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HATCHING PLASTICITY UNDER COMPLEX CONDITIONS: RESPONSES OF NEWT EMBRYOS TO CHEMICAL AND MECHANICAL STIMULI FROM EGG AND LARVAL PREDATORS

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ABSTRACT.—Environmentally cued hatching plasticity is a common attribute of the eggs of oviparous organisms that has been especially well studied in amphibians. Nevertheless, this process has been largely overlooked in species with complex natural histories. We exposed embryos of the rough-skinned newt (*Taricha granulosa*) to chemical and mechanical stimuli from multiple potential threats, including caddisfly larvae, a major predator on the egg stage of newts. Newt embryos did not exhibit hatching plasticity toward chemical cues from any treatment but, contrary to prediction, did delay hatching in response to mechanical stimuli from an egg predator. Observations of predation by caddisfly larvae on recently hatched newt embryos indicate that caddisflies may prey on multiple life history stages of *T. granulosa.* The results of this study indicate that hatching plasticity may be a complicated phenomenon in some taxa and that additional factors, such as toxicity of eggs or larvae and maternal behavior, may play an important role in the evolution of this phenomenon.

RESUMEN.—La plasticidad en la eclosión de los huevos originada por señales del ambiente es un atributo común de los huevos de organismos ovíparos que ha sido especialmente bien estudiado en anfibios. Sin embargo, en especies con historias naturales complejas, este proceso se ha pasado por alto. Expusimos embriones del tritón de piel áspera (*Taricha granulosa*) a estímulos químicos y mecánicos de múltiples amenazas potenciales, incluyendo larvas de insectos del orden Trichoptera, que son grandes depredadores de huevos de tritones. Si bien los embriones de los tritones no exhibieron plasticidad en la eclosión al exponerlos a estímulos químicos de ninguno de los tratamientos, a diferencia de lo que esperábamos, retrasaron la eclosión como respuesta a los estímulos mecánicos de un depredador de huevos. Las observaciones de depredación de embriones de tritones recién eclosionados por parte de larvas de tricópetros indican que los tricópteros pueden ser depredadores de los distintos estadios de la historia de vida de *T. granulosa*. Los resultados de este estudio indican que la plasticidad en la eclosión puede ser un fenómeno complicado en algunos taxa, y que ciertos factores adicionales, tales como la toxicidad de los huevos o las larvas y la conducta materna pueden desempeñar un papel muy importante en la evolución de este proceso.

Eggs produced by oviparous organisms are vulnerable to changing abiotic and biotic conditions due to their sedentary nature and minimal defenses (Orians and Janzen 1974). Other than toxic or noxious chemicals, the most important defense for developing embryos is likely the ability to adjust the time or developmental stage at which they hatch. Environmentally cued hatching plasticity is now recognized as an important mechanism by which organisms modulate the differential costs and benefits between these major life history stages (Warkentin 2011a).

For most organisms, the direction of plasticity depends on the specific threat perceived by the developing embryo. For example, the presence of egg predators often causes embryos to hatch early, thereby minimizing exposure to a potential deadly interaction (Warkentin 1995, 2000, Chivers et al. 2001). On the other hand, delayed hatching is often the response of eggs that co-occur with larval predators (Sih and Moore 1993). In this case, embryos exhibiting a plastic response may be larger and more developed, thereby conferring greater swimming ability that enables these individuals to escape early predation (Petranka et al. 1987, Sih and Moore 1993). Predation risk is not the only cue used by developing embryos to infer the relative costs and benefits between the egg and external environments. Studies have documented hatching plasticity in response to pathogenic bacteria and fungi (Warkentin et al. 2001, Wedekind 2002), food availability (Voronezhskaya et al. 2004), and environmental variables such as flooding, dehydration,

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and hypoxia (Miller 1992, Wedekind and Müller 2005, Warkentin 2011b).

