2002

The Embryonic World of Wood Frogs, Rana Sylvatica: Natal Pond Learning and Anti-Predator Behaviors.

Pamela J. Bryer

THE EMBRYONIC WORLD OF WOOD FROGS, *RANA SYLVATICA*: NATAL POND LEARNING AND ANTI-PREDATOR BEHAVIORS

By

Pamela J. Bryer

B.A. University of Maine, 1999

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Zoology)

The Graduate School

The University of Maine

December, 2002

Advisory Committee:

William E. Glanz, Associate Professor of Biological Sciences, Advisor

Mary S. Tyler, Professor of Biological Sciences

Malcolm L. Hunter Jr., Professor of Wildlife Ecology

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Thesis Advisor: Dr. William E. Glanz

An Abstract of the Thesis Presented
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Even while *in ovo* many amphibians can react to the world around them, as dissolved molecules are able to pass through their protective jelly matrix and interact with developing sensory systems. Although there are many potential signals dissolved in the water, two, natal-pond recognition cues and predator recognition signals (or kairomones), have been previously shown to be relevant to several species of developing anurans. My study used natural odorants in a test of natal pond learning, and in testing both short- and long-term effects of predator chemical cues on the development and behavior of wood frog, *Rana sylvatica*, embryos.

In 1992 Hepper and Waldman published a series of experiments on wood frog embryos. They exposed individuals to orange, lemon-like, and strawberry essence water during the embryonic period. After hatching, the tadpoles were tested for their stimulus preference, and whether or not embryonic exposure had changed their native response to the stimulus. In both the orange and strawberry essence trials, tadpoles that had been exposed to the stimuli as embryos significantly preferred those stimuli, as compared to embryos that had been raised in unadulterated water. These experiments demonstrate that embryonic wood frogs possess the ability to 1) perceive and 2) learn chemosensory information present. However, the stimuli used were highly artificial and not analogous to any dissolved molecules present in natural bodies of freshwater.

To test whether the tadpoles prefer orange extract water because it mimicked an "imprinting" experience on natal pond water, I exposed embryos to natural pond water in the laboratory. Tadpoles showed consistent preferences for water types high in turbidity in choice tests. However, the embryonic exposure to various natural water types did not play a part in larval forming preferences.

To test whether the reaction to orange-flavored water was analogous to a potential anti-predator behavior, I raised embryos in the laboratory in various predator-labeled waters. In the first year I used fish, earthworm (as a non-predator animal control), and well water

rearing treatments. These stimuli produced differences in timing and size at hatching, but for the earthworm control treatment only. However, at the time of metamorphosis the earthworm treatment showed no differences in size or anti-predator behavior, as compared to the fish and spring water treatments. In the second year, I expanded the treatments to include more realistic predators on wood frogs. I reared embryos in water containing fish, newts, larval caddisflies, larval dragonflies, and leeches; the control treatments contained earthworms or consisted of well water. The experiment ended at hatching, and no significant differences between any of the treatments were found.

These experiments imply that the embryonic experiences in wood frogs do not seem to play a major role in future habitat choices. Importantly though, this study shows that embryonic phenotypic plasticity may exist in wood frogs. Finally, differences seen at hatching do not transfer to differences at metamorphosis.

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Introduction

Wood frogs, *Rana sylvatica*, are medium-sized, terrestrial frogs of northern forests. At a site in Minnesota, Bellis (1965) found wood frogs to have a mean home range of 70.0 m^2, though there was great variation. Breeding habitat typically consists of ephemeral pools in the forest, which become dry annually. At a study site in Maryland, wood frogs were faithful to their natal pond 80% of the time, but once an individual frog bred successfully at a site, it was 100% faithful to that site (Berven and Grudzien1990). Despite the distance traveled in a year, wood frogs are able to select a specific water body typically out of many alternative breeding sites, each with a congregation of other calling conspecifics.

The exact cues that amphibians use to return to their breeding pond are not known, but most likely a combination of senses contribute, including celestial cues, polarized light, magnetism, humidity gradients, landmark-based visual cues, and auditory cues from calling males (Sinsch 1992, Stebbins and Cohen 1995; Freake *et al.* 2002; Bellis 1962; Stebbins and Cohen 1995). Chemosensory cues may also be used in the pool selection process. There is evidence that wood frogs will avoid pools with fish predators, presumably after sampling the water (Hopey and Petranka 1994). Gray treefrogs, *Hyla versicolor*, can discriminate between pools with and without heavy parasite loads, and avoid

ovipositing in pools containing parasites (Kiesecker & Skelly 2000). McGregor and Teska (1989) showed that *Ambystoma maculatum* shows a preference for mud removed from their home pond over mud removed from different ponds. Chemical cues available in freshwater habitats can be quite specific; Wisenden *et al.* (1994) showed that the brook stickleback, *Culaea inconstans*, can distinguish water taken from locations with and without predators within the same water body. Chemical cues learned early in life therefore may provide information that plays a role in natal pond homing.

Hepper and Waldman (1992) demonstrated learning of water-borne chemical cues by wood frog embryos. They raised embryos in water labeled with either orange, strawberry, or lemon-like extract until hatching, when all individuals were transferred to well water. Approximately 30 days after hatching, the tadpoles were given choice-tests of embryonic-rearing water versus non-familiar water (including well water). Wood frog tadpoles consistently chose the water in which they were reared, which suggests wood frog embryos may "imprint" on the chemical cues present in the embryonic environment.

In this study I tested whether this embryonic period of chemical-cue sensitivity and learning could be related to the ability of wood frogs to home to their natal pond. I replicated the design of Hepper and Waldman (1992), but I used natural water bodies as the sources of my chemical cues. I hypothesized that embryos exposed to odor of a specific

water body during development would learn that odor and prefer to associate with it later, as tadpoles. Because the water body stimuli also represented variation over a range of predator chemical cues, I recorded the time taken to reach hatching, as a possible developmentally plastic response to these predators.

Materials & Methods

To test whether wood frog embryos could learn to recognize the water body in which they developed, I set up a "common garden" embryo-rearing experiment. Embryos were raised in three different types of water: water collected from an agricultural field ditch, a river, and well water (hereafter ditch, river and well respectively). After hatching, the tadpoles were raised in well water until they were old enough for behavioral choice tests.

Eggs from five maternal contributions were collected from a roadside ditch on the University of Maine campus in Orono, Maine within six hours of oviposition, before full egg hydration had taken place (stage 1, Gosner 1960). The eggs were exposed to their true natal pond's cues for these few hours, but not at any other point in the experiment. The egg masses were carefully divided up to allow individual eggs to be placed in each of the three rearing treatments.

Sixty individual eggs were used in each of the three rearing treatments. After hatching, all tadpoles were placed into well water, which was used as the control water in later experiments. The well water

was obtained from a well in Greenbush, Maine (approximately 16 kilometers from the breeding site). River water was collected from the Stillwater River in Orono, Maine; the Stillwater River is a large permanently flowing river that contains an abundance of predators (both aquatic invertebrates and vertebrates). Wood frogs have not been observed breeding in it despite its proximity to their breeding habitat. The ditch water was collected along the University of Maine Witter Farm Road, Orono Me. The ditch represents a semi-permanent source of water close to wood frog wintering habitat, although no wood frogs were breeding there at the time of collecting. No vertebrate predators were detected in the area, but some invertebrate predators (most obviously larval dragonflies, Anisoptera) were noted (pers. obs.). The ditch water presumably represented the water most similar to typical breeding habitat for wood frogs.

