AN INVESTIGATION OF THE EFFECT OF MALATHION ON ADAPTIVE PLASTICITY OF *PSEUDACRIS SIERRA*

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ABSTRACT

An investigation of the effect of malathion on adaptive plasticity of *Pseudacris sierra*

Michael Jonathan Maples

This thesis is composed of two sections. Section one reviews what is known about adaptive plasticity in response to predators, describes the physiological systems involved in such plasticity, and outlines the evolutionary consequences of adaptive plasticity. Section two describes a scientific experiment that investigates how malathion may impact adaptive plasticity in the Sierran Treefrog, *Pseudacris sierra*.

Anuran tadpoles suffer high mortality rates due to predation. In response to strong selective forces relating to these high predation rates, tadpoles evolved the ability to adaptively respond to predators through morphological and behavioral plasticity. The morphological and behavioral responses are varied and depend on the hunting strategy of the predator, and the adaptive responses may be influenced by other biotic and abiotic factors. Tadpoles detect alarm cues released from tadpoles being eaten and kairomones that are released by predators. Tadpoles respond to these signals by changing tail and body shape along with a reduction of activity level, which enables tadpoles to escape predators more effectively. These changes in morphology can occur within a week, and behavioral changes can occur within 15 minutes. The adaptive responses are critical for increasing survival rates of tadpoles to metamorphosis and may have important evolutionary consequences for anurans.

Amphibians are in decline worldwide, and pollutants are considered to be a major contributor to these declines. Every year 5.2 billion pounds of active ingredients of pesticides are applied worldwide, and these application rates have led to ubiquitous low-level contamination of aquatic ecosystems. How low-level contamination of pesticides directly and indirectly affect how tadpoles respond to their predators is poorly understood. One potential indirect effect of pesticides is the inhibition of adaptive plasticity. Pesticides have been shown to modulate corticosterone levels in tadpoles. Corticosterone is the most likely mediator of the physiological response that results in adaptive morphological change. If the physiological system of tadpoles relies on corticosterone as the mediator of adaptive response, and pesticides can modulate corticosteone levels, then pesticides may inhibit or negatively impact adaptive responses to important biotic factors, like predators. Pesticides have been shown to weaken immune systems, affect developmental and physiological pathways that lead to malformations, and cause direct mortality in anurans. Adaptive phenotypic responses to predators increase survival rates to metamorphosis and are important in stabilizing amphibian populations through time. If pesticides influence the ecological interactions of tadpoles and their predators, this could play a part in amphibian declines.

In the experiment explained in section two, the following hypothesis was tested; malathion at a concentration of 0.1 mg/L inhibits anti-predator morphological and behavioral responses of *Pseudacris sierra* to the predatory dragonfly larvae *Anax junius*. The results of this experiment show that malathion alone caused the tail muscle depth to increase to the same magnitude as tadpoles that only experienced a predator's presence.

Malathion also caused a significant increase in tail depth, demonstrating that malathion directly causes morphological change. The experiment did not support the hypothesis that malathion inhibits adaptive plasticity, and malathion had no impact on behavioral plasticity. The results from this experiment give evidence that an ecologically relevant concentration of malathion can influence morphological components that are critical in escaping depredation events, which could affect predator-prey interactions.

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SECTION ONE

Introduction

Phenotypic plasticity is defined as the ability of a single genotype to produce multiple phenotypes in response to different environmental factors (Pigliucci 2001; West-Eberhard 2003). Phenotypic plasticity evolves when a phenotypic trade-off exists, where a phenotype experiences high fitness in one environment and the same phenotype has a low fitness in a different environment (Via et al. 1985). When an environment is unpredictable and organisms are unable to predict the most dangerous abiotic or biotic factor that is present, phenotypic plasticity enables organisms to express the phenotype that will maximize its chances of surviving (West-Eberhard 2003). Organisms can maximize fitness by producing phenotypes that have particular characteristics that are well suited to specific abiotic or biotic factors. The ability of organisms to modify morphology or behavior in response to changing environmental conditions is widespread and is thought to be important in balancing populations over time that experience unpredictable abiotic or biotic conditions (West-Eberhard 2003).

Predator-induced phenotypic plasticity is a common adaptation that anuran tadpoles have evolved in response to strong selection pressures related to predation (Benard 2004, 2006; Van Buskirk et al., 1997). Anurans are an r-selected species, and experience a 91 to 87 percent mortality rate before reaching sexual maturity. These high rates of mortality are mainly due to high predation rates and pond desiccation (Benard 2006; Kupferberg 1998; Jameson 1956, 1957; Relyea 2007; Wells 2007). In response to high predation rates, anurans have evolved a commonly expressed ability to respond through morphological and behavioral plasticity, which generally increase survival rates

to metamorphosis (Benard 2004; Relyea 2001a; Skelly et al. 1990; Van Buskirk 2001). There are many examples of amphibians utilizing plastic characters to increase survival rates in adaptive ways. For example anurans can change the timing of hatch in response to dangerous predators and pathogens (Touchon et al., 2006). Different species of spadefoot toads can decrease developmental time to escape a desiccating pond (Morey et al. 2000). Many species of tadpoles also show plastic responses in response to competition, by changing the size and shape of mouthparts and intestinal length to make them better competitors (Reylea 2002c).

The predator environment that tadpoles experience generally varies spatially and temporally over a geographic area. Predator assemblages vary among aquatic habitats in patterns associated with water availability linked to large-scale weather patterns (Richter-Boix et al., 2007; Van Buskirk 2002a, 2002b; Van Buskirk et al., 2005). Permanent ponds generally contain a more diverse set of predators. Temporary ponds, on the other hand, have varying densities of predators because of stochastic colonization and extinction dynamics, due to pond drying (Relyea et al., 2000). Furthermore, there is variation in the predator environment temporally, due to natural cycling of predator populations through life-stages. For example, some aquatic insects metamorphose into a non-aquatic adult form, thus their presence changes over time. This variability in predator environments has resulted in tadpoles from a wide range of species to respond in specific adaptive ways that increase survival rates to metamorphosis. The ability to adaptively respond through morphological and behavioral plasticity has been maintained due to the heterogeneous and unpredictable predator environments experienced by anuran species across their distributions and through time (Richter-Boix et al., 2007).

Tadpoles and their predators

The predators that tadpoles face exhibit two main types of hunting strategies. There are sit and wait predators, exemplified by dragonfly larvae and other types of aquatic insect predators. The other main strategy is an active hunting strategy, exemplified by fishes (Benard 2006). Additionally, there are gape-limited sit and wait predators, such as salamanders, which cause specific morphological changes in some anuran species (Kishida et al., 2007). These different hunting strategies place dissimilar predation pressures on tadpoles and can cause divergent selection in tadpole morphology (Benard 2006). Such divergent selection stems from differences in biomechanics elicited by the morphologies, which have varying capacities for escaping the different hunting strategies (Wilson et al., 2005). The morphological traits that change in response to predators include tail length and depth, tail muscle depth, and body depth and length (Relyea 2001a). The evolutionary history of the anuran species and the types of predators that occur in its species range determine the degree and the type of morphologically plastic response expressed by tadpoles (Benard 2004).

An anuran tadpole's chances of survival are often dependent on the result of a predator's initial strike. Once contacted, tadpoles elicit a characteristic startle reaction, which consists of a C-start response, where larvae turn quickly away from the strike and use several propulsive tail-beats to reach a maximum speed quickly (Wilson et al., 2005). Anuran tadpoles have two strategies to lower the lethality of a predator's strike, which relate to the different hunting strategies. Aquatic insect predators that employ sit and wait hunting strategies grasp at their prey, and the strikes are less deadly if the strike is directed to the tail of the tadpole (Van Buskirk et al., 2003). This is due to the tadpole tail

being fragile and easily torn, facilitating escape (Doherty et al., 1998). The most consistent and widespread predator-induced morphological trait change that occurs in response to dragonfly larvae and other insect sit and wait predators is an increase in tail depth (Relyea 2001a). The increase in tail depth has been shown to attract the strikes of sit and wait predators to the tail (e.g., away from the head), which increases survival rates in the presence of an active hunting predator (Benard 2006; Relyea 2001a, 2001b). Individuals with greater tail depths have slow burst swim speeds, giving more evidence that the increase in tail depth results in the tail acting as a lure, rather than being involved in burst swimming (Wilson et al., 2005). On the other hand, active hunting predators like fish select for faster swimming tadpoles. Tadpoles with small bodies, long, shallow tails, and greater muscle depth have high swimming speeds (Johnson et al., 2005; Wilson et al., 2005). This morphology is generally expressed in response to fish predators, and has been shown to increase survival rates when exposed to an actively hunting fish (Benard 2006).

Adaptive plasticity expressed by the Sierran Treefrog

In an experiment, Benard (2006) exposed Sierran Treefrog tadpoles (*Pseudacris sierra*), to a bluegill fish predator (*Lepomis macrochirus),* a diving beetle predator (*Dytiscus sp.)*, and no predator. Tadpoles exposed to a fish predator (bluegill-induced) developed shallower tails and bodies, whereas those exposed to a diving beetle predator (beetle-induced) developed deeper tails. Both groups of tadpoles that were exposed to predators also significantly decreased their activity levels. Benard then tested the survival rates of all three groups of tadpoles when exposed to active bluegill and diving beetle predators. The beetle-induced tadpoles had the highest survival rates when exposed to a

diving beetle predator, and the bluegill-induced tadpoles had the highest survival rate when exposed to an active bluegill predator (Benard 2006). When each predator-induced tadpole group was exposed to the predator it had not previously encountered, the tadpoles had comparatively much lower survival rates. These results support the hypothesis that adaptive plasticity in response to a specific predator confers higher fitness, through higher survival rates, when it involves the predator that caused the adaptive response.

