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Non-Adaptive Phentypic Plasticity: Morphology, but not Swim Speed, of Spotted Salamander Larvae is Affected by "Terrestrial" and "Aquatic" Herbicides

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NON-ADAPTIVE PHENOTYPIC PLASTICITY: MORPHOLOGY, BUT NOT SWIM SPEED, OF SPOTTED SALAMANDER LARVAE IS AFFECTED BY "TERRESTRIAL" AND "AQUATIC" HERBICIDES

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By:

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ABSTRACT

1. Phenotypic plasticity, although ubiquitous, may not always be advantageous. In cases where individuals expressing an induced phenotype outperform non-induced individuals, the phenotypic plasticity is considered adaptive. Conversely, if the individuals with an induced phenotype underperform relative to non-induced individuals, then the plasticity is maladaptive. A final possibility is that both induced and non-induced individuals perform equally well (or poorly). This would be a case of non-adaptive (i.e. neutral) phenotypic plasticity.

2. We investigated the mode of phenotypic plasticity induced by four glyphosate-based herbicides in larvae of the spotted salamander, *Ambystoma maculatum* (Shaw, 1802), by determining whether the herbicides induced different morphologies, if morphology was correlated with escape swim performance, and how induced individuals performed relative to non-induced controls.

3. Different herbicide formulations led to production of significantly different head and tail morphologies, and tail morphology correlated with fastest escape speed. However, escape speed did not vary among treatments. In addition, three out of four herbicide treatments experienced accelerated growth rates, in terms of lateral size of tails, but the tail shapes were either similar to preliminary controls or intermediate between preliminary and final controls.

4. These observations suggest that herbicide-induced morphology is case of non-adaptive phenotypic plasticity, and that there is potentially a trade-off between growth and development for larvae exposed to different formulations.
5. Understanding the functional significance of induced phenotypes is important for determining their importance in shaping an organism's ecological interactions and evolutionary trajectories. Under more natural conditions, our observed changes in morphology may dramatically affect salamander fitness and play a role in either mitigating or accelerating population declines.

Keywords: amphibian, morphometrics, pesticide, plasticity, swim

Dedicated to my ever supporting family and friends

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I would like to thank the Honors College for its continual support the past few years, which has provided such a supporting environment. Additional thanks are given to both the FUSE Grant Committee and the Ogden Research Committee for their financial support.

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CHAPTER 1

PREFACE

By Dr. Michael Collyer, Department of Biology, Western Kentucky University

This Honors Thesis is the culmination of a profound collaborative experience for Mitchell Schooler. Not only did Mitchell experience the scientific method through primary research, from project development through completion, he also experienced how good science results from the synergy created from the collaborative efforts of researchers with diverse skills. Mitchell's collaborators included Dr. Jarrett Johnson, a herpetologist and population geneticist in the Department of Biology at Western Kentucky University; Nicholas Levis, a former Masters Student in Dr. Johnson's lab and now a PhD student at the University of North Carolina; and Dr. Michael Collyer, a vertebrate evolutionary ecologist and biostatistician in the Department of Biology at Western Kentucky University.

The research project in this thesis was original conceived by Mr. Levis and Dr. Johnson, inspired by the experimental research Mr. Levis performed for his Masters Degree. Mr. Levis approached Dr. Collyer about collaborating on the project in the Spring 2014, to contribute expertise in morphometry and data analysis. At this time, Mr. Schooler was developing skills in 2D and 3D morphometry in Dr. Collyer's lab. All collaborators developed a research plan that had two phases. Phase one involved Mr.

Levis collecting salamander eggs, rearing salamander larvae, and subjecting larvae to environments with different herbicides. Mr. Levis performed swimming trials with these larvae before contributing specimens to the Collyer lab for morphometric analyses as phase two. Mr. Schooler developed appropriate methods for quantifying body shape, especially using a sychroscopic technique with a new digital microscope. Mr. Schooler performed all imaging and digitizing of specimens, plus performed shape analyses along with Dr. Collyer. Dr. Collyer was responsible for advanced statistical analyses.

