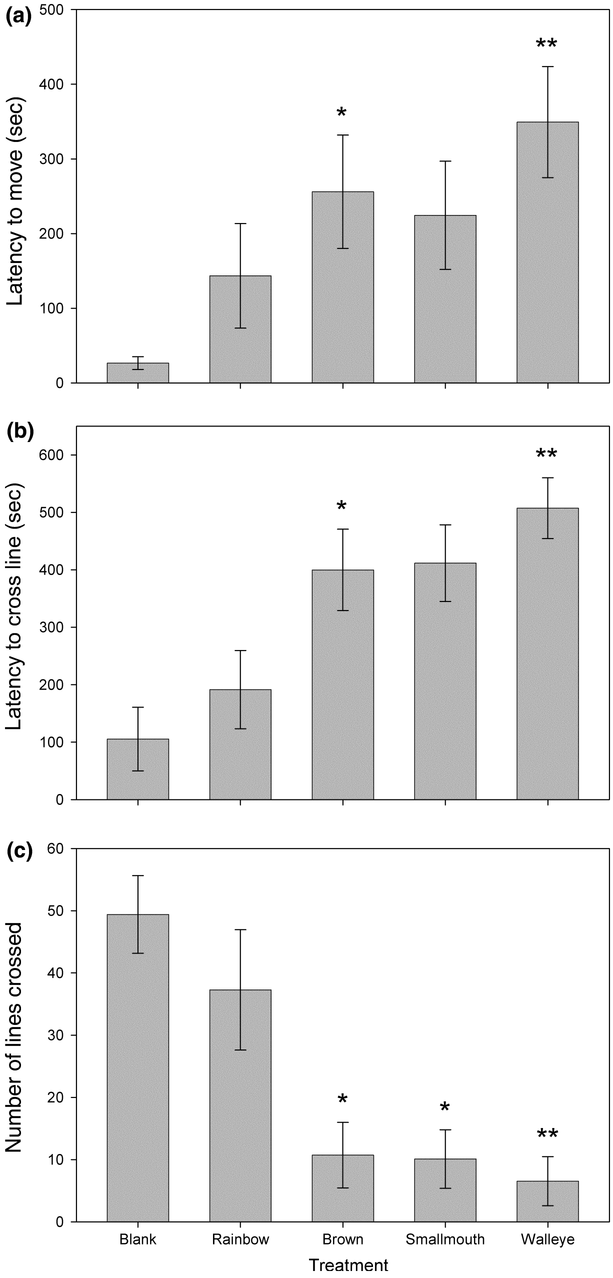

Fig. 3: Mean ± SE (a) latency to move (s), (b) latency to cross the first line (s), and (c) number of lines crossed for Ozark hellbender larvae exposed to dechlorinated tap water (blank), and to chemical stimuli from wild-caught rainbow trout (rainbow), wild-caught brown trout (brown), smallmouth bass (smallmouth), and walleye. Significant differences from dechlorinated water (blank) indicated by asterisk. $^{*}p < 0.05$; $^{**}p < 0.005$.

Table 3: (a) One-way Kruskal–Wallis anova results for three response variables for Ozark hellbender larvae exposed to predatory stimuli and a blank control; (b) nonparametric multiple comparisons (test statistic: *Q*) of predatory treatments vs. the blank control for three response variables

(a)

Response variable	Test statistic (*H*)	df	p-Value	Figure
Latency to move	128.00	10	<0.002	Fig. 3
Latency to cross line	122.99	10	<0.001	Fig. 3
Number lines crossed	113.81	10	<0.001	Fig. 3

(b)

Treatment	n	Response variable	$\bar{X} \pm$ SE	p-Value
Blank control	10	Latency to move	27 ± 9	
		Latency to cross line	105 ± 55	
		Number lines crossed	49 ± 6	
Rainbow trout	11	Latency to move	143 ± 70	>0.5
Oncorhynchus mykiss		Latency to cross line	191 ± 68	>0.5
		Number lines crossed	37 ± 10	>0.5
Brown trout	11	Latency to move	256 ± 76	<0.05
Salmo trutta		Latency to cross line	400 ± 71	<0.05
		Number lines crossed	11 ± 5	<0.02
Smallmouth bass	10	Latency to move	224 ± 73	>0.2
Micropterus dolomieu		Latency to cross line	412 ± 67	>0.05
		Number lines crossed	10 ± 5	<0.05
Walleye	11	Latency to move	349 ± 74	<0.005
Sander vitreus		Latency to cross line	507 ± 53	<0.002
		Number lines crossed	7 ± 4	<0.005

jerks for the following categories: Non-predatory controls (21%) vs. trout (9%) vs. native predatory fishes (0%) ($\chi^2 = 30.4$, df = 2, p < 0.001; Table 4).