In addition to morphological change, there is a general reduction in activity level when tadpoles are exposed to a predator's presence, which was demonstrated in the previous experiment and in others (Benard 2004, 2006; Relyea 2001b). Decreased activity levels limit the probability that a tadpole will come into contact with a predator (Orizaola et al., 2012). Behavioral responses to predators change over the larval period of tadpoles. The decrease in activity is strongest early in ontogeny and most likely reflects diminishing risk of predation as tadpoles grow larger (Relyea 2003a; Hossie et al., 2012). Behavioral changes in response to predators may also be dependent on context (i.e., there is an inverse relationship between activity level and the amount of risk posed by the predator environment). As the predator environment becomes more dangerous, tadpole activity decreases proportionately (Schoeppner et al., 2008).

Case studies of Local adaptations to predator environment

Geographic variation in predator composition and abundance can lead to population variation in plasticity as local adaptations. The next two studies demonstrate local adaptations occurring within species in response to different predator assemblages in their respective ranges. Laurila et al. (2008) investigated the differences in morphological and behavioral responses to predators of the European common frog

(*Rana temporaria*) along a 1,500 km latitudinal gradient. A general trend was found between high and low latitude predator environments. There were fewer predators at higher latitudes due to harsher climatic conditions, but a shorter optimum growing period for the tadpole larvae. At lower latitudes, there were more predators and a longer time available for tadpoles to develop. When the different populations of *R. temporaria* were raised in a common garden experiment, tadpoles that were collected from higher latitude ponds developed faster, had higher activity levels, and had more pronounced induced morphological traits than tadpoles from low latitude ponds (Laurila et al., 2008). The higher activity level reflects the local adaptations made by tadpoles in response to having shorter windows of larval development. The high activity levels caused tadpoles to be more susceptible to predation, but enabled higher growth rates. The more pronounced morphological anti-predator defenses in the populations from higher latitudes enabled tadpoles to escape predators more effectively and limited the costs associated with high activity levels. These results demonstrate how the context of the predator environment can shape how morphological and behavioral plasticity is expressed and how different environmental contexts can modulate the expression of adaptive morphological and behavioral plasticity (Laurila et al., 2008).

In another example of population-specific local adaptations, there is evidence that some populations of the wood frog *Rana sylvatica* have adapted to local predation pressures corresponding to different levels of canopy cover around natal ponds. Differences in predator assemblages among ponds in close proximity have strong effects on the evolution of population-specific reaction norms of *R. sylvatica* (Relyea 2002b). In a common garden experiment, wood frog larvae from eight different ponds were exposed

to dragonfly larvae predator cues, and behavioral and morphological responses were measured and compared to control larvae. Four of the ponds from which eggs were collected were closed, and the other four were open canopy. These ponds were within 0.3-8 km each other, representing local adaptations on a small scale. A dramatic pattern emerged from these two types of ponds. Closed canopy ponds contained a stable assemblage of salamander and predacious diving beetle predators. The open canopy ponds contained much more dynamic predator assemblages of salamanders, predacious diving beetles, hydrophilid beetles and dragonfly larvae. The results of a common garden experiment showed that spatially structured populations could express population-specific phenotypic plasticity on a very local geographic scale (Relyea 2002b). Tadpoles from open canopy ponds, which have large and variable predator assemblages, expressed pond-specific changes in morphological traits of muscle width and depth, tail depth and body length that varied from pond to pond (Relyea 2003a). Populations from closed canopy ponds, on the other hand, did not vary among each other in any of these traits in response to a dragonfly larva predator, although all developed deeper tail fins. These results show that predators have a strong impact on the evolution of phenotypic plasticity on a local scale. Expanding the geographical scale magnifies the potential differences that could be found (due to differences in predator assemblages), and this explains how so many different morphological responses can be detected using different species (Benard 2004).

The relationship between anuran tadpoles and their predators and other ecological factors is very complex. Tadpoles have to respond to dangerous predators, but a complex array of other abiotic and biotic factors are entangled in the phenotypic responses. For

example, competition, pond drying, water depth, temperature, food availability, and type of vegetation surrounding ponds have all been shown to affect phenotype expression (Lind et al., 2009; Michael 2011; Relyea 2002b, 2004a, 2004b, 2005b, 2007; Relyea et al., 1999; Richter-Biox et al., 2007). Since tadpoles in reality have to respond to a complex of environmental stressors, this could pose limits to the expression of phenotypic plasticity. These limits can come in the form of conflicts with other important interactions such as competition or pond drying (Relyea 2002d). Laurila et al. (2008) showed that differences in time for tadpole larvae to develop along a latitudinal gradient caused tadpoles to change their behavioral and morphological responses to a predator. These types of interactions between tadpoles and their complex environments could lead to the variation detected in morphological and behavioral responses. However, there is still evidence of a larger pattern: tadpoles across many species deploy specific and adaptive changes to their morphology and behavior in response to different predators, depending on those predators' hunting strategies and threat levels (Relyea 2004a). For this pattern to exist, tadpoles must be able to access signals in their environment that give accurate information on the predators present and how dangerous they are (Benard 2004).

Reliable signals are used to predict actual threat of predation

The chemical information required for the induction of predator-induced plasticity is a complex mixture of cues emitted by predators and prey (see Ferrari et al., 2010). Predator presence alone does not inform tadpoles about actual predation risk. Rather, tadpoles eavesdrop on species-specific kairomones that are passively released through the action of predators consuming and digesting conspecific or closely related species of tadpoles (Chivers et al., 1998; Schoeppner et al., 2009a). In addition, tadpoles release

chemicals while they are being consumed and these chemicals can be used as alarm cues by conspecifics (Schoeppner et al., 2009a). Tadpoles thereby utilize both alarm cues and kairomones, and the mixture of these chemicals present in the environment may confer information that distinguishes among different species of predators in their environment. Alarm cues have also been shown to be important in learning in anuran tadpoles (Gonzalo et al., 2007). When alarm cues are associated with kairomones of an invasive crayfish predator previously not present in the species range of the common toad, *Bufo bufo*, the tadpoles learned to respond behaviorally to a predator never experienced before by associating the danger of the predator with the alarm cues that were released during a depredation event (Ferrari et al., 2010).

Tadpoles have porous skin, allowing kairomones and alarm cues to be readily absorbed into their bodies (Hayes et al., 2010). This ability to receive environmental signals passively has been important in the evolution of adaptive plasticity. The consistent updating of the larval predator environment—reliable, accurate signals that are absorbed through the skin with little energy cost to the larva—allows the initiation and magnitude of the defensive response to be graded to actual threat. With increasing numbers of conspecifics being consumed, tadpoles can increase the magnitude of the anti-predator response, which plateaus with further increased predation risk (Schoeppner et al., 2009b; Van Buskirk et al., 2002).

The availability of accurate information that reflects actual predation risk has allowed tadpoles to evolve broad developmental windows for the induction of behavioral and morphological defenses throughout much of the larval period (Orizaola et al., 2012; Relyea 2003b). Tadpoles exhibit continuous growth and development and can easily

modify their morphology during ontogeny, because individuals can recycle tissues into novel structures leading to low costs of producing the adequate phenotype to match the predation risk experienced at a given time (Kisida et al., 2006). The reversibility of morphological defenses has been shown to occur very rapidly: in a week or less, a tadpole can change in response to a new threat, or completely lose its behavioral and morphological modifications and be no different than a tadpole that never experienced a predator (Orizaola et al., 2012; Relyea 2003b). In a time-constrained environment, organisms are less prone to maintain a costly defensive phenotype. As soon as the perceived predation risk is no longer present, the investment in anti-predator morphological or behavioral traits no longer increases fitness, and all available resources are re-invested in growth and development (Orizaola et al., 2012). As tadpoles reach later stages and are close to metamorphosis, the ability to express morphological plasticity diminishes. This is thought to occur because there could be a slowing of developmental rate once metamorphosis is imminent, and since large tadpoles are less vulnerable to predation events, the developmental window to express plastic changes closes as metamorphosis draws near (Relyea 2003b).