Collectively, these efforts resulted in a manuscript that was submitted to the journal, *Functional Ecology*, preceding defense of this thesis. This thesis includes that manuscript, which comprises the abstract and Chapter 3. The format of this thesis strays a bit from the traditional format. The abstract is presented in the format of the summary required for the journal, *Functional Ecology*. The Introduction, Materials and Methods, Results, Discussion, and References of the manuscript are subsections of Chapter 3 rather than separate chapters. Mr. Schooler has also contributed a Literature Review as Chapter 2 with relevant background to support the need for this research. The references for this Literature Review are also contained within the chapter rather than provided as a separate chapter. Finally, Supplemental Information pertinent to the manuscript in Chapter 3, with its references, is presented as Chapter 4. There has been no attempt to separate the collaborative spirit of this project from this thesis. The text of this thesis continually speaks from the perspective of "we" instead of "I". Nevertheless, this research is a result of Mr. Mitchell's persistent commitment and enthusiasm, and unlike most honors theses, his research was already submitted for publication prior to his graduation.

CHAPTER 2

LITERATURE REVIEW

The process of extinction is a natural event that occurs due to the system created by natural selection and evolution. There is typically considered a certain rate at which this process is meant to occur, however currently there are concerns that this rate is much greater than naturally would be expected. One major group that reflects recent concerns in conservation are amphibians that are, according to the International Union for Conservation of Nature (IUCN), seeing higher percentages of species being threatened than birds or mammals (Blaustein et al. 2011). This is very problematic as this major decline means not only a major loss of biodiversity in the world and all the benefits associated with biodiversity, but the rapid decline also means a loss of organisms that are widely regarded as important parts of ecosystems and monitors of environmental quality (Gibbons and Stangel 1999). Unfortunately, the cause of amphibian decline currently is not simply one problem, but a collection of issues that together effectively are hastening the extinction rates for amphibian species on a global scale. The issues and stressors that amphibian species in general are facing include climate change, contaminants, disease, competition, overexploitation, invasive species, predation, habitat destruction, and U/VB radiation (Blaustein et al. 2011). These stressors can affect amphibians on multiple levels including molecular, physiological, individual, population, and community, which independently or collectively lead to the major decline issue we have with amphibians.

Environmental contamination by pollutants is quite hazardous to many amphibian species and comes in many forms including insecticides, pesticides, heavy metals, and chemicals used to deice roads. A meta-analysis performed by Egea-Serrano et al. (2012) reviewed many studies related to this very subject and concluded that the overall effect of pollutants on amphibians globally to be moderately to largely negative. This backs up the earlier claims made that pollutants are one of the major factors contributing to amphibian decline and loss in biodiversity. The meta-analytical review also addressed that between all the studies examined, there was a 14.3% decrease in survival, a 7.5% decrease in mass, and a 535% increase in abnormality frequency seen in the amphibians studied in the reviewed studies (Egea-Serrano et al. 2012). These are staggering numbers in regards to how pollutants affect amphibians; however how they affect each individual amphibian species needs to be taken into consideration as well. There is a great variety of documented evidence on how different species react to different pollutants; some species suffer high mortality rates but others seem to be hardly affected by exposure (Bridges and Semlitsch 2000). Another study assessed how agricultural chemicals affected gold-striped salamander (*Chioglossa lusitanica*) embryos and found that exposure did not cause embryos to suffer from pollutant-related mortality or major sublethal effects (Ortiz-Santaliestra 2010). Contrasting results present major conservation implications. Research and conservation efforts need to focus on which species are especially susceptible to pollutants (Bridges and Semlitsch 2000).