Table 4: Number of trials per treatment in which either eastern or Ozark hellbenders exhibited head jerks

Treatment	No. trials with head jerks	Total no. trials	Trails with head jerks (%)
Blank control	9	38	24
Redhorse	5	28	18
Rainbow trout (H)	3	20	15
Rainbow trout	6	56	11
Brown trout (H)	4	20	20
Brown trout	1	56	2
Largemouth bass	0	29	0
Smallmouth bass	0	37	0
Rock bass	0	28	0
Walleye	0	26	0
Banded sculpin	0	38	0

Unless otherwise indicated, fishes were wild-caught.
H, hatchery-reared.

Discussion

Larval hellbenders exposed to chemical cues from native species responded by decreasing overall activity compared with blank and non-predator controls (Figs 1–3). Prey typically respond to the threat of predation in one of two ways: (1) by decreasing activity, and subsequently decreasing the probability of being detected by a predator or (2) exhibiting escape or avoidance behavior by increasing activity (Lima & Dill 1990). Decreased activity, or freezing, is a common antipredator mechanism among aquatic amphibians (Woodward 1983; Feminella & Hawkins 1992; Kiesecker et al. 1996; Kats & Dill 1998; Mathis et al. 2003; Bosch et al. 2006). In nature, hellbenders often remain motionless after their shelter rock is overturned (Nickerson & Mays 1973), and we conclude that reduced activity is an antipredator response for larval hellbenders in this study.

Because the larvae in this study were lab-reared, with no experience with fish predators, these data confirm that recognition of at least some native predators is innate, which is consistent with results from other studies of amphibians (Kats et al. 1988; Elliott et al. 1993; Sih & Kats 1994; Kiesecker & Blaustein 1997; Griffiths et al. 1998; Petranka & Hayes 1998; Gallie et al. 2001; Epp & Gabor 2008). Some studies have indicated that predator recognition by naïve individuals is facilitated by cues associated with the predators diet of conspecifics or members of the same prey guild (Mathis & Smith 1993; Wilson & Lefcort 1993; Laurila et al. 1997). Although we did not specifically control for the diet of the predator, several lines of evidence indicate this is unlikely to be important in the recognition of the native predators in our study. First, the predatory fishes were not fed between field and stimulus collection (approx. 24 h). Second, because each treatment stimulus was collected from three separate individuals, we would expect the variation in hellbender responses within predator treatment groups to be much greater than that observed if predator diet cues accounted for the antipredator behavior. Most importantly, the walleye collected for this study were obtained several months prior to stimulus collection and were fed exclusively with juvenile rainbow trout, eliminating the possibility of conspecific dietary cues confounding that antipredator response.

Mathis et al. (2008) recently demonstrated that amphibian embryos may learn to recognize predators prior to hatching. Because our eggs were collected from the field, it is possible that responses to fishes may have had a learned component. The mechanism of learned recognition proposed by Mathis et al. (2008) was through pairing of predatory stimuli with a chemical alarm cue. This type of associative learning requires predation on at least one nearby conspecific. Hellbender eggs are laid under large stones with typically only one entrance (Nickerson & Mays 1973), and male hellbenders guard the eggs. Therefore, predation by fishes on the eggs is unlikely. However, this possibility cannot be completely ruled out without additional data.

Although our data indicate that innate recognition of the most common hellbender predators is likely, learning may also play an important role. Innate responses can be fine-tuned by experience (Epp & Gabor 2008), and other species of salamanders have been shown to be capable of learning to recognize new predators (Woody & Mathis 1998; Ferrari et al. 2007b; Mathis et al. 2008).

For larval eastern hellbenders, responses to the non-native trout were substantially weaker than responses to native predators. Larvae did not discriminate statistically between chemical cues from trout and the non-predatory redhorse. This apparent failure to respond to trout could lead to increased larval predation in the wild. For antipredator mechanisms to evolve, predator and prey must co-occur over evolutionary time (Brodie et al. 1991; Kats & Ferrer 2003). There are no native members of the trout family (Salmonidae) in the state, so the relatively low-level response of hellbender larvae to trout is likely because of the limited time in which these species have co-occurred. Trout introductions to hellbender streams in MO were sporadic over the last century, but increased markedly beginning in the 1960's (reviewed by Alsup 2005). Hellbenders have a relatively long generation time (reproduction at approx. 5–6 yrs of age with a lifespan >40 yrs: Nickerson & Mays 1973), so responses to selection for innate recognition of introduced predators should be slow.