As the eggs came up to room temperature (23°C) they were placed individually into an opaque Nalge ® cup containing 400ml of the appropriate stimulus water. The rearing chambers were then positioned in a random stratified block design on two shelves in the laboratory. The room was kept on a natural photoperiod via windows and laboratory lighting throughout the experiment.

Once stage 16 (Gosner 1960) was reached, the eggs were observed every six hours to monitor for hatching. At hatching, each individual tadpole was removed from the cup temporarily, while the cup was

cleaned and refilled with well water. Additionally, the time of hatching was recorded, allowing for the analysis of effects of the stimuli on speed of embryonic development. Once the tadpoles were active, they were fed a diet of rabbit chow, occasionally supplemented with Tetramin® fish flake food. Tadpoles were raised to the point where they were able to perform in the choice tests, stage 24 (Gosner 1960), about 25 days post hatching.

The choice tests were conducted in long, shallow chambers (swimming area= 80 cm long, 5 cm wide by 3 cm deep) that allowed two stimuli to be placed at opposing sides for the tadpoles to choose between. The chambers were constructed from 6 cm diameter PVC tubing with 1/3 of the long axis removed to create an open top. Both ends of the chamber were blocked off (at 5 cm) with plastic mesh screening, to allow a zone for addition of the stimuli. The bottom of the chamber was marked with lines to create four equal-sized compartments.

Each trial consisted of an acclimation period, followed by pre-stimulus, stimulus addition, and post-stimulus periods. During the acclimation period (15 minutes) the chambers were filled with 300 ml of well water. I videotaped the pre-stimulus and post-stimulus periods (both 10 minutes long), as well as the intervening stimulus addition period (5 minutes). To add the stimuli I slowly and gently poured 200ml of the stimulus waters into the chamber ends simultaneously.

The three rearing treatments were divided into three groups for testing pairs of stimuli: river vs. ditch waters, river vs. well waters, and ditch vs. well waters. Each tadpole was tested only once. Dye trials conducted previously with an active tadpole confirmed that the stimuli at both ends diffused slowly toward the middle, but remained concentrated at the ends and that no detectable mixing occurred during a trial. The dye of one side typically remained slightly separated from the dye of the opposing side at the end of fifteen minutes, with an area of no dye (2-3 cm) in the middle of the test chamber. This created two compartments that were concentrated with dye (at the ends) and two (in the middle) that had a portion with no stimulus.

During video analysis, I noted tadpole position by recording which of the four compartments were occupied at 15-second intervals. For the data analysis, only positions at the extreme ends (closest to the stimuli) were used in calculating preference. Tadpole activity levels were measured by counting the number of line crosses. After the addition of the stimulus waters, I recorded the time taken to resume movement with a stopwatch.

For statistical analyses I performed Kruskall-Wallis analysis of variance tests on the line cross and time-to-move data. The choice test preferences were analyzed by creating a preference score ((time in the rearing stimulus compartment)/ (time in the rearing stimulus + time in the alternative stimulus)) and analyzing the scores with Kruskall-Wallis

analysis of variance tests. Time-to-hatch data were analyzed by a parametric analysis of variance test, using both rearing treatment and shelf position as independent variables. All statistical tests were performed with Systat 9 software.

Results

Embryonic rearing environment had no effect on the choices made by tadpoles, Figure 1.1, (Kruskall-Wallis test statistic=13.576, d.f.=8, p=0.093). Tadpoles responded to the stimulus waters similarly, regardless of their prior experience as embryos, for all three rearing treatments (ditch, river and well waters) when tested against each of the three choice test comparisons (ditch vs river, ditch vs well and river vs well). The tadpoles did show preferences for different types of stimulus waters, preferring the ditch water to the river water and both ditch water and river water were preferred to well water (median test statistic= 4.537, X^2_2= 5.991, p>0.05). Rearing treatment had no effect on activity patterns, as measured by change in the number of line crosses (calculated from the difference between the pre-stimulus and the post-stimulus) during the choice tests (Kruskall-Wallis test statistic = 3.635, d.f.= 2, p=0.162). Additionally, rearing treatment had no effect on time taken to resume movement after the disturbance of the stimulus being added to the test chamber (Kruskall-Wallis test statistic = 0.658, d.f.= 2, p=0.719).