Genetic basis of adaptive plasticity

The evolution of phenotypic plasticity requires a heritable genetic basis for the plastic trait itself (Benard 2004). Although there are a limited number of investigations, the general consensus is that there is a moderate to high level of heritability of morphological and behavioral plasticity in anurans (Kishida et al., 2007; Relyea 2002a, 2005). In a study investigating geographic variation in the expression of inducible morphological defenses, the genetic basis of phenotypic plasticity was supported

(Kishida et al., 2007). Kishida et al. (2007) studied the differences in the anti-predator morphological response between a mainland and island population of the Ezo brown frog, *Rana pirica,* to the predatory Ezo salamander, *Hynobius retardatus*. *Rana pirica* expresses a bulgy phenotype in response to the gape limited salamander predator. This inducible morphological defense increases survival rates in response to the gape limited salamander by preventing the predator from swallowing it (Kishida et al., 2004). The island population of the Ezo brown frog has been isolated from the mainland for several tens of thousands of years, and there are no salamander predators on the island. The mainland population of the Ezo brown frog occupies habitats containing larval salamanders, leading to strong differences in predator pressures between the island and mainland population. There are strong differences between the two anuran populations' ability to display anti-predator morphologies in response to the Ezo salamander. The island population still expressed a bulgy morphology that was significantly larger than tadpoles that were not exposed to a predator. The mainland population showed the greatest expression of the bulgy morphology, which was significantly larger than the induced bulgy morphology that was expressed in the island population. When there were selective crosses between the island and mainland population, the hybrids produced intermediate phenotypes. Furthermore, the parental origins and how they were crossed had no effect on the phenotype of hybrids. The lack of parental dependence of the expression of hybrid morphology gives support that the induced defenses are controlled by autosomal alleles. In addition, Mori et al. (2005) showed that the bulgy anti-predator response was linked with genes associated with fibrinolysis and intracellular assembly.

The results from Kishida et al. (2007) and Mori et al. (2005) strongly suggest that the population differences in the expression have a genetic basis.

The capability of *R. pirica* tadpoles to maintain the ability to respond to a predator that has not been present in its environment for thousands of years is an interesting finding from Kisida et al. (2007). The morphological response was diminished when compared to the mainland population, but the plastic response was maintained. The ability to express the bulgy morphology might be an ancestral character state that has been maintained even though the larval salamander predators have been absent for an extended period of time (Kishida et al., 2007). The reduction of the expression of the inducible state could be due to the costs associated with the plastic response, or the result of mutational degradation over time by genetic drift (Kishida et al., 2007). However, the plastic response is still expressed, which gives evidence that the plastic response is not a costly trait to maintain.

Stress hormones mediate environment-genotype interactions

The corticosteroid pathway is the most likely mediator of adaptive plasticity in anuran tadpoles in response to predators (Fraker et al., 2009; Hossie et al., 2010; Maher et al., 2013). The neuroendocrine stress system in anurans is composed of the hypothalamus, pituitary, and interrenal glands, which together form the hypothalamus-pituitary-interrenal axis (HPI axis). Corticotropin-releasing factor (CRF) is the dominant hypothalamic neurohormone, which regulates the HPI axis and functions as a neuromodulator to coordinate endocrine, behavioral, and autonomic responses to stressors (Yoa et al., 2007). CRF stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland, which then stimulates the biosynthesis and release of

glucocorticoids (i.e., corticosterone, CORT) (Fraker et al., 2009; Maher et al., 2013; Yoa et al., 2007). Glucocorticoids have a diverse set of actions on behavior and physiology including activity level and energy utilization; importantly, the hormones act at several points along the HPI axis to exert negative feedback on the hypothalamus and return the system to homeostasis (Fraker et al., 2009; Yoa et al., 2007).

Maher et al. (2013) used field surveys, mesocosm, and laboratory experiments to show that predators modulate the circulating concentration of CORT, which affects the neuroendocrine stress axis as a function of exposure time to a predator's presence. Tadpoles raised with non-lethal predators had significantly higher whole-body CORT levels than control tadpoles that were raised without predators (Maher et al., 2013). In the same study, treating tadpoles with CORT caused the expression of anti-predator morphology that was similar to that of tadpoles exposed to predators. Hossie et al. (2010) found similar results, which also supported CORT being a physiological mediator of adaptive morphological plasticity. Rather than exposing tadpoles to CORT and seeing the effects on morphology, Hossie et al. (2010) exposed tadpoles to a predator and a CORT inhibitor. When the tadpoles were exposed to both a predator and a CORT inhibitor, the development of anti-predator morphology was repressed, when compared to tadpoles only exposed to a predator. The results from Maher et al. (2013) and Hossie et al. (2010) together give strong evidence for CORT being a mediator of morphological plasticity.

Evolutionary consequences of adaptive plasticity

Phenotypic plasticity is widespread taxonomically and affects a diverse array of traits, and can impact ecological interactions (Kishida et al., 2010). The fact that plasticity is so widespread gives evidence to its importance in mitigating fitness-reducing

agents and impacting evolutionary trajectories of species and ecosystems (Abrams 2000; Fordyce 2006; Kelly et al., 2012; Miner et al., 2005). Very few empirical studies have documented the effects of phenotypic plasticity on ecosystem functioning, but many mathematical models have been put forward that support the idea that phenotypic plasticity reduces stochastic population fluctuations and balances ecosystems over time (Coquillard et al., 2012; Draghi et al., 2012; Ellers et al., 2010; Peacor et al., 2006; Thibert-Plante et al., 2011; Yamamichi et al., 2011).

An important role that phenotypic plasticity plays in evolution is generating a diverse set of phenotypes by the modification of developmental and physiological pathways (Fordyce 2006). Furthermore, plasticity enables anurans to expand geographic distributions and survive in the face of temporal and spatial environmental change. The increased phenotypic variation, which is filtered by selection, can produce well-tuned phenotypes in response to selection pressures and enable anurans to increase the chances of surviving in the face of unpredictable and harsh biotic and abiotic environments. The production of phenotypic variation through plasticity is similar to variation caused by genetic differences, but may occur faster (Pfennig et al., 2010). This would enable species to adapt to quickly changing environments (Fordyce 2006; Pfennig et al., 2002; Yamamichi et al., 2011). If an organism relied only on genetic changes, it would require time for new mutations to be produced within the population, with the threat of drift counteracting them at the same time. Furthermore, if a new mutation arose, it would take time for it to spread throughout a population over generations (Pfennig et al., 2010). Adaptive plasticity evolved deep in the evolutionary history of anurans and this has

enabled populations to quickly adapt to local environments and there is evidence that these adaptations can buffer populations from stochastic population fluctuations.

In a hypothesized predator-prey model, plasticity acts as a population stabilizer for both the predator and prey (Miner et al., 2005). This occurs through prey decreasing the per-capita consumption rates of the predators, by the prey becoming harder to capture through behavioral and morphological change. Overall, this results in a decrease in the population growth rate of predators, through a negative feedback cycle. If no adaptive response of the prey to the predators occurred, then predators would increase in population size, causing a larger decrease in prey density. Because there would be no impacts on the predator population growth rate until prey became limited, this could lead to more stochastic population fluctuations (Verschoor et al., 2004). This predator-prey model was tested using an aquatic system involving algae and herbivorous zooplankton. The results showed that algae that responded through adaptive plasticity to an herbivore had a significantly more stable population dynamic through time than a non-plastic alga (Verschoor et al., 2004). Boieng et al. (2010) conducted a similar experiment using *Daphnia pulex* as the prey species and a planktivorous fish as the predator. This experiment used two clones of *D. pulex,* one that responded through adaptive plasticity to the presence of a fish predator and the other did not. The results of this experiment showed that the *Daphnia* that responded through adaptive plasticity had a positive effect on predators and the prey of the *Daphnia*. The *Daphnia* that did not express adaptive plasticity quickly went extinct, and this in turn negatively affected the predators, which starved. This experiment gives another empirical example of how phenotypic plasticity can balance a predator-prey system, thus promoting a balanced ecosystem through

adaptive plasticity (Boeing et al., 2010).

Amphibians and pollution

Amphibians are experiencing major global declines (Beebee et al., 2005; Blaustein et al., 2002; Houlahan et al., 2000; Kiesecker et al., 2001; McCallum 2007; Stuart et al., 2004). There is no single cause that accounts for the declines, but rather a complicated web of interactions among a variety of factors (see Hayes et al., 2010). Environmental pollutants are widely cited as being one of the most influential factors involved in amphibian declines (Hayes et al., 2010; Wilcove et al., 2005). Amphibians are particularly sensitive to pollutants due to their porous skin, which allows chemicals to enter and distort their physiological systems (Willens et al., 2006). Pesticides, herbicides and fungicides are used to protect important crops and to limit human exposure to diseases, such as the West Nile virus. The estimated quantity of these chemicals used annually worldwide is 5.2 billion pounds of active ingredients (Grube et al., 2011). The widespread use of these chemicals has resulted in ubiquitous low-level contamination in aquatic ecosystems through run-off, direct spraying, and atmospheric deposition (Davidson et al., 2002; LeNoir et al., 1999; Relyea 2004c; Sparling et al., 2001). Understanding how low-level concentrations of these chemicals impact amphibians and their ecological interactions is of great importance due to the drastic declines in amphibian populations in the last thirty years and agrochemicals being implicated in population declines (Barinaga 1990; Corn et al., 1984; Carey 1993; Davidson et al., 2002, 2004; Vitt et al., 1990; Wake et al., 2008).

