

RESEARCH ARTICLE

Predator-Recognition Training: A Conservation Strategy to Increase Postrelease Survival of Hellbenders in Head-Starting Programs

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For species with declining populations, captive rearing with subsequent release into natural habitats (“head-starting”) is often used as part of a conservation strategy. One challenge to head-starting programs is that head-started individuals can suffer high rates of postrelease predation. Head-starting programs are currently being established for hellbenders (*Cryptobranchus alleganeinsis*), large aquatic salamanders that are experiencing population declines throughout much of the species’ range. Although hellbenders have innate recognition of many predators, inexperienced juveniles show only weak recognition of introduced trout. We used a classical conditioning protocol to train captive-reared hellbender larvae to show fright responses to the scent of trout. We exposed hellbender larvae to trout-scented water plus a hellbender distress secretion during training trials. In a subsequent test, these larvae responded to trout cues alone with a fright response; control larvae that were trained with the trout scent plus a blank control did not show a fright response to the trout cues. Learning was specific to trout because trained larvae did not respond to water that had been scented by a suckermouth catfish. Although a number of details remain to be addressed concerning standardized procedures, we recommend that head-starting programs for hellbenders include trout-recognition training. *Zoo Biol* 30:611–622, 2011.

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INTRODUCTION

Introduced predators can provide a particularly acute challenge for captive/release programs, because prey species have not had the opportunity to develop predator defenses through co-evolutionary processes. For example, when fishes are introduced into formerly fishless habitats, populations of amphibians can be quickly driven to extinction, presumably because the amphibians have no defense against fish predators [Knapp and Matthews, 2000; Vredenburg, 2004].

A more subtle effect of introduced predators can occur in cases where prey have well-developed antipredator defenses, but the prey fail to recognize that the introduced predator represents a danger. Individuals that were raised in captivity may suffer high initial postrelease rates of predation simply because they fail to take appropriate antipredator measures in time. A potential case in point is the hellbender (*Cryptobranchus alleganeinsis*), a large aquatic salamander whose populations have declined sharply in many parts of their range since the 1970s [Wheeler et al., 2003]. Because the decline is characterized by age distributions that are highly skewed toward older individuals [Wheeler et al., 2003], decreased survival of young—potentially via predation by introduced trout—is a possible contributing factor.

Although survival of young is difficult to study under natural conditions, recent laboratory data indicate that hellbender larvae seem to be particularly vulnerable to predation from nonnative predatory fishes (trout) [Gall and Mathis, 2009; Mathis and Crane, 2009]. In Gall and Mathis [2009], larval hellbenders gave strong fright responses to chemical cues from native predatory fishes but responded only weakly to nonnative rainbow (*Oncorhynchus mykiss*) and brown (*Salmo trutta*) trout. In addition, Gall [2008] further found that feeding by brown trout was stimulated by the presence of the hellbender secretions. These laboratory results suggest that predation by nonnative trout may be a serious issue with respect to survival of young hellbenders.

Establishment of head-starting programs is included as a potential conservation strategy in the US Fish and Wildlife Service's status assessment report for the hellbender [Mayasich et al., 2003], and captive breeding with re-introductions are included in the Hellbender Population and Habitat Viability Assessment report [Briggler et al., 2007]. The St. Louis Zoo has established a facility for captive rearing of hellbenders (The Ron Goellner Center for Hellbender Conservation), and a number of other zoos are investigating the possibility of establishment of similar programs [Briggler et al., 2007].

Numerous studies have shown that naive prey can learn to recognize unfamiliar predators, but relatively few studies have used this technique as part of a captive rearing–release protocol [Griffin et al., 2000]. However, three studies have shown that predator-recognition training can increase postrelease survival of lab-reared animals [Chinook salmon: Berejikian et al., 1999; brook charr: Mirza and Chivers, 2000; black-tailed prairie dogs: Shier and Owings, 2007]. Therefore, we propose that predator-recognition training might be an effective strategy to increase postrelease survival of head-started hellbenders. The first step in this process is to determine whether lab-reared hellbenders can be trained to recognize that trout are dangerous. In this study, we used a classical condition protocol similar to that used by Berejikian et al. [1999] and Mirza and Chivers [2000] to train larval hellbenders to respond to chemical cues from trout with a fright response. This training technique

has been effective with other species of salamanders [Woody and Mathis, 1998; Wildy and Blaustein, 2001].