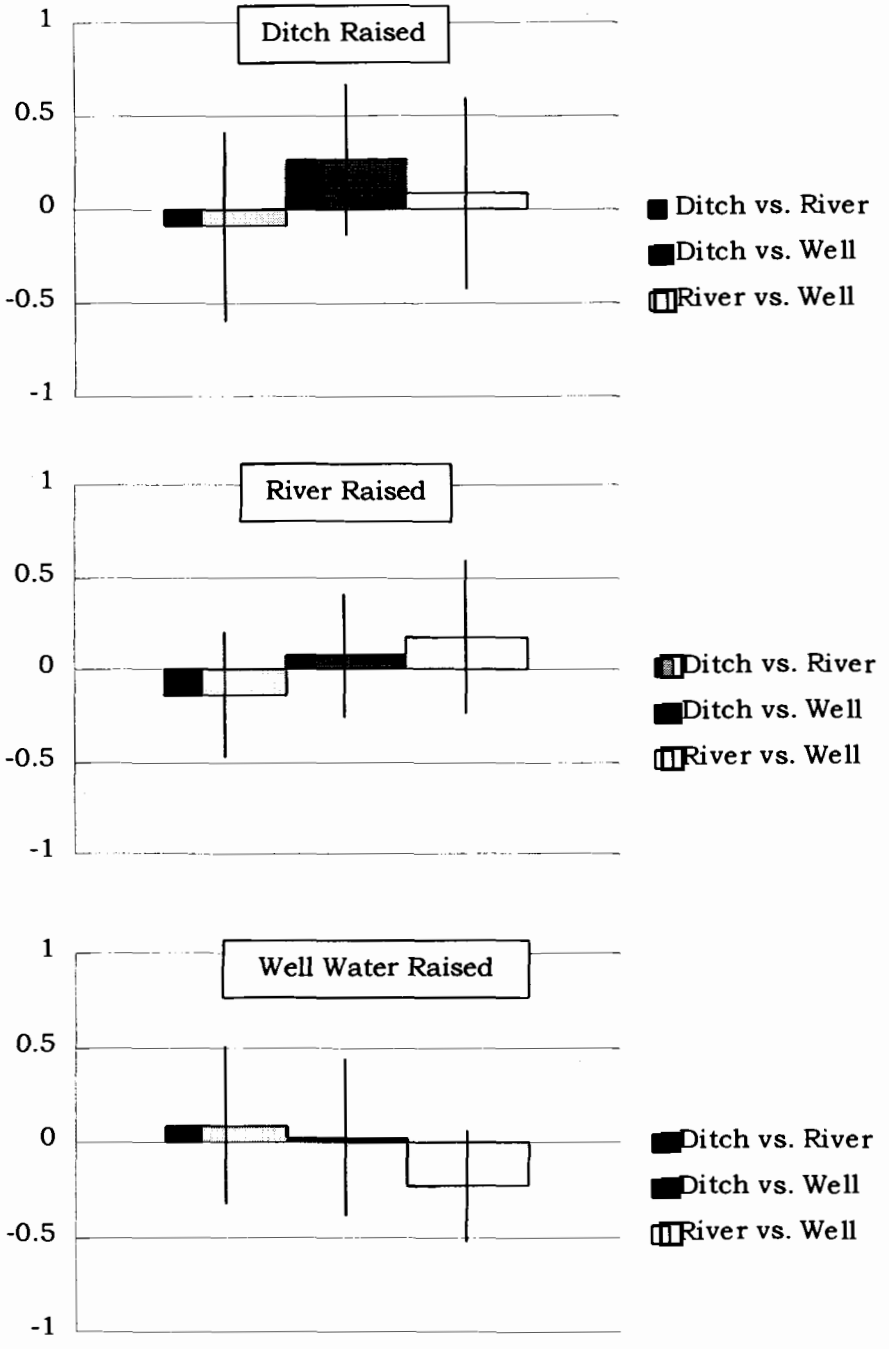

Figure 1.1 Choice test preference scores (mean ± SD). Positive values indicate movement towards embryonic rearing stimulus in paired choice tests, see legend for pairings. When neither choice test contained the embryonic rearing stimulus no choice was the predicted result. To calculate a preference in those cases a stimulus preference was assigned, from top to bottom: River; Ditch; Ditch.

Embryonic rearing environment did have a surprising effect on time taken to hatch, Figure 1.2. The three rearing treatments did not have any significant overall effect on time to hatching (Kruskall-Wallis test statistic= 5.540, n= 173, p= 0.063). However, the two laboratory shelves introduced unanticipated temperature differences, which resulted in different hatching times, with the lower shelf resulting in earlier hatching (Mann-Whitney U test statistic= 13455, n= 294, p< 0.001).

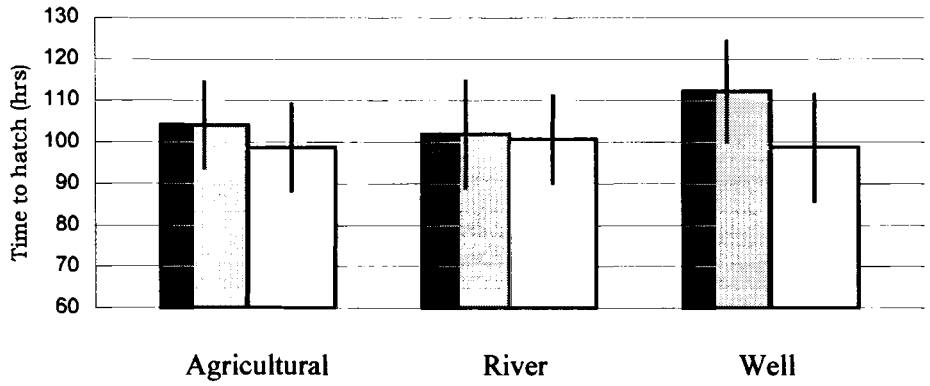

Figure 1.2 Effects of shelf position on time taken to hatch, by treatment (mean ±SD shown). Dark bars represent the top shelf; white bars represent the bottom shelf.

Discussion

The results here indicate that learning pond-specific chemical cues does not occur during the embryonic period in wood frogs. Tadpoles from all three rearing treatments showed consistent patterns in the water types that were preferred. Typically the more visually turbid water type of the two choices was preferred, as the ditch water (the most turbid) was preferred over others, and when given a choice, river water (moderately

turbid) was preferred over well water (clear). Additionally, I found that embryos did not alter their time to hatching in response to the predator (fish) chemical cues present in river water as compared to other rearing environments. This response by wood frog embryos may be due to the nature of these predator chemical cues (kairomones).

In this experiment time-to-hatch data were collected ancillary to the main purpose of examining embryonic natal pond learning. However, these data give us an opportunity to examine how realistic (natural strength) stimuli can be used to test for predator-induced hatching plasticity. Typically, in laboratory tests for hatching plasticity, predators are caged in a known volume of water for extended periods of time to create a proximate, fresh, and concentrated threat (Schalk *et al.* 2002, Chivers *et al.* 2001). Predator-induced phenotypic plasticity has been demonstrated in at least 11 species of amphibians (Sih & Moore 1993, Warkentin 2001, Chivers *et al.* 2001, Schalk *et al.* 2002, Warkentin pers. comm.), creating a pattern of expected responses to predator stimuli. The lack of response by wood frog embryos to chemical cues present in the waters tested may indicate that the embryos do not respond to weaker, generalized predation threats. Alternatively, wood frogs may not be able to respond to the predator kairomones at all (see Chapter 3), possibly because other constraints on their life history are more important.

The lack of a response to the natural strength pond cues is surprising, given that wood frogs have been shown to learn artificial cues embryonically. The series of experiments by Hepper and Waldman (1992) showed that wood frog embryos exposed to either orange- or strawberry-extract water would show a preference for those chemical cues later as tadpoles. When embryos were raised with an extract that naïve tadpoles found aversive (lemon-like), the resulting tadpoles were significantly more tolerant of the odor than lemon-like naïve individuals.

The protocols of the present study and Hepper and Waldman (1992) were similar, but the results are nearly opposite. In the current study tadpoles showed consistent preferences independent of rearing environment, whereas Hepper and Waldman showed that odor-naïve, tadpoles showed no preference for or against two of the extracts used. The contrast between these separate experimental results may illustrate an important difference, the nature of the stimuli.

Sneddon *et al.* (1998) performed a similar choice tests with chickens, in which they demonstrated that chicken embryos could imprint on strawberry-scented wood chips present only prior to hatching. Here the immediate utility of imprinting seems obvious, as chicks benefit from staying near the nest for several days following hatching. In contrast, the use of strawberry extract in wood frog embryos learning seems less relevant to the environment they experience as tadpoles.

The present study suggests that the purpose of an embryonic chemosense early in development is not natal pond water recognition, which is consistent with patterns of imprinting in other metamorphosing organisms. The mechanism of natal stream imprinting in salmonid fishes may suggest that pond learning by wood frogs could occur during metamorphosis, rather than during embryonic development. Wood frog tadpoles have an additional two to three months to acquire chemical cue information before leaving the pond as froglets. In salmonids the major period of imprinting is during the process of smoltification via the dramatic elevation in circulating thyroxine levels (Papi 1992). During amphibian premetamorphosis, thyroxine levels rise markedly, contemporaneous with continued exposure to the chemical cues of the natal pond by the larvae. Although most tadpole tissue is lost during metamorphosis, at least some of the same nasal epithelial neurons are retained in the adult (Higgs and Burd 2001). These receptor neurons would then be able to remember the unique suite of chemical cues that identify the pond during the breeding season.

Other explanations of the potential functions of an embryonic chemosense in wood frogs are beyond the scope of this chapter, but may include predation avoidance (discussed in chapters 2 & 3) and kin recognition. The benefits in kin discrimination (avoiding cannibalism and enhanced predator avoidance) soon after hatching support the development of early kin recognition. Rautio *et al.* (1991) showed that by

age 17 days, wood frog tadpoles prefer to associate with kin over non-kin. Hepper and Waldman (1992) tested whether or not the extracts used in their experiments interfered with the kin recognition process, which they did not.