There has been a recent focus on determining the impacts of environmental pollutants on amphibians in more realistic circumstances. Historically, experiments have only determined the concentration of a pollutant that is lethal to 50% of the experimental population of tadpoles. This approach is useful in determining lethal concentrations, but it is becoming clear that when pollutants are applied in addition to other stressors such as predators, the lethality of the pollutant may increase and other non-lethal effects can occur (Relyea 2004c; Relyea et al., 2008). For example, Relyea et al. (2008) simulated in a mesocosm experiment a diverse aquatic ecosystem that included 27 species of animals, phytoplankton, and periphyton. The experiment investigated how ecologically relevant concentrations of malathion impacted three species of tadpoles in relation to the community that they were in. One of the important findings is that malathion caused an increase in phytoplankton by reducing zooplankton diversity and abundance, which decreased the amount of periphyton due to the phytoplankton decreasing light transmission. The decrease in periphyton, which is a food source for tadpoles, ultimately resulted in tadpoles having significantly reduced mass (density-mediated indirect effect caused by malathion decreasing zooplankton density). Similar impacts on nutrition have been found in other experiments using other agrochemicals (Boone et al. 2004; Havens 1994, 1995; Mills et al., 2004). Relyea et al. (2008) also found an important trait-mediated indirect effect of malathion on predation rates, where predator foraging efficiency was affected. The concentrations of malathion used did not change the density of predators but decreased predation rates by impacting motivation and/or coordination, and this has been duplicated in other experiments (Relyea et al., 2008, 2010; Weis et al., 2001). The impacts of malathion and other agrochemicals on community dynamics and

predation rates may have large influences on amphibian populations and also reveals how low-level contamination can impact non-target organisms in ways not considered by traditional toxicology tests that determine a chemical's safety for non-target organisms.

Predation and pollution

How pesticides interact with predation rates is largely unknown, but the few studies conducted have shown that agrochemicals have both positive and negative impacts through indirect and direct effects on predation (Relyea et al., 2010; Qin et al., 2011). Sub-lethal concentrations of pesticides can impair tadpoles' ability to recognize and behaviorally respond to predators (Bridges et al., 1999). Pesticides may also alter the perceived palatability of noxious tadpoles to fish predators (Hanlon et al., 2013). Select pesticides have been shown to decrease mass and cause skeletal malformations which could increase predation rates, due to smaller tadpoles being more susceptible to predation and morphological abnormalities, resulting in irregular swimming behaviors, immobility, and generally impacting the biomechanics of the tadpole and how it escapes from a predator (Brunelli et al., 2009; Michael et al., 2011; Relyea et al., 2001a, 2005a; Relyea 2004c, 2009; Shenoy et al., 2009; Teplitsky et al., 2005; Widder et al., 2008). There also has been a well-documented direct effect of agrochemicals increasing mortality rates of predators. This decrease in predator density leads to a positive impact on tadpoles by decreasing predation rates (Boone et al., 2003; Cothran et al., 2011; Mills et al., 2004; Relyea et al, 2008).

Adaptive plasticity and pollution

There has been very little investigation of how agrochemicals can impact adaptive plasticity of anuran species in response to predators. Agrochemicals have been shown to inhibit adaptive plasticity in the planktonic cladoceran crustaceans *Daphnia* and *Bosmina* (Barry 1999; Sakamoto et al., 2009). Furthermore, there is evidence that copper pollution and acidic aquatic conditions can inhibit adaptive morphological responses in the Arabian toad *(Bufo arabicus)* and the Moor frog (*Rana arvalis)* (Barry 2011; Teplitsky et al., 2007).

The most relevant evidence that an agrochemical can impact adaptive plasticity in anuran tadpoles comes from Relyea (2012), who investigated the interaction between the herbicide Roundup® and morphological change in response to predators. The results of this experiment showed that Roundup®, by itself, caused an increase in tail depth at the same magnitude as the increase in tail depth in response to a predator's presence (Relyea 2012). These experiments give evidence that agrochemicals can directly augment morphological traits and potentially may interact with adaptive responses, through the chemical causing trait changes that could be in non-adaptive directions depending on the predator that is eliciting the adaptive response (Barry 1999, 2011; Relyea 2012; Sakamoto et al., 2009; Teplitsky et al., 2007).

Conclusion

Amphibians have evolved sensitive physiological systems that receive and process environmental signals that can determine the actual threat posed by the current predator environment. Tadpoles can then produce specific adaptive phenotypes that

increase fitness in response to the most dangerous predator. In natural aquatic ecosystems, tadpoles experience a complex of environmental stressors, and it is likely that populations often experience both predators and pollution at the same time. There is evidence that agrochemicals may be impacting aspects of the HPI axis, through modulation of corticosterone levels (Hayes et al., 2006). Anurans depend on specific environmental signals that are used by the HPI axis to elicit an adaptive response. If agrochemicals are modulating CORT concentrations, or impacting other important hormones of the HPI axis, then anurans may no longer be able to rely on predator kairomones and tadpole alarm cues to express the correct adaptive responses. This would occur because agrochemicals that modulate the HPI axis by changing the internal hormonal concentrations of anurans could result in non-adaptive changes. This could contribute to amphibian declines because adaptive responses likely contribute to the stability of amphibian populations through time (Fraker 2009, Miner et al., 2005).

SECTION TWO

Introduction

The class Amphibia is one of the most globally threatened groups of vertebrates, with 41% of its species in a documented decline (Bradford et al., 2011; Egea-Serrano et al., 2012; Hammer et al., 2008; Hayes et al., 2010; Sparling et al., 2009; Wake et al., 2008). Environmental pollution is considered to be the second most important cause, next to habitat modification, for the worldwide decline of amphibians (Hayes et al. 2010; Mann et al., 2009). Amphibians are considered particularly susceptible to the effects of chemical pollutants (Willens et al. 2006). Chemical contaminants may readily penetrate the permeable skin of amphibians and alter physiological processes underlying critical developmental and behavioral functions, which can reduce fitness (Hayes et al., 2010, Relyea, 2004a; Quaranta et al. 2009).

Malathion is one of the most widely used pesticides in the United States (Kiely et al., 2004), and concentrations of malathion in aquatic ecosystems, have been detected across a range from 0.001 to 0.6 mg/L (Giri et al., 2012; Relyea 2004b; Sparling et al., 2009). Malathion inhibits acetylcholinesterase (AChE), which hydrolyzes acetylcholine (ACh), a neurotransmitter utilized by the central and peripheral nervous systems that is critical for muscle activation and modulation of a diverse system of neural pathways (Gulledge et al., 2009; Relyea 2004b).

A potential non-lethal effect of malathion and other anthropogenic contaminants is the inhibition of amphibians' ability to morphologically and behaviorally respond to abiotic and biotic stressors. Anuran tadpoles can respond through adaptive morphological and behavioral plasticity to competition, pond desiccation, and predators, thereby

increasing survival rates to metamorphosis (Benard 2006; Boorse et al., 2004; Denver 2009; Gomez-Mestre et al., 2008; Kishida et al., 2010; Relyea 2002a, 2004c; Relyea et al., 2004). Hormones of the HPI axis have been shown to mediate adaptive plasticity in tadpoles (Denver 2009; Maher et al., 2013). The hormones involved in these adaptive responses are corticotropin-releasing factor (CRF), corticotropin (ACTH), and glucocorticoids (GC) (corticosterone (CORT)) (Denver 2009). Corticosterone is the most important mediator of tadpole anti-predator morphological defenses, and CORT has been shown to induce anti-predator morphologies (Denver 2009; Denver et al., 2010; Egea-Serrano et al., 2012; Fraker et al., 2009; Glennmier et al., 2002; Hossie et al., 2010; Maher et al., 2013). The inhibition of ACh, which can affect trophic hormone release at the hypothalamic level, may impact corticosterone levels. The modulation of plasma CORT concentrations could impede the proper functioning of the HPI axis (Cericato et al., 2008; Ghodageri et al., 2011; Ozmen et al., 1993; Rezg et al., 2010). The result could effectively inhibit the adaptive morphological and behavioral changes in response to predators and other environmental stressors.

The Sierran treefrog (*Pseudacris sierra*) is abundant and occupies a diverse set of habitats in California. The species' distribution results in a complex assortment of predator assemblages, with vaying levels of selection pressures for different phenotypes within the frogs' range. Predator assemblages also vary seasonally, leading to a dynamic shift in predation pressures temporally (Benard 2004). In response to variable predator environments, *P. sierra* tadpoles develop quantitatively different morphologies in response to specific predators (Benard 2004; Hossie et. al., 2010). Tadpoles that are exposed to fish predators have shallower tails and bodies, and tadpoles exposed to aquatic

insect predators have deeper tails (Benard 2006). These morphologies increase survival rates when tadpoles are exposed to hunting predators that elicited the specific morphology (Benard 2006). Furthermore, when naïve tadpoles were exposed to an active predator, there was a significantly lower survival rate (Benard 2006). The differential survival rates are thought to occur due to different adaptive values of the morphologies. The greater tail depth could lure a predator's strike from the head to the tail region, where it is much more fragile and is torn easily, facilitating escape from sit and wait predators such as dragonfly larvae (Dayton et al., 2005; Doherty et al., 1998; Van Buskirk et al., 2003). Shallower tails have been shown to increase swimming speed, allowing tadpoles to escape an attack from an actively hunting predator such as a fish (Wilson et al., 2005). There is also a general activity reduction in response to predators (Benard 2004; Relyea 2001). Tadpoles that decrease activity level may prevent detection by a predator or decrease the chance of coming into close contact with one (Relyea 2004a). These anti-predator morphological and behavioral responses occur in a wide range of anuran taxa and are important ecological responses that increase rates of survival to metamorphosis (Benard 2004; Relyea 2001a).