Associative learning protocols generally involve pairing a neutral stimulus (in this case, a trout stimulus) with an “unconditioned” stimulus that is known to produce the desired response (in this case, antipredator behavior). The test individuals then learn to associate the neutral stimulus with the desired response. The basic technique is similar to that used in the well-known example of “Pavlov’s dog,” which is described in any general psychology textbook.

One challenge to using this testing protocol is to determine a suitable unconditioned stimulus that produces the fright response in lab-reared hellbenders. The studies cited above for fishes and amphibians used a cutaneous chemical “alarm” cue that normally is released by damage to the skin during a predatory event; the alarm cue was presented to subjects of the learning studies in the form of skin homogenates. We did not choose to use skin homogenates as the alarm stimulus in our study because producing homogenates requires sacrifice of subjects, which is problematic for threatened species.

A potential alternative to skin homogenates for use as a training stimulus is the copious white secretion that is produced by hellbenders when they are disturbed [Nikerson and Mays, 1973; Gall et al., 2010]. Our preliminary data from captive-reared hellbenders housed at the St. Louis Zoo showed that hellbenders (approximately 3 years of age) seemed to respond to this secretion with a fright response [A. Mathis and T. Crow, unpublished data]; similar secretions produced by other salamanders without damage to the skin have been shown to function as an alarm cue [e.g. Mathis and Lancaster, 1997]. Therefore, we used this secretion as a training stimulus in our classical conditioning protocol.

We hypothesized that (1) adult hellbender secretion functions as an alarm cue and (2) naive (i.e. lab reared) larvae can learn recognition of unfamiliar predators through associations of predator chemical stimuli with the hellbender secretion. We chose hatchery-raised rainbow trout (*O. mykiss*) as the unfamiliar predator. In our experimental test of the above hypotheses, we divided the protocol into two stages: training and testing. During training, we exposed larvae to the trout stimulus paired either with hellbender secretions (trout+HB) or control water (trout+H₂O). After approximately 24 hr, we tested both groups with the trout stimulus alone. Note, however, that a fright response during the testing phase could indicate learned recognition that is specific for trout or learned sensitization to any new chemical in the water. To distinguish between these latter two interpretations, we also tested responses of a subset of trained larvae to an unfamiliar fish, the tropical suckermouth catfish (*Hypostomus plecostomus*). Demonstration of learned recognition of trout by larval hellbenders, therefore, requires a series of trials and associated responses (Table 1).

METHODS

Collection and Maintenance of Larvae

As part of a larger study, three egg clutches from eastern hellbenders were collected from the Big Piney River, during October 2007, by the Missouri Department of Conservation (MDC) biologists. Eggs were transported to Shepherd

TABLE 1. Results Required for Learning to be Unequivocally Demonstrated

Exposure stimulus	Result necessary for trout-specific learning	Interpretation
Training: Con. = Trout+H ₂ O Exp. = Trout+HB	Trout+H ₂ O ≠ Trout+HB	Larvae are not strongly afraid of trout [confirms Gall and Mathis, 2009]; larvae give fright response to hellbender secretion
Testing A: Trout only	Trout+H ₂ O-trained ≠ ← Trout+HB-trained	No learned sensitization based on disturbance; possible trout-specific learning
Testing B: Catfish only	Trout+H ₂ O-trained = Trout+HB-trained	No learned sensitization to new chemicals in the water

“Con.” = control, and “Exp.” = experimental. “Fright” will be measured as differences in activity levels. All experimental stimuli are chemical cues (water that has contained either trout or suckermouth catfish); HB is hellbender secretion and H₂O is river water that did not contain any stimulus animal.

of the Hills fish hatchery (SHFH) where they hatched (17 October–3 November). Eggs and larvae were housed in aluminum raceways with continuous flow well water at approximately 17°C. Larvae were fed daily with invertebrates (isopods, amphipods, and worms).

Collection of Stimuli

Hellbender secretions were collected from two adults (~30 cm SVL, 500 g mass), captured from the Eleven Point River by the MDC biologists. Each hellbender was placed in a plastic container (38 × 25 × 23 cm) with approximately 4 l of Eleven Point River water. The hellbender was restrained in a net until the milky secretion was produced (approximately 1 min). This process was repeated until the surface area of the water was milky white and a foamy layer was present; the entire process usually took about 5 min. The secretion was collected in plastic jugs and stored on ice for transport. The secretion was divided into 60 ml aliquots that were placed in separate plastic containers and frozen (−6°C). In the same manner, water from the Eleven Point River that lacked hellbender secretion was frozen to serve as a control.