Many embryos develop in complex environments where a balance must be reached between multiple threats. In aquatic environments where different stage-specific predators (e.g., egg predators and larval predators) may be present at the same time, embryos should assess the survival value of each strategy and respond with a single approach that minimizes immediate mortality, or they should integrate the approaches. Nevertheless, few studies have attempted to elucidate whether a single species can respond to different threats with alternative plastic hatching times. For example, Anderson and Brown (2009) exposed green frog embryos to stimuli from an egg predator, a larval predator, and both predators combined. Surprisingly, the embryos responded to all stimuli by hatching early, indicating that organisms may be limited in the type of plastic response they exhibit under complex conditions. Furthermore, additional factors such as multistage predators (consumes both eggs and larvae), chemical defenses, or maternal behavior could complicate an embryo's "decision" to initiate or delay hatching.

We conducted a series of experiments to examine hatching plasticity in a species that possesses multiple complex traits (chemical defenses and maternal oviposition behavior) that could influence the hatching response. Specifically, we tested for the presence of accelerated and delayed environmentally cued hatching plasticity in response to egg and larval predators in eggs of the rough-skinned newt (*Taricha granulosa*). Lehman and Campbell (2007) previously reported that *T. granulosa* eggs hatch earlier in the presence of caddisfly larval odor. However, the lack of randomization and use of females from a wide geographic range necessitates replication of this work. The rough-skinned newt is one of the most toxic organisms on the planet. The skin of individual adult newts may contain up to 28 mg of the neurotoxin tetrodotoxin (TTX), which is the lethal oral dose for as many as 56 humans (Stokes et al. in review). The toxin is secondarily deposited in the eggs (Wakely et al. 1966, Hanifin et al. 2003, Gall et al. 2012b) and is also present in larvae (Gall et al. 2011b). Tetrodotoxin protects newt larvae from predatory dragonfly nymphs (Gall et al. 2011b), but the eggs are extremely vulnerable to predation by TTX-resistant caddisfly larvae (Trichoptera; henceforth caddisflies; Gall et al. 2011a). These interactions suggest that there should be strong pressure to exhibit early hatching, and we hypothesize that newt embryos will hatch early in response to chemical stimuli from predatory caddisflies. We also conducted an experiment to determine if mechanical cues elicit early hatching in this species, as they do for other amphibians (e.g., *Agalychnis callidryas;* Warkentin 1995, 2000, Caldwell et al. 2010). Finally, we evaluated whether caddisflies are truly egg-only predators in predation trials with newly hatched newt larvae.

METHODS

Animal Collection

All female newts (*Taricha granulosa*) used in these experiments were collected in reproductive condition from Soap Creek ponds in Benton County, Oregon. Newts were transported to Utah State University and housed individually in 5.7-L plastic containers with 3 L of filtered tap water (henceforth water). They were maintained at 6 °C to prevent spontaneous egg deposition and were fed blackworms (*Lumbriculus variegatus*) weekly.

Caddisfly larvae (*Limnephilus flavastellus*) and dragonfly larvae (Anisoptera; henceforth dragonflies), including *Anax junius*, were collected from the same ponds as *Taricha.* Caddisflies were housed in 37-L aerated aquaria with 20 L water at 6 °C and fed maple-leaf detritus (see Gall et al. 2011a for a description of detritus preparation). Dragonflies were housed individually in 275-mL glass bowls and fed blackworms *ad libitum.* Mayfly larvae (Baetidae; henceforth mayflies) co-occur with *Taricha* at Soap Creek ponds, but at low densities. Therefore, mayflies were collected in Cache Valley, Utah, and housed in a 37-L aerated aquarium with a small amount of detritus. No organism was fed newt eggs or larvae prior to experimentation. No organism was reused within or between experiments.

Experiment 1: Response to Chemical Stimuli

Chemical stimuli are an important vector of information transfer in aquatic environments (Ferrari et al. 2010), and embryos may alter developmental timing in response to cues from egg (speed-up hatching) or larval (delay

TABLE 1. List of the cues, treatments, and treatment types from 2 experiments exposing rough-skinned newt (*Taricha granulosa*) embryos to various types of cues from control and predatory sources. Blackworms = *Lumbriculus variegatus*, caddisfly = *Limnephilus flavastellus*, dragonfly = Anisoptera, mayfly = Baetidae.