None of the rearing waters used, with their varying levels of predator cues, had any effect on time-to-hatch characteristics independent of temperature. The well water had no predator cues and was low in turbidity and nutrients. The ditch water was a natural strength environment without vertebrate predators, which is typical wood frog breeding habitat. River water, on the other hand, presumably contained predator chemical cues, and wood frogs did not breed there. Yet, tadpoles preferred river over well water, contradicting the anti-predator response predicted from lab studies. However, previous laboratory studies have used concentrated chemical cues from specific predators which complicates their translation to real world situations.

In summary, this paper does not describe the mechanism of wood frog philopatry. The results show that tadpoles can distinguish and show preferences for waters of different quality. However, these data indicate that wood frogs do not rely solely on embryonic memory of these water characteristics when selecting habitats later in life.

CHAPTER 2. SHORT-TERM EFFECTS OF EMBRYONIC EXPOSURE TO PREDATOR CHEMICAL CUES

Introduction

Because eggs of wood frogs (*Rana sylvatica*) are conspicuous, shell-less, and unguarded, they are easy prey for a variety of predators. To compensate for this vulnerable period, several mechanisms are used by wood frogs to avoid egg predation. A relatively rapid embryonic development shortens the exposure to predators (Rugh 1948). Explosive and communal breeding increases the predator dilution effect by concentrating the propagules in time and space (Duellmann and Trueb 1986, page 193). Another possible predation avoidance mechanism is phenotypic plasticity in developmental processes (*i.e.* developmental plasticity), wherein embryos change either the duration of development, the developmental stage at hatching or the size at hatching to effectively deal with the predation threat. Phenotypic plasticity is different from other predation avoidance mechanisms, because the embryo only responds to the threat when necessary. Without plastic responses, prey often face tradeoffs; for example, tadpoles from communal breeders in small pools that lack predators potentially face decreased fitness as a result of competition and crowding (Lynn & Edelman 1936 in Alford 1999). Alternatively, for tadpoles in ponds that contain predators, fewer individuals survive but those individuals that do often have access to more resources (DeBenedictis 1974, Cortwright 1988 both in Alford

1999). In contrast, developmental plasticity allows individual embryos to either, when no egg predators are present, stay in the safety of the egg capsule, or, when egg predators are present, change the timing of hatching to reduce the exposure to the predator.

Many species of amphibians display developmental plasticity during egg and tadpoles stages. Warkentin (2001) has shown that the embryos of the red-eyed treefrog, *Agalychnis callidryas*, can respond to snakes, wasps, or a fungus by escaping out of the arboreal eggs and falling into the water below. This response may be unique in that the anti-predator reaction can be induced by mechanical stimuli, in contrast to the chemical stimuli typical of the other examples of phenotypic plasticity. The embryos under attack benefit immediately by escaping predation, but the young hatchlings face risks in the water. Predation by the benthic shrimp, *Macrobrachium americanum*, on the young hatchlings may be severe. The shrimp are also responsible for phenotypic differences observed in the tadpoles, which develop deeper tails for better shrimp escaping capabilities.

Many examples of embryonic plasticity in amphibians involve early hatching in response to chemical cues of aquatic predators or threats. Chivers *et al.* (2001) showed that both *Hyla regilla* and *Rana cascadae* can hatch sooner in response to cues of leech predation threat. In contrast, the streamside salamander, *Ambystoma barbouri*, delays hatching in response to predatory flatworms, *Phagocotus gracilis*, and

sunfish, *Lepomis cyanellus*, (Sih & Moore 1993; Moore *et al.* 1996). By delaying hatching, the larvae hatch at a larger size and are better able to escape attack (Sih & Moore 1993).

Schalk *et al.* (2002) found that *Rana clamitans* embryos delay hatching, and hatch at a larger size, when reared with leeches (*Macrobdella decora*). By staying motionless within the egg capsule, the embryos may not attract the attention of the leech and its vibration-dependent foraging, and thus the embryos' time to hatching is prolonged.

To better understand the embryonic life of wood frogs, *Rana sylvatica*, I attempted to test: 1) whether wood frogs had the ability to facultatively alter the timing or size at hatching; and 2) what types of predation-threats provoke developmental responses in wood frogs. In the first year's experiment (2000) I tested embryos' responses to visually-hunting tadpole predators and control treatments. In the second year (2001) I expanded the treatments to cover a wider range of likely-to-be-encountered sympatric predators, which included egg predators, tadpole predators, and predators that feed on both eggs and tadpoles.

Materials & Methods

Year 2000 Experiments

To test whether wood frog embryos would respond to chemical cues of predators with phenotypic plasticity, I raised eggs in three types of stimuli: fish-labeled, a novel-odor control, and a blank control. In both

years the bioassay remained the same; in the laboratory I incubated embryos in different chemical stimuli until hatching, when I noted the time taken to hatch and then measured the individuals.

In both years the same egg collection site was used. Eggs were collected from a recurrent temporary pool that forms alongside a gravel road on the University of Maine Orono campus. The pool has dried each year for five years (pers. obs.). The only predators observed in the pool are dytiscid beetle larvae, though other invertebrates may be present.

Eggs from five maternal contributions were collected within six hours of oviposition, *i.e.*, before full egg hydration had taken place (stage 1, Gosner 1960). The egg masses were carefully divided up to allow five individual eggs to be placed in each container. Eighty containers total were used (n_{FISH} =28, n_{WORM} =27, $n_{CONTROL}$ =25).

The fish-labeled cues were generated by placing a single fish (*Perca flavescens*) into 30L of aerated well water for 24 hours. A total of three fish (mean total length of approximately 150 mm) were used throughout the study; each was selected haphazardly for stimulus collection. The fish were fed a diet of brine shrimp and tubifex worms for 12 months prior to the experiment. To provide a novel-odor stimulus I generated earthworm-labeled cues by placing approximately 75 ml of loosely packed earthworms (*Lumbricus sp.*) in 30L of aerated well water for 24 hours. The blank (control) cue was generated by placing 30L of well water in an aquarium with aeration overnight. This ensured that the

temperature and any chemical cues left by the equipment were standardized between the treatments. After hatching, all tadpoles were raised in well water for use in another study (Chapter 3). The well water was pumped from a well 16 kilometers from the egg-collection site and transported to the laboratory.

As the eggs adjusted to laboratory temperature they were placed into an opaque Nalge ® cup containing 400 ml of the appropriate stimulus water. These rearing containers were then positioned in a random stratified block design (*i.e.* each row of rearing containers contained at least one container from each treatment, but the order within each row was randomly determined) set within a cold water bath (11°-13°C). The room was kept on a 14L: 10D photoperiod throughout the experiment.

Once the embryos reached stage 16 (Gosner 1960) the eggs were observed every three hours to monitor for hatching. At hatching each tadpole was measured under a dissecting microscope with calipers to the nearest 0.05mm; both total length and body length were recorded. The developmental stage of the hatchling was recorded according to the staging table in Gosner (1960), supplemented by descriptions in Rugh (1948). Malformations were recorded as encountered. When the observations involved going into the laboratory during the dark portion of the photoperiod, the room was kept dimly lit and a flashlight was used where needed.