Anuran morphological and behavioral adaptations to predators occur in response to alarm pheromones and predator kairomones. Alarm pheromones are released from tadpole skin cells when a predator consumes tadpoles, and predators release specific kairomones as they consume their prey (Schoeppner et al., 2005). The two distinct signals trigger a stress response, ultimately increasing whole body CORT concentrations in tadpoles (Maher et al., 2013). The actions of CORT on gene transcription, as well as its role in regulating hormone activity through negative feedback on the HPI axis, results in

the expression of the adaptive morphological and behavioral responses in response to the predator (Denver 2009; Denver et al., 2010; Egea-Serrano et al., 2012; Fraker et al., 2009; Maher et al., 2013). The presence of malathion in addition to the predator has been shown to interact synergistically, increasing the lethality and genotoxicity of malathion. The synergism that exists between predators and malathion may inhibit adaptive responses of tadpoles to their predators (Relyea 2004a). There has been very little investigation of how agrochemicals can impact adaptive plasticity of anuran species in response to predators. Malathion has been shown to inhibit adaptive plasticity in the planktonic cladoceran crustacean *Daphnia*, but it is not known if malathion impacts adaptive plasticity in anurans (Barry 1999).

In this study, I tested the hypothesis that malathion inhibits anti-predator behavioral and morphological responses of *P. sierra* to dragonfly larvae (*Anax junius*). I predicted that in response to the aquatic insect predator, tadpoles will have an increased tail depth and decreased activity levels. When tadpoles experience *A. junius* and malathion together, there should be a significant decrease in the magnitude of the expression of a greater tail depth, leading to shallower tails, and there should be no decrease in activity level.

Methods

Tadpole collection and care

On 2 March 2012, 28 *P. sierra* egg masses were collected from a permanent pond in San Luis Obispo County (NAD83 N 35.28703, W 120.47890). All egg masses were between the Gosner developmental stages of 18 through 20 (Gosner, 1960). The egg masses were separated and individually housed in 1-liter plastic containers. Each container had

constant aeration and was maintained at 20±1 ^{o}C. The egg masses were kept in artificial pond water (25% Holtfreter's solution) and 25% water changes were performed every five days. All tadpoles were free swimming on 9 March 2012. The tadpoles remained in the 1-liter plastic containers until 14 March 2012 when the tadpoles were moved to the experimental tanks. The tadpoles were randomly distributed throughout all the experimental tanks using a random number generator (N=10 per tank), and no two tadpoles in each tank came from the same egg mass.

The experimental tanks were 56 identical 15-L plastic containers. The containers held 7.8 liters of charcoal-filtered deionized water that was supplemented with salts to make 25% Holtfreter's solution. Air bubbled vigorously and evenly in all of the tanks. Tadpoles were fed Purina rabbit chow ad libitum every two days. The food was allowed to sit in the tank for approximately 24 hours and then was removed to avoid water fouling. Every five days, two-thirds of the water was replaced with fresh 25% Holtfreter's solution (Benard 2006).

Four experimental tanks were randomly selected on 21 March 2012. The tadpoles in these tanks were euthanized using 20% Benzocaine, weighed, and photographed to be used to estimate the size of the tadpoles at the beginning of the experiment. The initial mean wet mass was 16.27±1.4 mg and all tadpoles were at Gosner stage 25.

Treatments

Treatments began on 21 March 2012. A 2 by 2, fully crossed experimental design was used, with the following variables: predator cues (absent or present) and malathion (absent or present) (Fig. 1). The treatments were randomly assigned to the experimental tanks, resulting in 13 replicates for each treatment (N=10 tadpoles per replicate).

Predators

On 18 March and 19 March 2012, 20 *Anax junius* larvae were collected from Stenner Pond on the California Polytechinic State University campus in San Luis Obispo, CA. The larvae were maintained in 0.5-L plastic cups with artificial pond water and a 2-in^2 piece of screen to add structure. On 2 April 2012, eight more dragonfly larvae were collected to replace dead larvae or those that stopped feeding due to imminent metamorphosis.

Every two to three days, all *A. junius* predators were fed two to four tadpoles, depending on how large the dragonfly larvae were. One hour after feeding, the water from all the plastic cups was mixed into a single 5-gallon container (= predator cue water), and 200 ml of predator cue water were distributed to all the predator present treatments. The predator absent treatments received 200 ml of Holtfreter's solution to control for the effect of adding liquid to the containers, at the same time the predator cues were added.

A static renewal procedure was implemented for malathion treatments in this experiment. To distribute the malathion to the malathion present treatments, 1.6 µl of Ortho Malathion 50 Plus (50.6% active ingredient) was added to each experimental tank. The tanks that did not receive malathion had an injection of 1.6 µl of Holtfreter's solution to control for the addition of the treatment. This procedure was performed every 5 days, after water changes occurred.

Tadpole activity

Behavioral assays were done on 9 April 2012 and 11 April 2012, starting at 1700 hours each day. On 9 April 2012, observations of tadpole activity levels were made

before and after predator cues were added to the predator present treatments and water added to the tanks in which predator cues were absent. The observations made on 9 April 2012 determined if the presence of predators had an effect on activity level. On 11 April 2012 observations of tadpole activity level were conducted before and after predator cues, malathion, and water were added to the appropriate treatments. The observations on 11 April 2012 were made to determine the effect malathion had on anti-predator behavioral responses. Tadpole activity level was calculated by observing the number of tadpoles moving out of the total number of tadpoles in a tank. Each tank was observed for five seconds and the proportion of moving tadpoles out of the total number in the tank was calculated. Each tank was observed five separate times. The five observations were than averaged to determine each tank's average activity level. A constant of one was added to the tank averages so that there would be no zero levels for activity when performing data analysis.

Morphological measurements

On 21 April 2012, when the tadpoles reached Gosner Stage 37, the experiment ended and each tadpole was euthanized using 20% Benzocaine, weighed (wet mass), developmentally staged, and laterally photographed using a Canon Powershot SX500 IS 16.0 megapixel camera. The staging was done using a magnification lens of 10X. The digital images were analyzed using TpsDig2 v2.05, using a 13-landmark method (Hossie et al., 2010). The TpsDig file with landmark and scale factors for all individual tadpoles was uploaded to http://www.morpho-tools.net/measLMs_online.html, an internet-accessible Landmark Measurement Morpho-tool program, which calculated Euclidean distance for each linear measurement (Krieger, 2006). The morphological measurements

taken were body length, body depth, tail length, tail depth and tail muscle depth. All of these morphological measurements have been shown to be involved in morphological plasticity in response to predators (Van Buskirk et al., 1998; Dayton et al., 2005; Benard, 2004; 2006).

Data analysis

All analyses were performed in JMP 11.0.0 software (SAS Institute Inc.). All data met the assumptions of normal distribution and equal variance, except where described below. In all cases where assumptions were violated, the statistical test employed was fairly robust to the violation (see below).

Statistics for tadpole survival

Survival rate was calculated as the proportion of surviving tadpoles for each tank in each treatment. This proportion was arcsine transformed, and there was no significant difference in survival rate among the treatments. Survival rates were analyzed using an ANOVA, using presence of predator cue and presence of malathion and their interaction as independent variables. The overall survival rate of all the tadpoles in the experiment was 92.5%. The Control, Malathion, Predator and Predator-Malathion treatments had an average number +/- standard error of tadpoles per tank of 9.38 +/- 0.31, 9.15 +/- 0.15, 9.23 +/- 0.34 and 9.15+/- 0.35 respectively.

Statistics for tadpole activity level

Tadpole activity was analyzed using a Repeated-Measures ANOVA model with predator and malathion as main effects, and their interaction term. Time was included in the model, as was the interaction between time and the predator and malathion treatments. Furthermore, the three-way interaction between time, malathion, and predator

was included in the model. The same model was used to analyze the tadpole activity level for the observations made with and without malathion being added at the same time as the predator cues (See Methods: Tadpole Activity Level).

The observations of activity level before and after the addition of malathion and predator cues on 11 April, 2012, did not meet the assumption of equal variances. An inverse transformation was used for the data to meet the equal variance assumption. All other assumptions for the models were met for the activity level data.

Statistics for tadpole morphological measurements

The linear morphological response variables and mass were analyzed using a MANOVA and an ANCOVA model. The two models analyzed the data differently. The MANOVA model analyzed the data at the tank level and the ANCOVA analyzed the data at the tadpole level. Both approaches were used to determine how the treatments affected mass and the morphological measurements. For the MANOVA model, the data for each response variable were averaged for each tank. This analysis represents a conservative analysis. The ANCOVA model included individual tadpoles in the analysis, which is more liberal, but analyzed the data at the correct level for this experiment, because how individual tadpoles responded behaviorally and morphologically to the treatments was the focus of the experiment.

The MANOVA model included the treatments of predator and malathion as fixed effects, and the interaction term of predator and malathion. Significant multivariate effects were followed by univariate tests. Mass was the only response variable that violated the assumption of normal distribution. No transformation would make mass meet this assumption, but a log10 transformation came the closest to satisfying this

assumption. All other assumptions were met for the MANOVA model. Normality was determined by using the Kolmogrov-Smirnov test and the Brown-Forsythe test established homoscedasticity.