To obtain the trout stimulus, four individuals (total length = 20–30 cm; mass = 180–240 g) from SHFH were kept in separate 19 l plastic containers (dimensions: 34 × 21 × 24 cm), containing dechlorinated tap water (25 ml/g of fish) at 18–20°C for 24 hr; the stimulus water was then poured through Aqua-Pure filter fiber (HBH Pet Products, Springville, UT) to remove solid particles before being frozen in 60 ml aliquots. The control solution from the tropical catfish (mass = 80 g) was collected and stored in the same manner as the trout stimulus. The trout from SHFH had been raised on a diet of floating trout feed (Silver Cup; Nelson and Sons, Inc., Murray, UT) and the tropical catfish consumed algae that grew in the aquarium.

Training Procedures

Training occurred in March 2008 when larvae were 21–25 weeks old ($n = 84$; total length = 86.8 ± 1.2 mm). The stimulus solutions were thawed in warm water immediately before each trial [following Gall and Mathis, 2009]. Individuals were

removed from the raceways and were placed individually into small plastic training containers (dimensions: 10 × 10 × 9 cm). After a 90 sec acclimation period, either trout+hellbender or trout+H₂O stimulus solution was poured into the container, and behavior was recorded every 30 sec for 12 min. We recorded the following behaviors: freezing (no movement for 2 sec) or swimming (body entirely off container surface or climbing container walls) and latency to move (time from the beginning of the trial until the first movement, not counting movement of the gills only). The most common antipredator response for amphibians is decreased activity [Ferrari et al., 2007; Gall and Mathis, 2009; Wildy and Blaustein, 2001], although increased activity (presumably reflecting escape or search for hiding places) also has been reported [Smith, 2000]. After each trial was completed, hellbenders were returned to a compartment in the raceway with treatment groups partitioned. During the interval between training and testing, some larvae breached the partition between two adjacent compartments, making it impossible to identify training treatments for individuals in these compartments; these 12 individuals were eliminated from the testing trials.

Testing Procedures

To determine whether learning occurred, we tested the trained larvae approximately 24 hr after training, which is a similar time frame as used in other studies on predator recognition for amphibians [e.g. Woody and Mathis, 1998; Mirza et al., 2006a]. Testing arenas (5.71, dimensions: 31 × 17.5 × 10.5 cm) were filled with fine gravel (1 kg, 3–5 mm diameter) and well water (3 l), and the outer walls had lines drawn at 5.5 cm intervals and were covered to minimize visual disturbances. We used a testing arena that was different from the training arena to ensure that the larvae did not learn to associate some aspect of the training procedure with the stimulus [e.g. Mathis and Smith, 1993]. Testing followed the procedures of Gall and Mathis [2009]. The stimulus solutions were thawed in warm water before each trial. Each hellbender was removed from the raceway and placed individually into a plastic holding cylinder positioned in the center of the testing arena. The cylinder (dimensions: 7.5 cm diameter, 8 cm height) had 24 circular holes (1–2 mm diameter) allowing the stimulus water to enter freely. Each larva was allowed to acclimate in the cylinder for 90 sec, before 60 ml of either the trout or tropical catfish stimulus was poured around the perimeter of the container. In preliminary trials with dye, the dye entered the cylinder in about 60 sec; thus, larvae were given 90 additional seconds to detect the stimuli before the cylinder was removed and latency to move and the number of lines crossed were recorded as a measure of activity.

RESULTS

Training

During training, larvae exposed to the trout–hellbender secretion spent significantly less time freezing (Mann–Whitney $U = 511.5$, $P < 0.001$) and more time swimming (Mann–Whitney $U = 257.5$, $P = 0.013$) than in the trout–H₂O treatment ($n = 42$ per treatment; Fig. 1). Therefore, both of the requirements for training (Table 1) were met: larvae in the control (trout–H₂O) treatment did not show fear of the trout, and the hellbender secretion does seem to serve as an alarm function.