For statistical analyses I performed separate analyses of variance on duration of the embryonic period, total length at hatching and developmental stage at hatching. Where significance was found, I used Bonferroni multiple comparisons for treatment comparisons. All analyses were preformed on Systat 9 software.

Year 2001 Experiments

In 2001 I attempted to retest the same population of wood frogs for developmental plasticity in response to predator chemical cues by expanding the list of predators. The predators used represent predators that can be sympatric with wood frogs, though none were observed at the collection site. All were collected locally and contemporaneously with the start of the experiment (with the exception of the previously collected fish). I tested whether wood frog embryos would respond to chemical cues of the following: sunfish (*Lepomis gibbosus*), newts (*Notophthalmus viridescens*), leeches (*Macrobdella decora*), caddisfly larvae (*Ptilostomis* sp.), dragonfly larvae (Anisoptera), earthworms (*Lumbricus* sp.), and blank well water. The experiment ended when I recorded the time and stage at hatching and measured total length for each individual.

The methods in 2001 were similar to those for 2000; the modifications in procedure and stimulus collection follow. Three sunfish were used instead of yellow perch; the mean total length was approximately 175 mm. The fish-labeled water was created by placing one fish in 20L of

well water overnight. Four eastern spotted newts were collected (mean weight 3.1 grams), reared separately, and fed wood frog eggs. One newt was placed in 10L of well water overnight to generate the stimulus. Three leeches were collected and housed together in the laboratory, where they were observed to consume large numbers of the wood frog eggs provided. Leech stimulus was created by placing a single leech (mean weight 5.0 grams) in 10L of well water overnight. The dragonfly-nymph stimulus was created with five individuals of unknown species (weighing a total of 1.9 grams) placed in 10L of well water overnight. The caddisfly-larvae stimulus contained 10 individuals of the genus *Ptilostomis* (weighing 6.5 grams total) that were placed in 10L of well water overnight. The earthworm treatment differed from that in 2000; in 2001 fewer worms were used than previously. The mass of the earthworms was set to closely match the mass of the leeches; five individual earthworms weighed a total of 6.2 grams. Earthworm stimulus was created by placing the five individuals in 10L of well water overnight.

Five eggs (from five maternal contributions) were placed in a single rearing container, holding 400ml of the appropriate stimulus. Twenty rearing containers were used for each treatment (n per treatment= 20, total number of eggs per treatment= 100) and arranged in a stratified random block design. The eggs were observed every five hours to monitor for hatching.

For statistical analyses I performed a Kruskall-Wallis analysis of variance for both time taken to hatch and length at hatching, on rearing container means. All statistical tests were performed on Systat 9 software.

Results

2000

Wood frogs embryos showed both developmental and phenotypic plasticity in this experiment. Wood frog embryos exposed to fish-labeled water and blank water took a similar amount of time (Figure 2.1) and

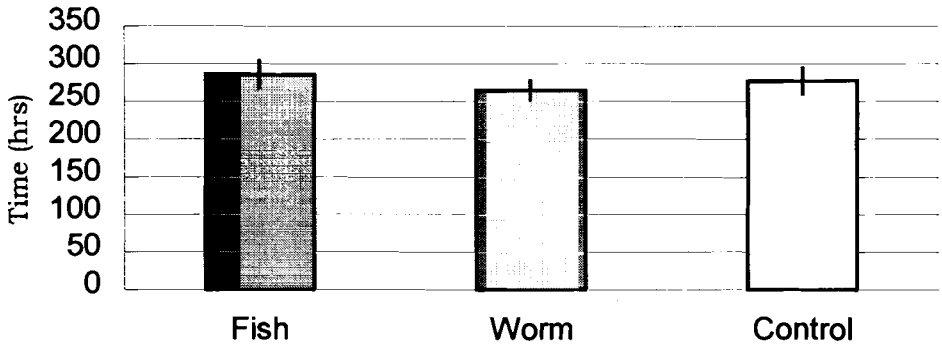

Figure 2.1 Mean time to hatching in hours from start of experiment (± SD).

were the same length at hatching (Figure 2.2). The embryos that were raised in the worm-labeled water hatched significantly smaller and earlier as compared to the other treatments ($ANOVA_{length}$ d.f.=2, F=11.547, p=0.000 ; Bonferroni multiple comparisons: fish vs worm p=0.000, worm vs blank p=0.0.011, blank vs fish p=0.206; $ANOVA_{time}$

d.f.=2 , F=11.758, p=0.000 ; Bonferroni multiple comparisons: fish vs worm p=0.000 , worm vs blank p=0.02, blank vs fish p=0.107). No statistical test was performed on the stage at hatching data, as there was virtually no variation, and almost all wood frog embryos hatched during stage 20.

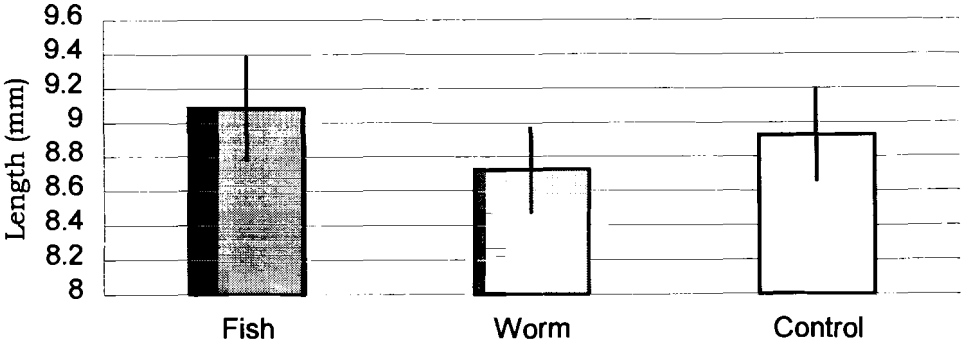

Figure 2.2 Mean length in mm (± SD) at hatching.

2001

In contrast to the previous year's results, wood frogs did not display any plastic responses to the treatments in 2001. There were no significant differences in length between the treatments (Figure 2.3); mean hatchling length was 7.6 mm (± 0.2 SD) (Kruskall-Wallis analysis of variance: d.f.=6, test statistic=9.537 , p=0.146). There were no significant differences in the time taken to hatching (Figure 2.4); the mean elapse time to hatching was 105.7 hrs (± 10.6 SD) from the start of the test period (Kruskall-Wallis analysis of variance: d.f.=6, test statistic=5.490 , p=0.483). No statistical test was performed on the stage at hatching

data, as again there was no variation (only three of 674 hatchlings were not in stage 20 when they hatched).