The ANCOVA model used the treatments of predator and malathion as fixed effects and also included the interaction between the two treatments. To account for any tank effect, tank was included in the model as a random effect. The covariate, centroid size, was also included in the model to account for the allometric effect of larger tadpoles having larger morphological measurements (Relyea 2012, 2004; Zelditch, 2004). Centroid size is a measure of geometric scale and was calculated independently of the linear measurements in TpsDig2 v2.05 using 13 landmarks to quantify tadpole shape. Centroid size has been commonly used as a covariate in similar experiments (Johansson et al., 2011). An important assumption for this model is that there are no two- or three-way interactions between the covariate and the main effects in the model. This assumption was violated by a significant interaction being detected between centroid size and the predator treatment for tail muscle depth. The significant interaction is the result of larger tadpoles having a more pronounced plastic response to a predators' presence, compared to smaller tadpoles (Fig. 2). A muscle depth by centroid size interaction plot was made using JMP 11.0.0. The results of the plot show that the two treatments that included predator cues had steeper slopes, than the treatments that did not include predator cues, giving evidence that larger tadpoles have a greater ability to express morphological anti-predator defenses (Fig. 2). The increased ability of lager tadpoles to respond through morphological plasticity has been documented in other experiments (Relyea 2004a). Body length and mass were the only response variables that did not have

a normal distribution. No transformation made the distribution normal for either measurement. There was no difference between the results of mass and body length when using either the untransformed or transformed data, so the untransformed data were used in the analysis. Normality was determined by using the Kolmogrov-Smirnov test and the Brown-Forsythe test established homoscedasticity. The Student's t and Tukey HSD pairwise comparison tests were used to determine the direction of the effect of the treatments that had a significant p-value.

Results

Tadpole activity level

Predator cues significantly affected tadpole activity level (Table 1). The Predator and Predator-Malathion treatments significantly decreased activity level after predator cues were added (Table 1;Fig. 3). The Predator and Predator-Malathion treatments decreased activity by 20% and 18% respectively, when compared to the Control treatment. The Malathion treatment did not differ significantly from the Control treatment in activity level (Table 1, Fig. 3). There was also a significant time and a predator by time interaction, which further supports that the predator treatment decreases activity level.

When malathion was added at the same time the predator cues were distributed, there was also a significant change in activity level in response to the predator treatment (Table 2). The Predator and Predator-Malathion treatments significantly decreased activity level by 15.5% and 14.6%, respectively, when compared to the Control treatment (Table 2, Fig. 4). The Malathion treatment did not significantly alter activity level.

Tadpole morphology

The MANOVA model detected significant effects on body morphology caused by the predator treatment and a significant interaction between the predator and malathion treatments (Table 3). When univariate tests were performed, tail depth and tail muscle depth were significantly affected by the treatments and/or the interaction between them (Table 4). The predator, malathion and interaction between the two treatments had a significant affect on tadpole tail depth. For tadpole tail muscle depth, only malathion had a significant effect in the model. However, the predator fixed effect was marginally non-significant for tail muscle depth, which was 0.06.

The ANCOVA model revealed significant treatment and/or interaction effects for tail depth and tail muscle depth, which concurs with the results of the MANOVA model. Univariate analyses of tadpole morphology found significant effects of the predator treatment on tail depth and tail muscle depth (Table 5). There was also a significant effect of the malathion treatment on tail muscle depth. Furthermore, a significant interaction between the predator and malathion treatment on tail depth was detected, with a non-signficant interaction for tail muscle depth (P-value= 0.07).

When pairwise comparisons were done using the Student's t-test, it was found that the predator treatment significantly increased tail depth and tail muscle depth. These changes in morphology in response to *Anax* predators are well-documented and correspond to the directions predicted by previous experiments and the predictions made in this experiment (Benard 2004; Relyea 2000). The pairwise comparison also found that the malathion treatment significantly increased tail muscle depth.

There was a significant interaction between the predator and malathion treatments for tail depth (Table 5, Fig. 5). The Predator group had significantly greater tail depths than the Control and Malathion groups. The Predator-Malathion group did not differ significantly in tail depth from the Predator group and was significantly different from the Control and Malathion groups. The Malathion group had a significantly larger tail depth than the Control group, and had a significantly smaller tail depth, when compared to the Predator groups (Table 5, Fig. 5).

Tail muscle depth was significantly affected by the predator and malathion treatments. Predator, Predator-Malathion and Malathion groups all had significantly greater tail muscle depths than the Control group (Table 5). Predator, Predator-Malathion and Malathion groups did not differ significantly from each other in the expression of muscle depth (Fig. 6).

Discussion

In this experiment, the effect of malathion on behavioral and morphological change of *P. sierra* in response to an *Anax* predator was investigated. I hypothesized that the interactions between malathion, predator kairomones, and tadpole alarm cues would impact physiological pathways involved in adaptive behavioral and morphological responses, leading to an inhibition of adaptive anti-predator plasticity. Data collected here, however, did not support this hypothesis for malathion inhibition of behavioral and morphological plasticity. However, there is evidence that malathion impacted physiological pathways involved in morphological change. Malathion alone increased tail muscle depth, suggesting that malathion causes developmental changes in morphology that mimic predator-induced adaptive plasticity in this species. Furthermore, there was a

significant predator by malathion interaction for tail depth, which is explained by malathion itself increasing tail depth, to the point that it did not differ from the Predator-Malathion group. The Predator-Malathion group did not significantly differ from the Predator group, giving evidence that when tadpoles experience both a predator and malathion, there is no inhibition of adaptive plasticity.

Amphibians have evolved the ability to respond through adaptive morphological plasticity to a diverse set of predators, which increases survival rates to metamorphosis (Benard 2004, Relyea 2001). *Pseudacris sierra* exemplifies this ability by expressing fine-tuned phenotypes in response to specific predators that have different hunting strategies. The morphologies that are produced are important because they confer different biomechanical attributes that aid in escaping the initial strike of a predator (Wilson 2005). The different hunting strategies of predators have resulted in a trade-off, under which tail morphologies confer higher fitness for one hunting strategy, and at the same time cause a greater vulnerability to a different hunting strategy. For example, a shallow tail increases survival rates when the most dangerous predator is an active hunter, like a fish, where burst swimming speed is more important in escaping a predator. This shallow tail, however, increases capture efficacy of sit and wait predators such as dragonfly larvae, because they strike tadpoles on their bodies more often (Benard 2006; Van Buskirk et al., 2003). In contrast, if tadpoles had deeper tails, dragonfly larvae would be lured to the tail, which is torn easily, facilitating escape (Van Buskirk et al., 2003). However, this comes at a cost of slower burst swimming speeds, making fish more dangerous (Wilson 2005). Tadpoles have evolved the ability to "eavesdrop" on the predator environment using signals that predators passively release as they consume and

digest other tadpoles. This ability establishes the current most dangerous predator in their environment (Schoepper et al., 2005). The tadpole HPI axis utilizes the predator information and translates it into predator-specific morphologies to mitigate their predator environment by increasing survival rates (Schoepper et al., 2005).

Agrochemicals such as malathion may be modulating tadpole CORT concentrations (Hayes et al., 2006; McMahon et al., 2011), impeding an adequate stress response and the ability to maintain homeostasis, which can indirectly or directly feedback into changes in HPI axis reactivity. Such malathion-induced changes in HPI function could have major consequences for tadpole populations that depend on reliable signals in order to express correct adaptive morphological responses that decrease predation rates through the action of predator kairomones and tadpole alarm pheromones on the HPI axis (See Chapter One for review of consequences of adaptive plasticity). The results of this experiment provide evidence malathion, may be disrupting the ability of tadpoles to interpret environmental signals correctly. A specific adaptive response may no longer be attainable, due to the influence of agrochemicals either mimicking signals, or influencing the internal hormonal environment, resulting in morphological change to occur, when it is not adaptive. Here, malathion caused tail muscle depth to increase to the same magnitude as if only a *Anax* predator was present. This morphological change was not an adaptive response, however, since there was no predator actually present in the environment. There may be a cost associated with investment in tail muscle growth when it is not favored by selection. The energy invested in increasing tail depth could be used for feeding and body growth, which is associated with higher fitness, because size at metamorphosis is strongly correlated with adult survival (Benard 2004).

Data obtained here also reveals that malathion exposure directly affected tail depth. Malathion alone increased tail depth in the same direction as caused by *Anax* predators. These malathion-induced changes would be in the opposing direction to the adaptive response to some predators, such as a fish, which has a different hunting strategy. The modulation of morphology as a result of ecologically relevant concentrations of malathion may have detrimental consequences for tadpole populations. This could occur because tadpoles might not be able to accurately eavesdrop on their predator environment due to malathion indirectly impacting the same physiological pathways that are vital for decreasing predation rates. This could result in tadpoles developing increased tail depths and tail muscle depths, when the most dangerous predator favors shallow tails. The direction of morphological change would not be in an adaptive direction if this were the case. The results from this experiment can only show that malathion does impact the same morphological characteristics involved in adaptive morphological change in response to a predator.