Experiment	Cue	Treatment	Treatment type
1	None	Filtered water	Control
	Chemical	Blackworms	Control
	Chemical	Caddisfly larvae	Egg & larval predator[a]
	Chemical	Dragonfly larvae	Larval predator
	Chemical	Macerated blackworms	Control
	Chemical	Macerated newt eggs	Simulated egg predation
	Chemical	Macerated newt larvae	Simulated larval predation
2	None	Filtered water	Control
	Chemical & mechanical	Mayfly larvae	Control
	Chemical & mechanical	Caddisfly larvae	Egg & larval predator[a]

[a]Experiment 3 of this study indicates that in addition to consuming newt eggs, caddisfly larvae may also be able to capture and consume newt larvae.

hatching) predators or the presence of alarm cues (chemical stimuli from damaged eggs or larvae; Warkentin 2011b). We exposed developing newt eggs to water conditioned with chemical stimuli from one of 7 treatments simulating the presence of egg or larval predators (Table 1). These treatments included chemical cues from live (1) blackworms (control), (2) caddisflies, or (3) dragonflies; cues from macerated (4) blackworms (control), (5) newt eggs, or (6) newt larvae; or a (7) blank control.

Chemical cues from uninjured blackworms, caddisflies, and dragonflies were collected in 275-mL glass bowls filled with 200 mL of water. A piece of plastic screen was placed in the bottom of the bowl to provide an inert substrate. The blank control was treated the same except no animal was added. A single caddisfly or dragonfly, or 20 blackworms were placed in each glass bowl. After 48 h, the invertebrates were removed and the stimulus from all bowls (within one treatment) was combined to eliminate variation in cues from individual donors. The solutions were transferred to 50-mL centrifuge tubes in 40-mL aliquots and immediately frozen at –80 °C.

We collected cues from macerated blackworms, newt eggs, and newt larvae by macerating 3.0 g of the appropriate tissue with a mortar and pestle and combining it with 4 L of water. This homogenate was thoroughly mixed and frozen in 40-mL aliquots. For the larvae alarm cue treatment, 0.75 g of larvae (approximately 50 larvae) was collected and combined from 4 females. Larvae hatched 2 weeks prior to stimulus collection were free-feeding on *Daphnia* and possessed no remaining yolk. The egg alarm cue treatment was prepared by combining 0.75 g of eggs from 4 different females (approximately 60 eggs). These eggs were deposited 1–6 days prior to preparation and had been separated from the female shortly after oviposition.

Twelve gravid female newts were transferred to an environmental chamber at 14 °C and 12L:12D cycle, injected with 10 µL LHRH (de-Gly10, [d-His(Bzl)6]-Luteinizing Hormone Releasing Hormone Ethylamide; Sigma #12761) to induce egg deposition, and provided a small piece of polyester fiber as an oviposition site. The females were monitored at 07:00 and 19:00 for egg deposition, upon which the eggs were carefully removed from the fiber and placed into 2-mL numbered cups in groups of 5. After all the eggs from one female were placed into cups, each cup was randomly assigned to one of the 7 treatments such that a group of 7 cups received all 7 treatments and the eighth cup from one female started a new random sequence; if more or fewer than 7 cups worth of eggs were present during one deposition event (i.e., at 07:00), then the random sequence of all 7 treatments was completed with the subsequent batch of eggs (i.e., at 19:00). In total, 444 cups were filled with 2220 eggs. Each treatment comprised at least 305 eggs, of which at least 15 eggs came from a single female. Because females deposited different numbers of eggs and because some eggs died prior to hatching, the number of cups and number of individual eggs per female was not equal across treatments. The total number of eggs used from an individual female ranged between 110 and 285. A single female was removed from all analyses because only 37 eggs were deposited during the experiment, and that amount did not allow implementation of all 7 treatments. Dead eggs

were removed each day throughout the course of the experiment.

Eggs were exposed to the appropriate treatment stimuli at 12:00 each day for 9 days following deposition. A randomly chosen stimulus vial was thawed in a warm water bath at 14 °C. Five hundred μL of stimulus solution was pipetted slowly down the side of the cup to minimize disturbance to the eggs; this cue concentration is more than twice that necessary to elicit predator avoidance responses in other amphibians (Takahara et al. 2008). This process was repeated until all eggs in the appropriate treatment had received stimulus, whereupon the pipette tip was changed and the process was repeated with the next randomly chosen stimulus. The water in each cup was replaced with clean water every 3 days. Water was changed by withdrawing the water into a pipette and immediately replacing it with clean water; this process minimized disturbance to the eggs. On the tenth day, larvae were nearing hatching; a final water change was performed and no additional stimuli were introduced; if hatching plasticity occurs, exposure to potential predators during the first 24 h of development is likely to be the critical phase in facilitating hatching plasticity in newt embryos (Lehman and Campbell 2007).