Although MO does not have any native salmonids, hellbender populations from the eastern United States have co-evolved with a member of the salmonid family (brook trout: *Salvelinus fontinalis*). Because some prey can generalize predator recognition to closely related, but otherwise unfamiliar, predators (Ferrari et al. 2007a, 2008), hellbenders from these Appalachian populations may show stronger fright responses to trout cues. In a preliminary study of larvae from two clutches of eggs from a MO population of hellbenders and a single clutch from North Carolina (NC), the NC larvae showed a strong response to chemical cues from brown trout while those from the MO population did not (Mathis & Crane 2009). Although the results of their study needs to be verified with replication from additional clutches, these preliminary responses are consistent with the hypothesis that co-evolution with trout is necessary for innate predator recognition; Appalachian larvae may be able to generalize recognition of brook trout to the related brown trout.

In addition to changes in activity levels, larval hellbenders also performed a behavior that we termed 'head jerks'. Although we did not make prey available during the trials, some sediment particles may have entered the testing chamber in the stimulus water, and head-jerk behavior may result from larvae snapping at those particles (i.e., feeding behavior). Head-jerking is similar to the behavior observed when hellbenders subjugate and consume prey (Green 1933; personal observation). Alternatively, head-jerking may be a form of chemosensory sampling. Larval hellbenders did not perform the head-jerk behavior when exposed to stimuli from native fish predators, presumably because they perceived a high-risk environment; like other forms of activity, head jerks would likely draw the attention of nearby predators. However, larval hellbenders did perform head jerks when exposed to stimuli from all non-native predatory fishes, presumably because they did not perceive that these stimuli represented danger.

A few species of larval anurans that have co-evolved with stream fishes have been shown to be differentially affected by native and introduced fish predators. In simple field enclosures, Gillespie (2001) found that tadpoles of the stream-breeding amphibians *Litoria spenceri* and *L. phyllochroa* were preyed upon heavily by introduced trout, but few or none were eaten by two native fish predators. Additionally, in more natural stream enclosures incorporating alternative prey and natural refuges, trout reduced survivorship of these same species. The lack of evolutionary history with trout apparently is responsible for poor survival of these two species in the face of trout predators (Gillespie 2001). Bosch et al. (2006) compared habitat use of native brown trout (*Salmo trutta*), introduced brooke trout (*Salvelinus fontinalis*), and the Iberian frog (*Rana iberica*) and found that although all three species had similar habitat preferences, tadpoles only occurred in fishless regions of the stream. In addition, when exposed to chemical cues from these fish predators, tadpoles decreased activity to both predators but cues from the native predator elicited stronger responses.

In general, larvae of the two subspecies responded similarly to the predatory stimuli. However, there was one notable difference in their responses: Ozark hellbenders responded to brown trout in a similar manner as their responses to native fishes. Because the trout stimulus for Ozark hellbenders was collected from wild-caught trout that were larger than the brown trout used for stimuli in the trials with eastern hellbenders (Table 1), we hypothesize that the difference in responses between the two subspecies to wild-caught brown trout may be as a result of dietary affects because of the different body sizes. Invertebrates are important dietary components for small brown trout (Ellis & Gowing 1957; Brynildson et al. 1963; Pflieger 1997), but as fish length increases (approx. 22 cm), brown trout shift to foraging on vertebrates (Evans 1952; Brynildson et al. 1963). The level of response to predatory chemical cues is often influenced by the predator's diet, with prey responding more strongly to chemical stimuli from predators whose diet is composed of conspecifics or other members of the same prey guild (Mathis & Smith 1993; Wilson & Lefcort 1993; Laurila et al. 1997). If the large wild-caught brown trout in this study had been consuming primarily fish prey, then the larvae may have perceived them as more dangerous than rainbow trout that had been consuming only invertebrate prey; small fishes are in the same prey guild as larval amphibians. It is also possible that the wild-caught trout in this study had consumed larval hellbenders, which also would account for a dietary effect. Nevertheless, the role dietary cues play in antipredator behavior and the differences observed between hellbender subspecies requires further investigation.

The role fish introduction and stocking have had on amphibian declines are well documented, and in many cases are responsible for extensive declines or extinctions (Burger 1950; Bradford 1989; Knapp & Matthews 2000; Gillespie 2001; Kats & Ferrer 2003; Vredenburg 2004; Denoel et al. 2005; Bosch et al. 2006; Welsh et al. 2006; Barr & Babbitt 2007). These studies typically focus on permanent but historically fishless waters. Our study demonstrates that even amphibians occupying permanent streams where fish predation is part of the evolutionary history may be threatened by these same introductions because of limited opportunity for evolution of antipredator responses.

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