Previous research has shown that insecticides induce anti-predator morphology in the cladoceran *Daphnia* and can also inhibit anti-predator morphology in a different cladoceran species, *Bosmina fatalis* (Barry 1999; Hanazato 1991; Sakamoto et al. 2006). Furthermore, the herbicide Roundup® has been shown to cause anti-predator morphology in the wood frog (*Rana sylvatica)* and leapard frog (*Rana pipiens)* (Relyea 2012). The results from these experiments show that pesticides can impact adaptive plasticity, but more research needs to be done to determine the mechanisms that cause morphological change in response to agrochemicals, and if these changes can impact survival rates to metamorphosis by impeding adaptive changes that increase survival

rates in the faces of the strong selection pressure of predation. It is critical to understand how aquatic ecosystems are impacted by the common low-level contamination of agrochemicals. More research needs to be done to determine if the chemicals are significant contributors to global amphibian declines by impacting important ecological responses of tadpoles to environmental stressors. Future research should incorporate the testing of concentrations of corticosteroids in tadpoles in different treatment groups that include a sit-and wait predators, active hunting predators and no predator environments. Furthermore, this experiment used a concentration of malathion that is on the lower spectrum of the commonly found concentrations of malathion in natural aquatic ecosystems which ranges from 0.001 to 0.6 mg/L. It would be worthwhile to use higher concentrations within this range, and in addition, use different pesticides and herbicides, and also the combination of different agrochemicals to recreate more realistic circumstances.

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APPENDIX

	rm-ANOVA		
Treatment	DF	F-Ratio	P-Value
Predator	1, 48	25.0035	<0.0001
Malathion	1, 48	0.0171	0.8965
Predator x Malathion	1, 48	0.0315	0.8599
Time	1, 48	18.6385	<0.0001
Predator x Time	1, 48	10.1201	0.0026
Malathion x Time	1, 48	0.791	0.3782
Predator x Malathion x Time	1, 48	0.9664	0.3305

Table 1: Univariate outputs of the activity level of *Pseudacris regilla* tadpoles before and after the addition of predator cues, when malathion was not added.

	rm-ANOVA		
Treatment	DF	F-Ratio	P-Value
Predator	1, 48	56.08	<0.0001
Malathion	1, 48	0.4267	0.5168
Predatcor x Malathion	1, 48	0.5429	0.4648
Time	1, 48	14.3282	0.0004
Predator x Time	1, 48	9.5864	0.0033
Malathion x Time	1, 48	1.2515	0.2688
Predator x Malathion x Time	1, 48	1.3609	0.2491

Table 2: Univariate outputs of activity level of *Pseudacris regilla* tadpoles before and after the addition of predator cues, when malathion was added at the same time as predator cues.

	Multivariate test (Wilk's Lambda)		
Treatment	DF	F-Ratio	P-Value
Predator	5, 44	9.7322	<0.0001
Malathion	5, 44	1.8375	0.1252
Predator x Malathion	5, 44	4.604	0.0018

Table 3: MANOVA results for the whole model using Wilk's Lambda.

	Tail Length			Body Depth			Tail Depth			Muscle Depth			Body Length			Mass		
Treatment	DF	F-Ratio	P-Value	DF	F-Ratio	P-Value	DF	F-Ratio	P-Value	DF	F-Ratio	P-Value	DF	F-Ratio	P-Value	DF	F-Ratio	P-Value
Predator	1, 48	0.0902	0.7652	1, 48	2.2355	0.1914	1, 48	9.4496	0.0035	1, 48	3.593	0.0641	1, 48	0.256	0.6152	1, 48	1.3392	0.2529
Malathion	1, 48	1.9624	0.1677	1, 48	0.0023	0.9618	1, 48	5.6292	0.0217	1, 48	4.7538	0.0342	1, 48	3.7563	0.0585	1, 48	0.0246	0.8761
Predator x Malathion	1, 48	0.3735	0.544	1, 48	0.0002	0.9895	1, 48	7.0492	0.0107	1, 48	0.0004	0.9847	1, 48	0.0211	0.8852	1, 48	0.639	0.428

Table 4: Univariate tests for the MANOVA model.

	Tail Depth			Muscle Depth		
Treatment	DF	F-Ratio	P-Value	DF	F-Ratio	P-Value
Predator	1, 48.49	46.5835	<0.0001	1, 42.15	11.2115	0.0017
Malathion	1, 48.69	0.8656	0.3568	1, 42.37	6.0358	0.0182
Predator x Malathion	1, 48.69	7.6669	0.0079	1, 42.37	3.3708	0.0734
Centroid	1, 431	3020.551	<0.0001	1, 444.4	3426.028	<0.0001
Centroid x Predator	1, 431	3.3808	0.0666	1, 1444.4	13.0365	0.0003

Table 5: ANCOVA results for Tail Depth and Tail Muscle Depth of *Pseudacris regilla* tadpoles.

Control	Predator
Predator Cues Absent	Predator Cues Present
Malathion Absent	Malathion Absent
Malathion	**Predator Malathion**
Predator Cues Absent	Predator Cues Present
Malathion Present	Malathion Present

Figure 1: Treatment designation for the experiment. The presence or absence of the main effects are given for each treatment.

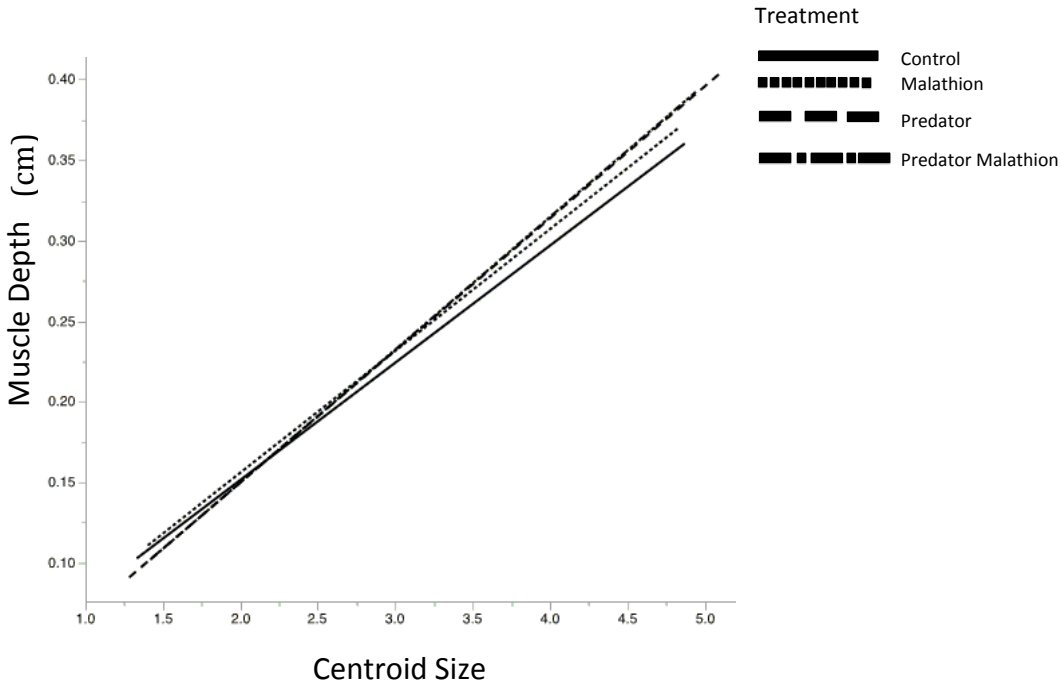

Figure 2: Muscle depth by centroid size interaction plot for all treatments. *Pseudacris sierra* tadpoles that are larger have a greater ability to express anti-predator morphology when compared to smaller tadpoles.

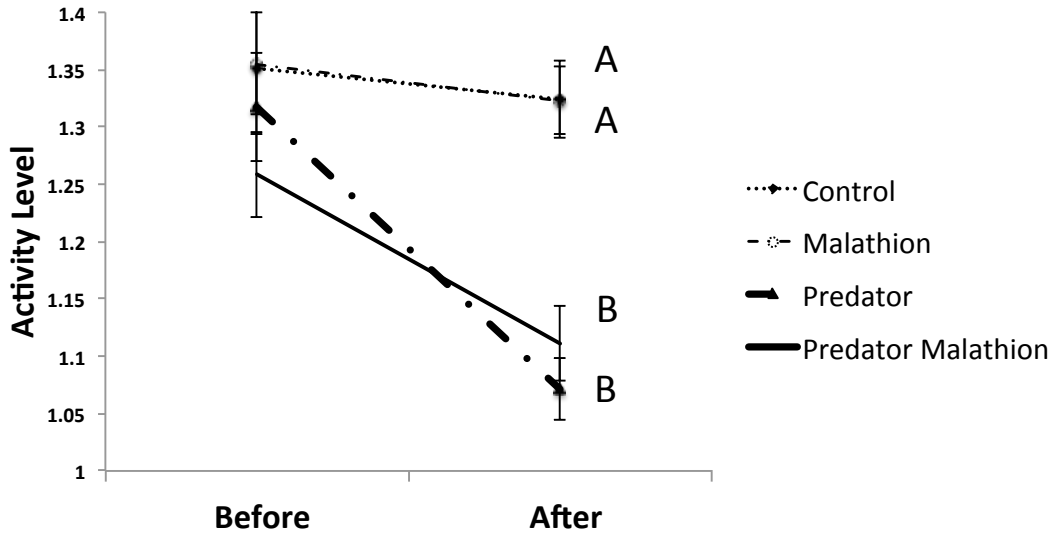

Figure 3: Average proportion of *Pseudacris sierra* tadpoles moving during five second observations before and after the addition of predator cues. Data plotted as the mean $^{+}/_{-}$ standard error of the mean. The Predator and Predator-Malathion treatments significantly reduced activity level after the addition of predator cues, when compared to the Control and Malathion treatments. Significant differences determined by Tukey HSD.