Eggs were monitored for hatching at 07:00 and 19:00. When a larva had completely hatched and was free-swimming (evident by straightening of the body), it was removed from the cup with a pipette, and the time to hatching (hours) and developmental stage were recorded. Developmental stage (Harrison 1969) was recorded using an Olympus stereo microscope. The larva was then photographed (Nikonä D70 digital camera with a AF Micro Nikkor 105 mm lens) to determine total length at hatching. Total length was calculated from the photos using the photo analysis software ImageJ (U.S. National Institutes of Health, Bethesda, MD).

We examined the effects of treatment on hatching time, developmental stage, total length of recently hatched newt embryos, and proportion of embryos that died during the experiment. Data were analyzed using a generalized linear mixed model with treatment as a fixed-effect factor. Female was treated as a random factor, with cup and eggs within a cup nested within female. Hatching time, developmental stage, and total length were analyzed with a normal distribution with the identity link function. Egg mortality was analyzed using a binomial distribution with the logit link function. A cup of 5 eggs was considered the replicating unit. Eggs within each cup were incorporated into the model as subsamples. Assessments of distributional assumptions were based on graphical analysis of residuals; all assumptions appeared to be adequately met for all response variables. Analyses were performed using the GLIMMIX procedure in SAS/STAT software version 9.2 (SAS Institute, Inc., Cary, NC).

Experiment 2: Response to Mechanical Stimuli

To determine if mechanical stimuli (i.e., the physical presence) of a predator affects hatching plasticity in newt embryos, we exposed newt eggs to an egg predator (caddisfly larvae; $n = 46$), a nonpredator (mayfly larvae; $n = 46$), or a blank control ($n = 49$) in a specially designed experimental chamber that prevented the invertebrates from consuming the eggs but permitted some contact and vibrational stimuli (see below for details).

Several days prior to experimentation, 5 females were injected with 10 μL LHRH. Females were given plastic mesh (500 μm aperture) as oviposition sites. Females preferred to deposit eggs on the edges of the mesh, so we cut off small pieces of mesh that contained an egg and attached these pieces to the center of a circular piece of mesh (6 cm diameter) by using hot glue. The hot glue was positioned at the edges of these small pieces and did not contact the eggs. Three eggs were attached to each circular piece of mesh.

The experimental container consisted of a 237-mL plastic cup that had most of the bottom cut out; a 5-mm section around the outside was left intact to support the circular piece of mesh (see below). Two pieces of wire were attached in an X-pattern to the top of each cup, and the cup was then hung inside a larger container (946 mL). The wire served to suspend the bottom of the small cup 3.8 cm off the substrate. The large container was filled with 800 mL of water. One circular piece of mesh, with 3 attached eggs, was placed upside down inside the small cup. This experimental apparatus exposed eggs to chemical as well as mechanical stimuli from predators, such as the vibrational stimuli an egg would likely experience when a caddisfly climbs the vegetation

on which an egg is attached. In addition, the holes in the mesh were large enough to permit contact by the tarsal claws of each invertebrate, yet prevented the invertebrates from actually consuming the eggs.

Each experimental apparatus was placed in an environmental chamber at 16 °C and was randomly assigned to a blank control, mayfly (nonpredator), or caddisfly (predator) treatment. Once the newt embryos exhibited physical movements inside the egg (15 days after egg deposition), one of the appropriate invertebrate (or no invertebrate) was placed inside the small cup. A small piece of maple-leaf detritus (see Gall et al. 2011a for a description of detritus preparation) was also placed inside each cup to provide a food source for stimulus animals. The eggs were monitored daily, and the hatching date was recorded for each egg. We were unable to record developmental stage at hatching or morphological characteristics of hatchlings because this would have required removal of the cup, which would have disturbed the remaining unhatched eggs.