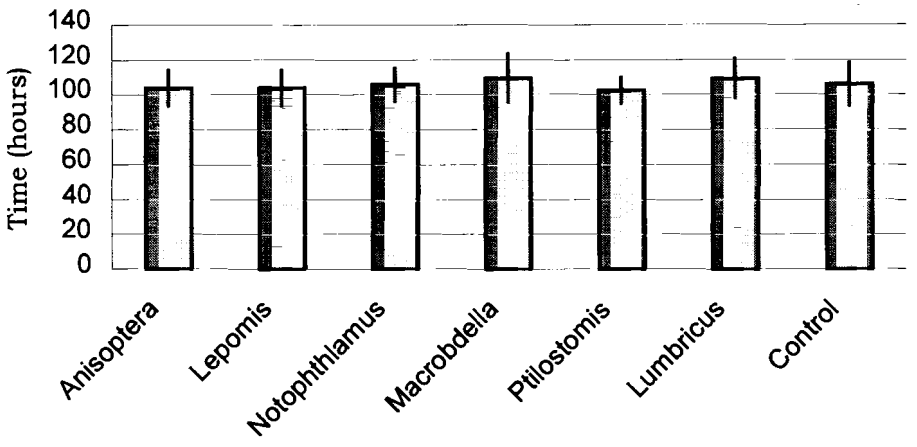

Figure 2.3 Mean time taken to hatch, in hours (±SD). Embryonic rearing treatments consisted of dragonfly nymphs, fish, newts, leeches, caddisflies, earthworms and control treatments.

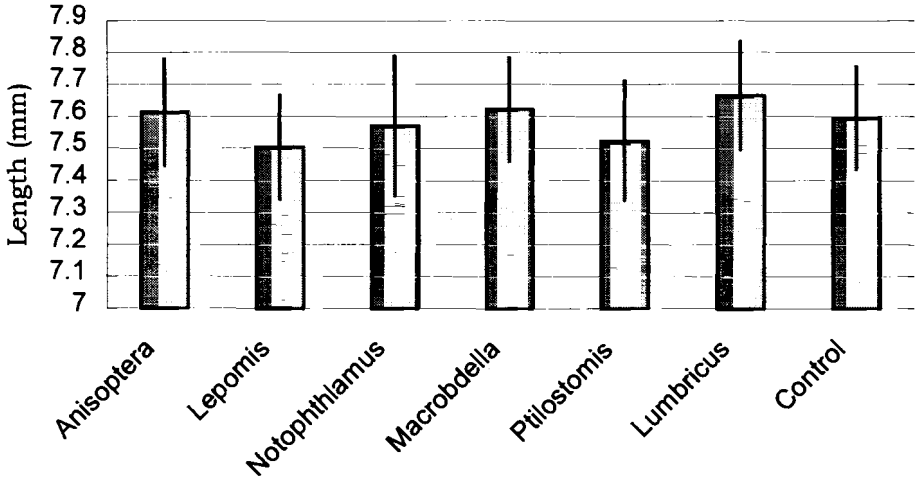

Figure 2.4 Mean length of hatchling (±SD). Embryonic rearing treatments consisted of dragonfly nymphs, fish, newts, leeches, caddisflies, earthworms and control treatments.

Discussion

This study produced no clear patterns in developmental or phenotypic plasticity in wood frog embryos. During the first year (2000) embryos showed plasticity in both length and timing at hatching. In response to a novel-odor control treatment (earthworm), wood frog embryos hatched sooner and at a smaller size when compared to the hatchlings from the other treatments (fish-labeled and well water). In the second year (2001) wood frog embryos showed no significant plastic reaction to any of the stimuli (fish, newt, caddisfly, dragonfly, leech, earthworm, or well water). All the embryos hatched at the same approximate time, and the hatchlings were all similarly sized.

Given the patterns in other studies of specific embryonic responses to threats, these results are disappointing. My prediction that wood frog embryos would delay hatching in response to the visually-hunting predators of tadpoles (the dragonfly and fish treatments) was not supported. These results contrast with what is know about hatching patterns in *A. barbouri,* the salamander embryos that delay hatching into a dangerous larval environment (Sih and Moore 1993; Moore *et al.* 1996).

The embryos exposed to an egg predator (the caddisfly larvae treatment) were predicted to respond to that predator with a shortening of the embryonic period, similar to the studies of Warkentin (2001) and Chivers *et al.* (2001). Warkentin found that embryos of red-eyed treefrogs could respond to the threat of predation by hatching

immediately. However, the caddisfly treatment did not produce the predicted shortening in embryonic period. The caddisfly species chosen, *Ptilostomis sp.*, has been observed to devastate wood frog clutches (MB Koloszvary pers. comm.). The lack of response to a known, frequently syntopic predator like *Ptilostomis* caddisflies was inconsistent with expectations based on Warkentin (2001) and Chivers *et al.* (2001).

The fish (two species), leech, and newt treatments potentially represented both egg and tadpole predators. The direction of the response to predators that attack more than one of their prey's life history stages would be difficult to predict. Despite these capacities for oophagy and predation by fishes, leeches, and newts as well as the inducible defenses of the wood frog, I did not document any reaction to predation cues. Interestingly, I did not observe these predators at the egg collection site even though they are present nearby (within 0.5-1 km). When predation-threat cues become unreliable within a population, the likelihood of an anti-predation reaction evolving becomes less probable (Harvell 1990). Because of the high levels of philopatry in wood frogs it is possible that at this specific breeding site the population does not ever encounter these particular predators.

Clearly, this study has not entirely resolved the importance of predator chemical cues in the embryonic development of wood frogs. It demonstrates that, yes, they can alter developmental timing (in response to the novel-odor control cues created with earthworms in 2000), but we

do not understand the nature of those cues. Given responses to predator chemical cues by larval wood frogs (Petranka & Hayes 1998, Chivers & Mirza 2001) and given that this experimental protocol closely follows other successful demonstrations of developmental plasticity, it is surprising that no predator-induced plasticity was observed. One possible explanation is that such responses vary greatly among populations. Relyea (2002) demonstrated that in as few as 13 generations wood frog populations could adapt to changes in a pond's predator composition. He found that wood frogs from ponds with variable predator composition showed a greater capacity for phenotypic plasticity. Alternatively, wood frogs in ponds with consistent predator composition between years showed few phenotypic differences from one another and lower capacity for phenotypic plasticity.

Another possible explanation for these results is that the available chemical cues are not reaching their targets, or not stimulating them sufficiently. Despite several attempts, the Sih laboratory has not successfully duplicated their *A. barbouri* results from Sih and Moore (1993); water chemistry is thought possibly to play a part in the difference (Warkentin pers. comm.). In another freshwater predator-prey system, Brown (pers. comm.) has shown that a fish alarm pheromone, hypoxanthine-3-N-oxide, is rendered inactive in acidic waters. The increased acidity adds a hydrogen ion to the signaling compound, thereby changing its efficacy. All chemical signals are at mercy of their

medium. Perhaps the wood frog embryos used here could not respond to the informative predation-threat chemical cues because of water quality. Future investigations should consider the differences in water quality among field sites and between the field and the laboratory.

The variability of anti-predatory responses between populations may help explain the results of this study in context of what is currently understood about phenotypic plasticity in amphibian propagules. The lack of specific embryonic responses may reflect the conflicting nature of the various survival demands that developing wood frogs face, such as changes in hydroperiod, water quality, intra- and interspecific competition, as well as the variations in predator composition and abundance. The patterns of responses observed are results of an integration of all the demands confronting growing frogs.