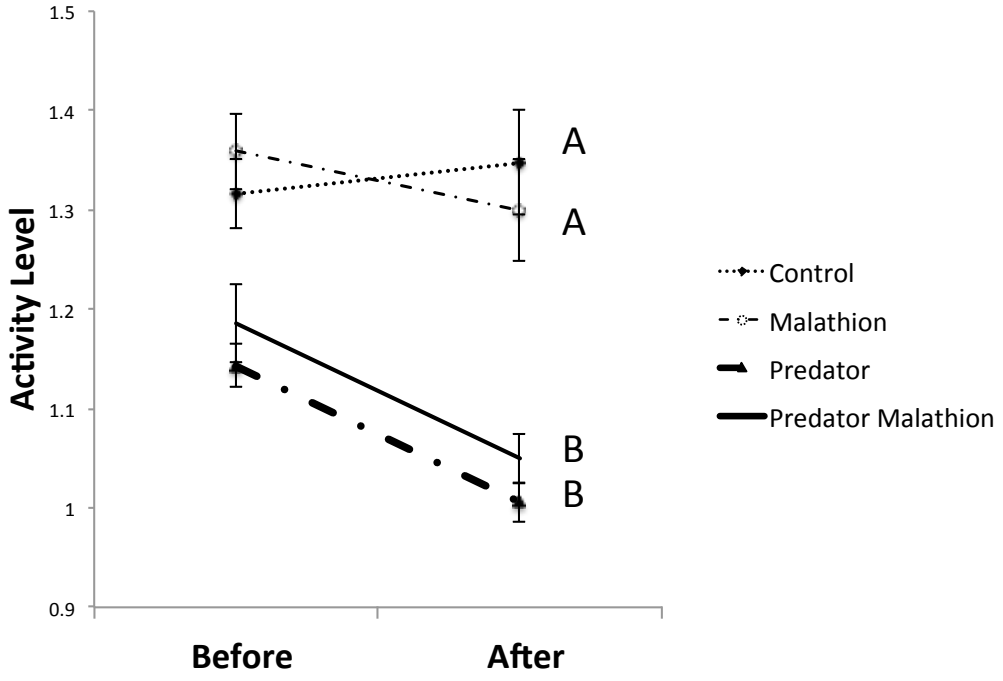

Figure 4: Average proportion of *Pseudacris sierra* tadpoles moving during five second observations before and after the addition of predator cues, and malathion. Data plotted as the mean $^{+}/_{-}$ standard error of the mean. The Predator and Predator-Malathion treatments significantly reduced activity level after the addition of predator cues and malathion, when compared to the Control and Malathion treatments. Significant differences determined by Tukey HSD.

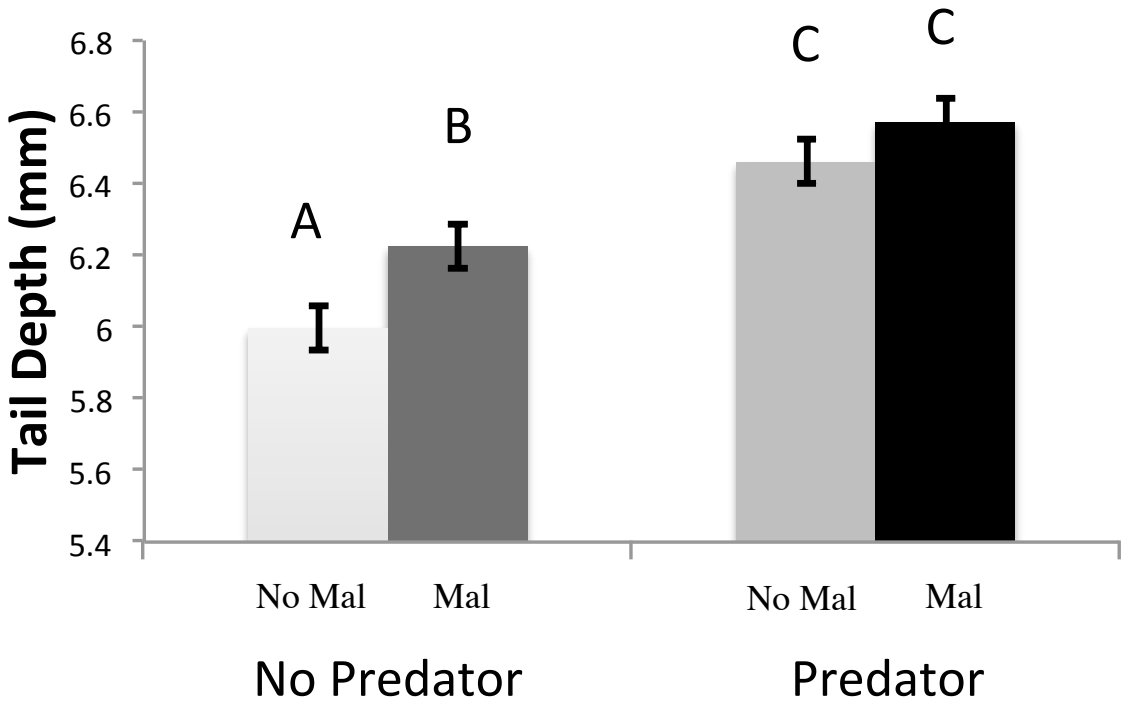

Figure 5: Bar graph of the tail depth size in all treatment groups of *Pseudacris sierra* tadpoles. Data plotted as mean $^{+}/_{-}$ standard error of the mean. Tadpoles exposed to predators significantly increased tail depth and malathion did not significantly affect the expression of adaptive plasticity when combined with predator presence. The Malathion treatment group also significantly increased tail depth when compared to the Control group. No mal refers to the treatment groups that did not receive malathion. Mal refers to treatment groups that did receive malathion. Predator refers to the treatment groups that did receive predator cues. Significant differences determined by Tukey HSD.

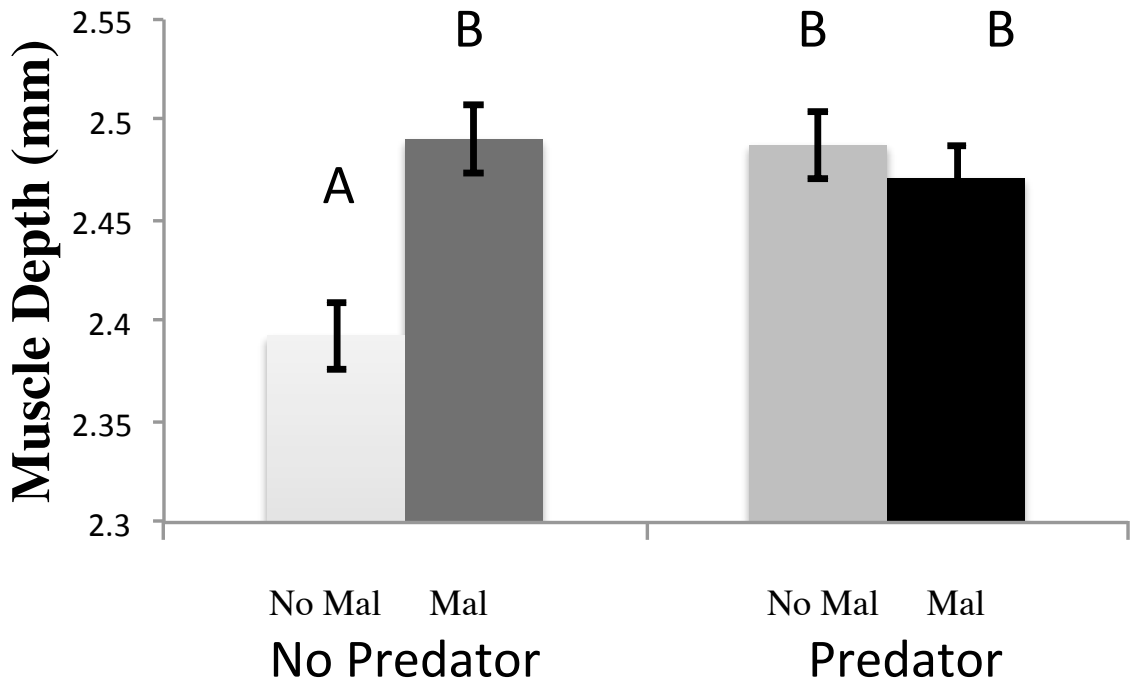

Figure 6: Bar graph of the muscle depth size in all treatment groups of *Pseudacris sierra* tadpoles. Data plotted as mean $^{+}/_{-}$ standard error of the mean. Tadpoles exposed to predators significantly increased muscle depth and malathion did not significantly affect the expression of adaptive plasticity when in combination with predator presence. Tadpoles that were only exposed to malathion significantly increased muscle depth to the same magnitude as tadpoles exposed to predators. No mal refers to the treatment groups that did not receive malathion. Mal refers to treatment groups that did receive malathion. Predator refers to the treatment groups that did receive predator cues. Significant differences determined by Tukey HSD.