To determine whether exposure to mechanical stimuli from potential egg predators affected time to hatching, we compared the days to hatching between the 3 treatments by using a generalized linear mixed model, with cup considered as the replicating unit and eggs within each cup incorporated into the model as subsamples. Pairwise comparisons among the treatments were adjusted for family-wise type I error using the Tukey method. The GLIMMIX procedure in SAS 9.2 (SAS Institute, Inc.) was used for all calculations. Assessments of distributional assumptions were based on graphical analysis of residuals; all assumptions appeared to be met for all response variables.

Experiment 3: Predation on Newt Larvae by Caddisflies

We gave caddisflies a recently hatched newt larva to determine whether caddisflies are able to consume this toxic prey. Individual caddisflies ($n = 16$) were placed in 237-mL mesh-bottom cups. Eight cups were placed in a 5.7-L container with 3 L of water and an aerator. One recently hatched newt larva was placed in each cup; newt larvae had hatched fewer than 7 days prior to experimentation and were free swimming. We recorded the behavior of the larva when it was initially contacted by a caddisfly. We checked each cup after 24 h and recorded whether the newt larva was alive and apparently uninjured or completely or partially consumed by the caddisfly.

RESULTS

Experiment 1: Response to Chemical Stimuli

Exposure to chemical stimuli from potential egg or larval predators did not affect time to hatching ($F_{6,65} = 0.93$, $P = 0.479$; Fig. 1), total length at hatching ($F_{6,65} = 0.46$, $P = 0.798$; Fig. 1), or embryo mortality ($F_{6,65} = 1.88$, $P = 0.098$) for newt embryos. Treatment had a marginally significant effect on developmental stage at hatching ($F_{6,65} = 2.17$, $P = 0.057$; Fig. 1); however, the difference in mean developmental stage between all 11 females was very small (ranging between 40.11 and 40.29), and post hoc comparisons did not yield any significant differences between treatments (all adjusted $P > 0.25$). Because of the error associated with visually assigning developmental stage, this difference is unlikely to represent biologically meaningful responses to the different treatments. See Hopkins et al. (2012) for a description of the female effects.

Experiment 2: Response to Mechanical Stimuli

There were significant differences in hatching time between newt embryos exposed to mechanical stimuli from predator, nonpredator, and control treatments ($F_{2,86} = 4.23$, $P = 0.017$; Fig. 2). Post hoc comparisons indicated that newt embryos exposed to mechanical stimuli from predatory caddisflies hatched significantly later than newt embryos exposed to a blank control ($t = -2.89$, $P = 0.013$; Fig. 2). Eggs exposed to mechanical stimuli from nonpredatory mayflies exhibited hatching times intermediate between the control and caddisfly treatments. Hatching time in response to mayflies was not significantly different from either treatment ($P > 0.29$; Fig. 2).

Experiment 3: Predation on Newt Larvae by Caddisflies

Six larval newts were completely or partially consumed by caddisflies within 24 h (Fig. 3). The remaining 10 newt larvae responded to stimulation with a probe by swimming away and were seemingly uninjured. When a caddisfly initially touched a larval newt, the larva