CHAPTER 3. LONG-TERM EFFECTS OF EMBRYONIC EXPOSURE TO PREDATOR CHEMICAL CUES

Introduction

Adaptations may be mediated via either short-term or long-term effects and it is possible that an adaptation may increase fitness in the short-term while potentially decreasing it in the long-term (Harvell 1990). For example, if an amphibian embryo hatched from its egg sooner than typical for its species in response to an immediate predation threat, it has increased its fitness by surviving in the short-term. However, this younger-than-average tadpole may not be as strong a swimmer, or as vigilant as it needs to be, and thus, it may over the long-term suffer increased risk of predation and greater competition than longer-developing, larger conspecifics. Warkentin (1999) demonstrated this tradeoff in red-eyed treefrogs (*Agalychnis callidryas*) by showing that, although early hatchlings avoided snake predation, they were more likely to be preyed upon by the freshwater shrimp, *Macrobrachium americanum*, than later-hatching individuals.

For phenotypic plasticity in any trait to be maintained in a species, the benefits of the plasticity must outweigh its costs (Newman 1992). Despite the potential of predation by shrimp, Warkentin (1999) showed that there were no other long-term effects (measured as size at metamorphosis) on growth or survivorship for individuals that hatched earlier in response to snake attack. Size at metamorphosis is a useful

measurement of the cost of plasticity, as it is related to lifetime fitness in several species. In female wood frogs, *Rana sylvatica,* size at metamorphosis is related to first year survivorship and fecundity (Berven 1990). Size at metamorphosis therefore may be used as an indicator of the costs or benefits of predator-induced phenotypic plasticity.

Aside from strict survivorship, embryonic and larval experiences with predators may influence adult frog behavior. Adult wood frogs in the process of choosing oviposition sites have been observed to behaviorally avoid ponds containing predatory fish (Hopey and Petranka 1994). How the aversion to fish cues develops is unknown, but it may develop based on early life experiences. Semlitsch and Reyer (1992) showed that *Hyla chrysoscelis* tadpoles reared with predator cues were significantly less responsive to predators when compared to tadpoles raised in isolation from predators. Depending on the species, early experience with predators may either teach them to avoid predators or habituate them to predator presence.

This study had two aims: 1) to test whether or not hatching plasticity in wood frogs was linked to changes in size at metamorphosis (a measure of fitness) and 2) to test whether early exposure to predator chemical cues changed the individual's behavioral response to those cues later in life.

Materials & Methods

To examine whether embryonic exposure to predator stimuli had long-lasting effects on individuals, I reared embryos in treatments with and without predator stimuli, followed by raising tadpoles in predator-free water until metamorphosis. At metamorphosis, tadpoles were measured, and froglets were tested for preferences to predator vs non-predator stimuli. To test for phenotypic plasticity in growth to metamorphosis, I measured total length, body length, tail depth (at its maximum point), and I recorded the time taken to reach metamorphosis. In the froglet behavioral experiment, I assayed for differences in anti-predator behavior between embryonic rearing treatments by observing reactions to predator stimuli (fish chemical cues versus either earthworm chemical cues or spring water).

All individuals used in these experiments were initially used in another experiment testing embryonic reactions (during the egg stage) to predator stimuli, and the methods through hatching are described in Chapter 2. Briefly, wood frog embryos were individually separated out of five different clutches and five eggs were placed (one from each of the five maternal contributions) into rearing containers. Each rearing chamber contained one of three possible stimuli: predator chemical cues (fish, *Perca flavescens*, n=28), novel-odor control chemical cues (earthworm, *Lumbricus sp.*, n=27) and blank control (spring water, n=25). At hatching, the embryos were measured and placed individually into

different rearing chambers (Solo ® 16 oz. plastic party cups) containing between 300-500ml of spring water. The tadpoles were maintained at room temperature (21°-23°C) and kept on their embryonic photoperiod of 14L: 10D. All tadpoles were fed the same amounts of rabbit chow suspension, supplemented with fish flake (Tetramin ®). The chambers were cleaned (water changed) regularly to prevent fouling, approximately every 3-6 days, as needed.

Once metamorphosis began, the tadpoles were checked every 12 hours. Metamorphosis was defined as forelimb emergence (stage 42 Gosner 1960). Metamorphs were measured (total length, body length and maximum tail depth) to the nearest 0.05 mm, the time was recorded, and the metamorph was placed into transitioning aquarium.

All choice tests were run within 1-4 days of tail absorption. The stimuli (fish, earthworm and spring water) were prepared in the manner typical of the embryonic stimulus production, using the same equipment and organisms. All three stimuli were frozen and thawed simultaneously. The tests were conducted in 5-liter plastic buckets lined on the bottom with moistened paper towels. The paper towels were soaked in the stimulus water for five minutes, then placed onto the bottom half of each bucket. Each bucket contained one fish-labeled paper towel alternated with either earthworm or spring water, and each towel was used only once. Froglets were carefully placed in the center of the bucket and allowed five minutes to acclimate. Each froglet was used

for one trial. The entire assay ran for 120 minutes, with froglet position (fish side or not) recorded every 5 minutes. To avoid biases of lighting and room effects, the buckets were rotated 180 degrees every 30 minutes.

The analyses on time to metamorphosis and morphometrics were run by first calculating means for each of several embryonic rearing chambers, then Kruskall-Wallis analysis of variance tests were performed. To analyze the choice test data, the proportion of time spent in the fish-labeled side was calculated and Mann-Whitney U tests were run on each of the choice test pairs (fish vs spring water and fish vs earthworm). Since the effects of embryonic exposure could hypothetically have produced either a lessening or an increase in fish-odor preference, two-tailed tests were preformed. All data were analyzed with Systat 9 software.

Results

Growth to Metamorphosis Experiment

Despite treatment differences at hatching (chapter 2), by the time of metamorphosis individuals from each of the three treatments were similar, in every measured way. There were no differences in time taken to reach metamorphosis, (see Figure 3.1, KW test statistic 0.294, d.f.=2,

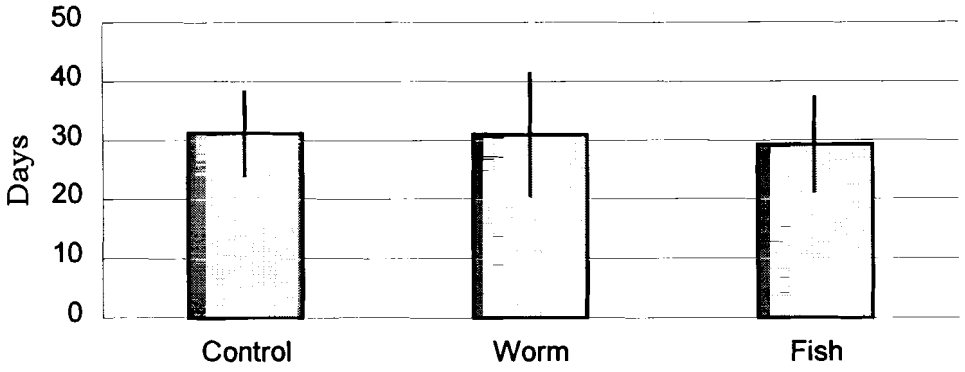

Figure 3.1 Mean (±SD) time taken to reach metamorphosis. Embryonic rearing treatments are as follows: spring water, *Lumbricus* sp., *Perca flavescens*.

p=0.863) or time of day during which the metamorph was detected (KW test statistic 0.062, df=2, p=0.969). There were no differences in any of the body proportions measured, Table 3.1 (KW_{TL} test statistic 3.977, df=2, p=0.137, KW_{Body} test statistic 1.550, df=2, p=0.461, KW_{Depth} test statistic 4.791, df=2, p=0.091).