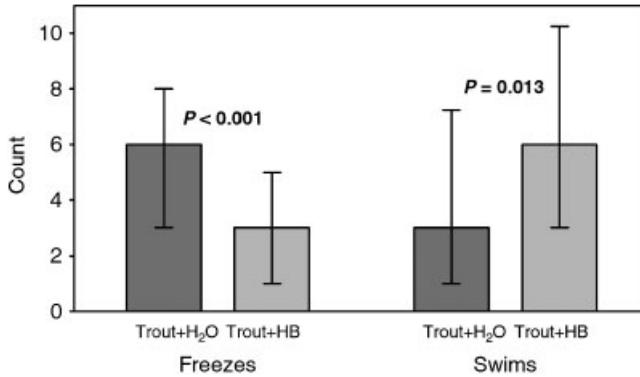


Fig. 1. Bouts of freezing and swimming for hellbender (*Crytobranchus alleganeinsis*) larvae ($n = 36$ per treatment) exposed to rainbow trout (*Oncorhynchus mykiss*) stimulus paired with either adult hellbender (HB) secretion (light gray bars) or blank river water (H₂O) (dark gray bars). Vertical and error bars represent medians and quartiles, respectively.

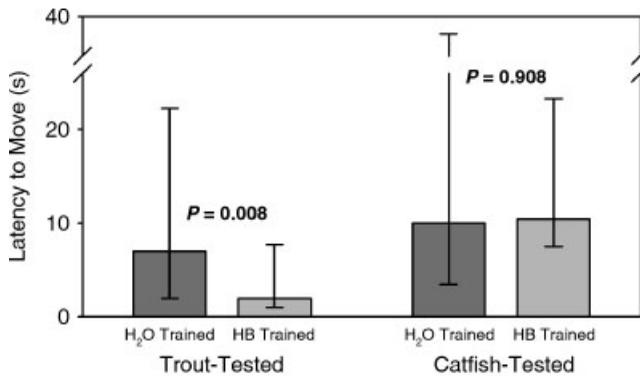


Fig. 2. Latency to move for hellbender (*Crytobranchus alleganeinsis*) larvae previously trained with pairings of either trout (*Oncorhynchus mykiss*) stimulus and adult hellbender (HB) secretion (light gray bars) or trout stimulus and blank river water (H₂O) (dark gray bars) and then tested with either trout ($n = 24$ per group) or suckermouth catfish (*Hypostomus plecostomus*; $n = 12$ per group) stimulus. Vertical bars and error bars represent medians and quartiles, respectively.

Testing

When exposed to the stimulus from the suckermouth catfish during subsequent testing, responses of larvae previously trained with the trout–hellbender secretion did not differ from responses of larvae trained with the trout–H₂O stimulus ($n = 12$ per treatment; lines crossed: Mann–Whitney $U = 12.5$, $P = 0.298$; latency to move: Mann–Whitney $U = 8.5$, $P = 0.908$; Fig. 2). These results indicate that larvae trained to the trout–hellbender secretion did not exhibit general sensitization responses in the training trials (see Table 1).

When exposed to the trout stimulus alone, the groups were not different in the number of lines crossed (Mann–Whitney $U = 23.5$, $P = 0.820$), but larvae that had been previously trained with trout–hellbender stimulus were significantly faster to move (Mann–Whitney $U = 123.5$, $P = 0.008$) relative to the larvae previously trained

with the trout-H₂O stimulus ($n = 24$ per treatment; Fig. 2). Therefore, larvae successfully learned to recognize that trout are dangerous (see Table 1).

DISCUSSION

Implications for Head-starting Programs

These results demonstrate that larval hellbenders can learn to associate danger with chemical cues from trout. Our results have particular relevance for head-starting programs, where animals are raised in captivity before release in the wild. One criticism of head-starting programs is that individuals may have low postrelease survival or reproduction owing to a lack of the behavioral competencies required to integrate into the natural habitat [e.g. Synder et al., 1996; Alberts, 2006]. Although prerelease training—including predator-recognition training [Berejikian et al., 1999]—has been shown to be successful at increasing postrelease survival in some species, head-starting programs involving amphibians and reptiles have historically lagged behind studies of birds and mammals in implementation of such training [Beck et al., 1994]. We, therefore, recommend—with a few caveats—that trout-recognition training be included in all head-starting protocols for hellbenders whose ranges occur outside of the native range of trout; presumably, hellbenders with co-evolutionary histories with trout will show innate recognition of trout [Mathis and Crane, 2009].