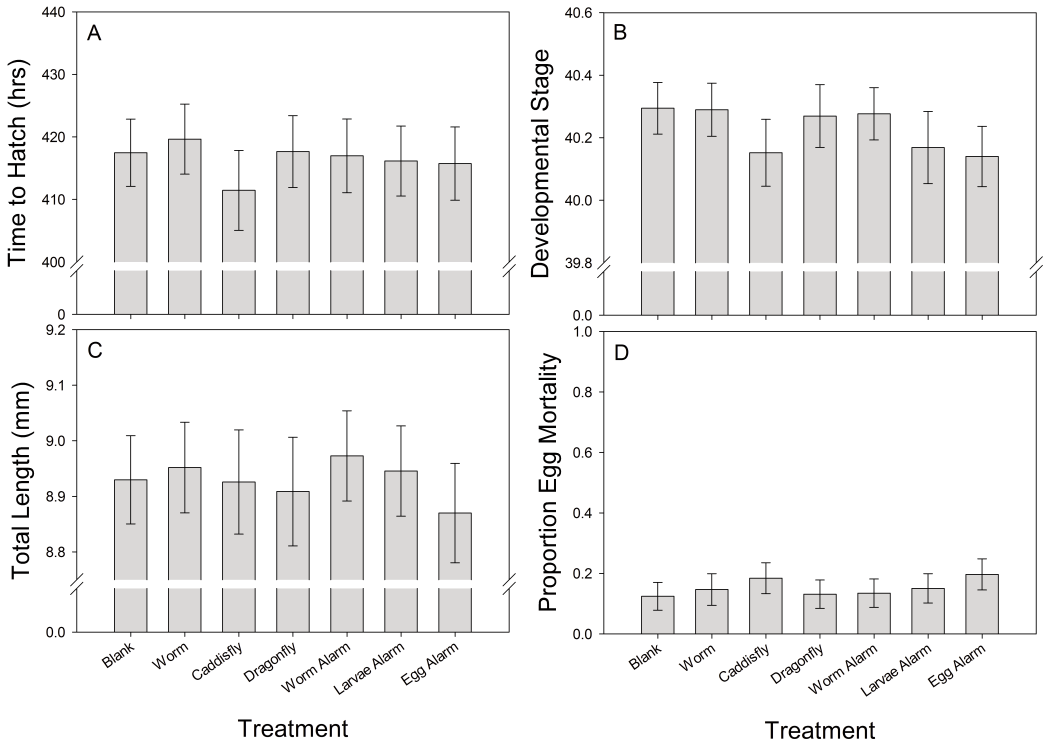

Fig. 1. The mean (±2 SE) time to hatch (A), developmental stage at hatching (B), total length at hatching (C), and proportion mortality (D) for newt embryos exposed to chemical stimuli from one of 7 treatments. Hatching plasticity was not observed in newt embryos in response to any treatment stimulus.

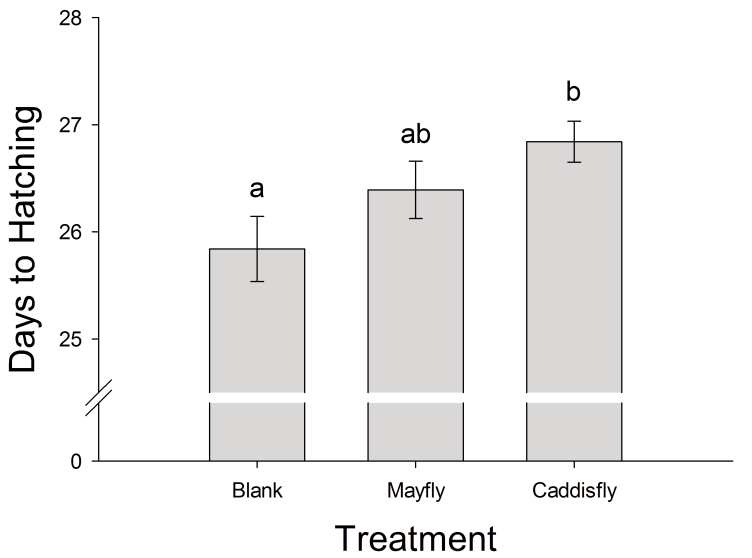

Fig. 2. Mean (±SE) days to hatching for newt eggs exposed to mechanical stimuli from a blank control, nonpredatory mayflies, and predatory caddisflies. Newt eggs exposed to a caddisfly took significantly longer to hatch than eggs exposed to a mayfly or a blank control ($F_{2,86} = 4.23$; $P = 0.017$). Different letters indicate significant differences between treatments ($P < 0.02$).

Fig. 3. Recently hatched newt larva (*Taricha granulosa*) partially consumed by a caddisfly larvae (*Limnephilus flavastellus*). The larva was alive and free swimming at the start of the trial.

rapidly swam away. Although the newt larvae may have died and subsequently been scavenged by the caddisflies, we find this explanation unlikely. We have reared thousands of newt larvae and find them to be exceedingly hardy in response to food deprivation for 24 h and to predatory attacks by other invertebrates (Gall et al. 2011b; personal observation).