Table 3.1 Morphometric values (in mm) for metamorphs by treatment.

	Total Length mean (±SD)	Body Length mean (±SD)	Tail Depth mean (±SD)
Control	36.0 (1.4)	12.8 (0.5)	5.3 (0.5)
Worm	35.4 (1.4)	12.6 (0.3)	5.0 (0.3)
Fish	35.8 (1.4)	12.5 (0.4)	5.1 (0.3)

The length of time taken to reach metamorphosis across all treatments, mean (± SD), was 47 ± 7 days from the latest hatching date. The mean (± SD) metamorph body length across all treatments was 12.6 ± 0.33 mm.

Froglet Predator-avoidance Experiment

Embryonic exposure to predator chemical cues did not alter the responses to those same cues later in life. The proportion of times froglets spent on the fish-labeled side of the container was compared between the two rearing treatments: fish reared and spring water reared. Two types of choice tests were performed: fish vs spring water and fish vs earthworm control. Froglets exposed to fish chemical cues, responded with the same intensity and direction as metamorphs exposed only to spring water during both types of choice tests (Mann-Whitney $U_{vs.worm}$ test statistic=270.5, df=1, p=0.787 ; Mann-Whitney $U_{vs.water}$ test statistic=303.0, df=1, p=0.791). Overall, there was little preference for, or avoidance of, any of the chemical stimuli (see Table 3.2).

Table 3.2 Proportion of time spent by froglets on fish-labeled substrate in choice tests.

	n	Mean	±SD
Water raised			
Fish versus Water	20	0.44	0.298
Fish versus Worm	21	0.57	0.274
Fish raised			
Fish versus Water	19	0.44	0.335
Fish versus Worm	20	0.49	0.339

Discussion

This study offers little support for long-term consequences of embryonic exposure to predator chemical cues. Despite size and timing differences at hatching (originating in response to earthworm chemical cues), there were no such differences at metamorphosis. Similarly, embryonic exposure to predator chemical cues did not change the behavioral responses of froglets to predator cues at metamorphosis.

The most significant result of this study is the reversal of the potentially negative impact of embryonic developmental plasticity. Wood frogs that hatched earlier and smaller (earthworm treatment) were no longer different at the time of metamorphosis. In wood frogs, size at metamorphosis is positively related to fecundity (in females) and survivorship to the first breeding attempt (Berven 1990). Possibly by accelerating their developmental and growth rates, it is possible for wood frog tadpoles to compensate for small size due to early hatching. If earlier hatching were to lead to a later date of metamorphosis, there might be no overall selective advantage to hatching plasticity in species of temporary-pool breeding amphibians like wood frogs. These results are congruent with the results of Warkentin (1999), who showed that, in *Agalychnis callidryas,* early induced hatching was not related to delayed metamorphosis. Phenotypic plasticity therefore may be a short-term adaptive mechanism for survival with no discernible long-term effects.

The lack of significant results in the froglet predator avoidance experiment indicates that predator odor is not learned embryonically. Hepper and Waldman (1992) demonstrated that wood frog embryos possess the ability to sense novel chemical cues (orange extract water) present in their environment. Thus, embryonic sensation could be an opportunity to learn predator chemical cues. Conversely, predator cues in the absence of danger or mortality cues might actually cause the embryo to learn predator cues as harmless or simply habituate to them. There were two alternative predictions for the outcome of the froglet choice tests in this study: 1) embryonic exposure to predator chemical cues would teach or reinforce the "odor" of a predator, or 2) embryonic exposure to predator chemical cues would lessen the response to predators in the future.

Unlike most fishes, many larval amphibians have an innate recognition of the chemical signature (kairomones) of their predators (Gallie *et al.* 2001, Lauria *et al.* 1997, & Sih and Kats 1994). Predator chemical cues, especially in combination with diet cues, are enough to cause predator-naïve wood frog tadpoles to reduce activity, a typical anti-predator defense mechanism (Chivers and Mirza 2001). In both *Bufo americanus* (Gallie *et al.* 2001) and *Ambystoma barbouri* (Sih and Kats 1994) larval experience did not affect anti-predator behaviors. However, Bridges and Gutzke (1997) showed two species of *Rana* tadpoles to require conditioning before anti-predator reactions were evoked. In

contrast, Semlitsch and Reyer (1992) showed the opposite effect, *Hyla chrysoscelis* tadpoles showed a weakened anti-predator reaction after they had been conditioned with predator chemical cues. Despite innate recognition of predators, early exposure to kairomones may alter anti-predator behavior in some species.

The specificity of anti-predator reactions and population differences may provide an alternative explanation to the results from this study. While most amphibians studied show innate recognition to predator chemical cues, the responses are often very precise. In a study by Puttlitz *et al.* (1999) Pacific treefrog (*Hyla regilla*) tadpoles were shown to display anti-predator reactions in a threat-sensitive manner. As tadpoles grew larger they lost their anti-predator responses to predators (larval *Ambystoma gracile*), which were gape-limited. If that specificity is present in wood frogs, my testing metamorphs may have missed the expression of learning that occurred embryonically, because I used a tadpole predator and the individuals were tested as metamorphs. Additionally, because the only predator cue tested was sunfish, a fish not likely to be encountered by this population of wood frogs in its typical temporary-pool environment, selection for recognition of that predator may not have occurred in the population (Relyea 2002).

These results show that embryonic exposure to predator kairomones does not have any long-term effects on wood frogs, at the metamorph stage. The size and time differences seen at hatching

disappeared by the time the tadpoles reached metamorphosis. The mechanism that caused this compensatory growth is not understood. Although the embryonic rearing treatments did not appear to change metamorphs' decisions, the study ended when the frogs were only days out of the water. The follow-up choice test experiment would be more difficult, but it is only when the females start breeding (typically at two years) that the effect of embryonic exposure to predators becomes relevant for anti-predator behavioral choices and oviposition site selection.

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BIOGRAPHY OF THE AUTHOR

Pamela Bryer was born in Peterborough, New Hampshire on the chilly night of February 17, 1972. After growing up in the Hancock-Greenfield-Antrim-Bennington-Peterborough area she graduated in 1990 from Contookcook Valley Regional High School, in Peterborough. That year she attended her first year of college, Sterling College in Craftsbury Common Vermont, where she later earned a Grassroots Certificate (1991). Before arriving in 1997 at the University of Maine to complete her Bachelor of Arts degree in zoology she attended several other colleges: Pima Community College in Tucson, AZ; University of Massachusetts at Boston; Keene State College in Keene, NH; and Brookdale Community College in Lincroft, NJ. In 1999 Pam finished her Bachelor's and started work on her Master's degree in zoology.

Since Pam likes animals a great deal she looks forward to getting a job somewhere that allows her to sit back, drink coffee, and talk about animals. To this end, she hopes to earn a doctorate and pursue a career as a professor. Pam is a candidate for the Master of Science degree in Zoology from The University of Maine in December, 2002.