The trout-recognition training that we have demonstrated in this study is practical for implementation in most zoo or hatchery facilities. Young hellbenders could easily be trained in groups by personnel with minimal training, and these procedures are not costly. Below is a list of our recommendations and other issues to consider with respect to training:

- (1) Collection of the alarm secretion should be accomplished by trained professionals who are approved by either the U.S. Fish and Wildlife Service or state conservation agencies, because the collection procedure requires some stress to the donor animal.
- (2) Secretions and trout-water should be kept on ice if they are not used immediately, and should be frozen if not used quickly (within, say, an hour). We do not know the length of time until the stimuli are no longer chemically active (although it does seem to survive freezing for some weeks), so the stimulus should generally be used as quickly as possible.
- (3) Is there an ideal/critical age for the training to occur? We do not know the answer to this question. However, similar training protocols have been shown to be effective for adults of other species of salamanders [Woody and Mathis, 1998], so it seems unlikely that there is a critical time period beyond which such learning would not occur, although the duration of memory may be longer if larvae are conditioned during a critical period as is the case with young salamonids [Ditmann et al., 1996]. Based on preliminary studies, we do know that larvae that are both younger [14–17 weeks old: A. Crane and A. Mathis, unpublished data] and older [approximately 3 years old [response was less consistent]: T. Crow and A. Mathis, unpublished data] than those in the study reported here responded to the alarm secretion in the laboratory.

- (4) How long should training exposures last? To mimic occurrences in natural habitats, exposure to stimuli (trout water+hellbender secretions) should be relatively short. We recommend a period of 10–20 min, after which water should be slowly replaced (ideally through a flow-through system such as the one in place at SHFS).
- (5) Is reinforcement training necessary before release? Most studies of predator-recognition training in amphibians [Woody and Mathis, 1998; Wildy and Blaustein, 2001; Mirza et al., 2006a; this study] and fishes [e.g. Chivers et al., 1995; Ferrari et al., 2005] used only a single training session. However, there was typically a relatively short time period (days) between training and testing, so the retention time without reinforcement is not known. One study demonstrated that fathead minnows, *Pimephales promelas*, can retain trained responses in the laboratory for at least 2 months [Chivers and Smith, 1994]. Given the uncertainties about retention abilities of salamanders, we recommend that either larvae be trained just before release or that a timetable of retraining sessions be established. If the latter option is selected, we recommend that retraining events include negative consequences, such as larvae being chased by a net or a trout model after exposure to the secretion+the trout water; without negative consequences, larvae could habituate to the trout stimulus. In previous studies, the effect of repeated training trials without negative consequences has not been consistent. Both Mirza et al. [2006b] and Berejikian et al. [2003] found that repetition of exposures eliminated fright responses for some fishes, but Sih and Kats [1994] reported no influence of exposure repetition on magnitude of the fright response in *Ambystoma barbouri*, and Vilhunen [2006] found that repeated conditioning enhanced the survival of Arctic charr (*Salvelinus alpinus*).
- (6) How can we know that trout-recognition training is successful at increasing postrelease survival? We recommend that initial efforts involve release of trained and untrained larvae that are marked for individual recognition. Success of this protocol can be determined by comparing recaptures for the two groups in subsequent population censuses.
- (7) Can hellbenders trained in captivity “train” other individuals following release? Cultural transmission of information used in predator-recognition has been demonstrated in the laboratory for both frog (*Pseudacris maculata*) tadpoles [Ferrari et al., 2007; Ferrari and Chivers, 2008] and several species of fishes [e.g. Mathis et al., 1996], and in natural habitats for fathead minnows [e.g. Brown et al., 1997]. However, both woodfrogs and minnows are known to school, whereas young hellbenders are likely more solitary, at least after some unknown initial period of time. Owing to the lack of information on the behavior of juvenile hellbenders in natural habitats, the possibility that information transfer between trained and naive individuals should be investigated.
- (8) Can hellbenders be trained to recognize multiple predators in the same training trial? Maybe. For example, glowlight tetras, *Hemigrammus erythrozonus*, were simultaneously exposed to tetra alarm cues and a “cocktail” of stimuli from three novel heterospecifics and later exhibited fright responses to all three species [Darwish et al., 2005]. If a similar ability applies to hellbenders, then training captive individuals to recognize multiple potential predators could be an efficient process.