DISCUSSION

Exposure to chemical stimuli from an egg predator, larval predator, injured eggs, or injured larvae had no effect on the hatching time, developmental stage, total length at hatching, or mortality in newt embryos. This result is surprising given the diversity of organisms that exhibit hatching plasticity in response to similar cues (Warkentin 2011a), as well as studies that indicate exposure to kairomones or alarm cues alone is sufficient to induce these shifts (Moore et al. 1996, Touchon et al. 2006). These data add to the growing body of literature indicating that hatching plasticity is variable both between and within taxa. For example, Anderson and Petranka (2003) showed that another salamander species (*Ambystoma maculatum*) failed to delay hatching in response to dragonflies, an important predator of salamander larvae in pond communities. Nevertheless, some populations of a closely related species, *Ambystoma barbouri,* delay hatching when exposed to predator kairomones alone (Moore et al. 1996). A similar study on rough-skinned newts found embryos hatched approximately one day early when exposed to chemical cues from predatory caddisfly larvae (Lehman and Campbell 2007); however, the authors did not randomly assign eggs from a specific female or population across treatments. There is tremendous variation among females (even within a single population) in the time their embryos take to hatch, which may account for the observed differences (Hopkins et al. 2012).

The ecological interactions between newt embryos and their potential predators are complex and may explain the failure of rough-skinned newts to adjust hatching timing according to the simulated predation risk. Female newts deposit large quantities of tetrodotoxin in the yolk of their eggs (Wakely et al. 1966, Hanifin et al. 2003, Gall et al. 2012b). This toxin is retained by developing embryos through hatching and metamorphosis and is present in sufficient quantities to deter predation by dragonfly larvae at all larval stages (Gall et al. 2011b). Failure to delay hatching in response to dragonfly kairomones may therefore be due to alternative antipredator mechanisms that preclude selection on developmental plasticity.

Unlike dragonflies, the caddisfly *L. flavastellus* is resistant to the negative effects of TTX (Gall et al. 2011a). Moreover, these predators are extremely abundant and, under optimal conditions, could consume the entire reproductive output of a newt population in only 36 h (Gall et al. 2011a). Despite the apparent strength of predation on newt eggs, the behavior of the female newt may mitigate predation risk for its eggs. Caddisflies are benthic organisms, and *L. flavastellus,* in particular, do not generally utilize the upper portions of aquatic vegetation (Gall et al. 2012a). This behavioral limitation has yielded a microhabitat that is used by female newts as an oviposition site that provides protection from egg predators (Gall et al. 2012a). Given that newt eggs are deposited in such a way that reduces predation pressure, we hypothesized that a plastic hatching response may only occur when newt eggs are exposed to mechanical stimuli from potential predators. This type of stimulus occurs

immediately prior to a predation event and is indicative of imminent risk (Warkentin 1995, 2000, Caldwell et al. 2010). In this system, newt embryos should adjust the timing of hatching only when the threat of predation is extremely high because chemical cues alone may not accurately reflect the level of risk to each egg. Newt eggs did adjust the time of hatching when exposed to mechanical stimuli from caddisflies; however, the response was opposite of the predicted direction. Newt embryos hatch underdeveloped (relative to other salamanders), and young larvae respond to stimulation with uncoordinated movements (Gall personal observation). Further, once hatched, newt larvae would fall to the bottom of the pond, which is the primary habitat of these caddisflies. These benthic "detritivores" were able to prey on mobile newt larvae in the laboratory, indicating that these insects may be predators of both newt eggs and newt larvae. Therefore, newt embryos are likely to either not exhibit hatching plasticity in response to chemical cues (as demonstrated in this study) or delay hatching until the risk of predation is imminent (i.e., when a caddisfly is breaking into the egg).

We demonstrated that, although newt embryos do not respond to chemical stimuli from potential predators, they do delay hatching in response to the physical presence of caddisflies. Surprisingly, this response was in the opposite direction as expected, and lab studies indicated that the egg-only predator may in fact be a threat at multiple life history stages. Combined, our results indicate that hatching plasticity is a complicated phenomenon in *Taricha granulosa.* Hatching plasticity is likely to be dependent on the selection regime from different predators, as well as the life history stages that they prey upon. Moreover, other factors such as chemical defenses and maternal behavior are likely to influence the evolution of hatching plasticity.

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