Contributions to Our Understanding the Basic Biology of Hellbenders

Our data support the hypothesis that stress secretions from adult hellbenders function as an alarm pheromone. Voluntarily released secretions from stressed individuals have also been shown to perform an alarm function in some *Plethodon* [Mathis and Lancaster, 1997; Watson et al., 2004] and *Ambystoma* [Hedberg, 1981] salamanders, and likely other genera as well [other studies have used damage-released extracts rather than voluntarily produced secretions: *Cynops*: Marvin and Hutchison, 1995; *Desmognathus*: Lutterschmidt et al., 1994; *Eurycea*: Marvin et al., 2004; *Rhyacotriton*: Chivers et al., 1997; *Notophthalmus*: Woody and Mathis, 1997; *Triturus*: Margolis, 1985]. One important difference between the studies of salamanders in other genera and our study is that the subjects of the above studies have all been postmetamorphic adults. Our study is the first to document that larval salamanders respond to adult distress secretions with an alarm response.

The nature of the alarm response (increased activity) was surprising. In Gall and Mathis' [2009] study, hellbender larvae responded to predatory chemical stimuli by freezing; reduced activity is a common fright response of amphibians [e.g. Skelly, 1994; Lefcort, 1996; Mathis et al., 2003]. In contrast, the response of the larvae in our study was to increase activity; qualitatively, the larvae seemed to be searching for shelter. There are several hypotheses that could explain the difference between the nature of the fright response of larval hellbenders reported by Gall and Mathis [2009] (decreased activity) and by our study (increased activity). First, different testing conditions may lead to different behavioral responses; for example, aquatic *Eurycea tynerensis* salamanders decrease activity under laboratory conditions, but increase activity (flight) under field conditions [Hickman et al., 2004]. This explanation can be rejected because we used the same methods for our testing trials as were used by Gall and Mathis [2009]. Second, alarm cues provide different information than cues directly from the predator, and so may elicit a different type of response. We also reject this hypothesis based on our preliminary data on hellbender larvae that were a few weeks younger than those in this study; these younger larvae showed decreased activity in response to the alarm secretion (Mann-Whitney $U = 222$, $P = 0.001$, unpublished data). Third, hellbender larvae may exhibit an ontogenetic or size-based shift in the nature of their responses to alarm cues; similar shifts in response to predatory cues have been reported for fishes (although in those studies, experience cannot be ruled out) [Brown et al., 2001; Harvey and Brown, 2004] and ambystomatid salamanders [e.g. Mathis et al., 2003]. If this hypothesis is correct, then the behavioral shift occurs at some point between 14–17 weeks (our preliminary data) and 21–25 weeks (this study), at least under laboratory conditions. Data from Gall and Mathis' [2009] study of 8–9 week old larvae are consistent with this hypothesis. Mathis et al. [2003] suggested that flight responses by larger *A. annulatum* may be more effective than for smaller individuals, which showed reduced activity. These behavioral shifts may result from increased motor capabilities of prey, as was suggested by Fronzuto and Verrell [2007] for *Ambystoma tigrinum* larvae. Interestingly, in a post hoc analysis, we found that for trained individuals, there was a significant positive correlation between body size and activity (Spearman $r^2 = 0.18$, $P = 0.016$), indicating that larger individuals are more active (faster latencies to move).

Opportunities for predator-recognition learning may occur in natural habitats. If larvae occur in close proximity, predation on nearby larvae might result in the release of distress chemicals that could “warn” nearby larvae. Larval salamanders typically lack the glands required to produce such secretions [Formanowicz and Brodie, 1982; Mathis and Vincent, 2000], but we have recently observed productions of large quantities of the secretion by larvae during disturbance (captured with a net) [Gall et al., 2010]. In addition, it is possible that larvae could learn from adults; recently, an adult was filmed apparently guarding a group of larvae in a river in Georgia [Jones, 2006]. Thus, there is a large potential for learning to play an important role in the development of antipredator responses for hellbenders.

CONCLUSIONS

1. Because of population declines, hellbenders (*Cryptobranchus alleganiensis*) are currently being reared in captivity with a goal of releasing head-started individuals. However, hellbenders released from captivity may be vulnerable to predation from introduced trout.
2. We used a classical conditioning technique (pairing hellbender stress secretions with a trout stimulus) to train larval hellbenders to associate fright with cues from nonnative trout. Trained larvae responded to trout cues with appropriate antipredator behaviors.
3. Our study shows that hellbender secretions function as a chemical alarm cue, and larval hellbenders can learn to respond to novel predators in future encounters after being exposed previously to the predator’s scent paired with the hellbender alarm cue.
4. In areas where there are no native trout, we recommend that similar protocols be used by head-starting facilities to train captive-reared hellbenders to recognize trout as predators before their release.

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