Often when determining the cause of decline or extinction in a particular amphibian species the stressors that lead to decline or extinction tend not to be limited to just one stressor, with those suffering from mainly just one stressor being the exception to

the rule rather than being the general rule (Blaustein et al. 2011). The fact that decline in the majority of amphibian species' are due to multiple stressors makes it more difficult to fully understand and therefore limit the adverse effects of these stressors. One such example is an experiment that studied the effects that herbicide (atrazine) exposure had on streamside salamanders (*Ambystoma barbouri*) in conjunction with climate change. The results demonstrated that embryonic and larval exposure to the pesticide led to change in behavior and water retention as well as increased dehydration rates in postmetamorphic salamanders found in conditions similar to natural or anthropogenic climate variation (Rohr and Palmer 2004). The results represent the detrimental effect of synergistic forces or stressors that is causing mass decline rates. The synergistic effect created by multiple stressors means even small independent stressor effects can contribute to harmful impacts; therefore long-term sublethal effects of one stressor may have greater implications than originally considered indepdently in experimental settings (Rohr and Palmer 2004).

The concern of synergistic effects however are not limited to synergistic relationships between the major stressors leading to amphibian decline mentioned earlier, but even the effects of multiple aspects of a single stressor or factor. An example would be that of the synergistic effects of multiple pesticides (herbicides, insecticides, and/or fungicides) on amphibian species. One study looked at the aforementioned example and examined the consequences of amphibians being exposed to such combinations. The amphibians being studied were exposed to a mixture of four herbicides, two fungicides, and three insecticides and found that the amphibians not only suffered from larval growth and development retardation, but also neutralized or reversed the positive correlation

between time to metamorphosis and size at metamorphosis (Hayes et al. 2006). In other words, larvae exposed to the mixture who took longer to metamorphose were also smaller than those who metamorphose earlier. The most serious result of the amphibians being exposed to the nine pesticide mixture was damage to the thymus, which resulted in immunosuppression and contraction of flavo-bacterial meningitis (Hayes et al. 2006). Another researcher found that the mixing of ten pesticides had a dramatically greater effect on certain amphibian species than any of the pesticides did on their own. On their own, each pesticide showed only some direct or indirect effects with the greatest direct effect being 84% mortality in the leopard frogs being studied, but when exposed to the mixture there was 99% mortality in leopard frogs (Relyea 2008). Even though it is relatively unknown how the components of the mixtures worked together to be so potent, it is clear that the additive/synergistic effect of pesticides and the role of pesticides in amphibian decline should not be underestimated (Hayes et al. 2006). This thesis examines the effects of herbicides on amphibian species as they play a major factor in global amphibian decline by themselves and certainly in synergistic relationships with other major stressors related to amphibian decline.

Pesticides and herbicides (henceforth both referred to as pesticides, inclusively) are a major concern as they have a powerful impact on amphibians, as discussed in some earlier examples, are quite prevalent, and are rising in popularity in the agriculture industry. In a U.S. survey of streams and ground water, a large portion of streams contained pesticides with 97% of streams found in agricultural and urban areas containing pesticides, 94% of mixed streams contained pesticides, 61% of agricultural ground water contained pesticides, and 55% of urban groundwater contained pesticides

(Gilliom et al. 2001). The same survey also found that the potential for effect was widespread for aquatic wildlife that resides in these streams (Gilliom et al. 2001). Streams and other major bodies of water where amphibians can be found are susceptible to exposure to pesticides through runoff or by prevailing winds from nearby agricultural areas (Fellers 2004). Contamination is also quite common due to how common the practice of using pesticides in agricultural lands for agricultural use and disease prevention (Hua and Relyea 2012). Contamination may also occur due to the biphasic lifestyle of most amphibians which could lead to terrestrial exposure to pesticides (King and Wagner 2010). The problem is only supplemented by the fact that agricultural development is projected to increase in the upcoming decades (Hua and Relyea 2012). Another consideration when examining the effects of pesticides is that there appears to be a stratification of pesticides in bodies of water based on stratifications of temperature which could have important implications in consideration of habitat choice (Jones et al. 2010 B).

The effects of pesticides also vary drastically depending on factors like amphibian species exposed (as described earlier), type of pesticide, concentration of pesticide exposure, and potential concern with direct and indirect effects as well as lethal and sublethal effects of a particular pesticide or pesticides. As mentioned earlier exposure to pesticides can have powerful direct effects in terms of mortality on certain amphibian species, especially at higher concentrations where pesticides like Roundup® and Vision® when studied with larval anuran and larval salamander species found that high concentrations were moderately toxic to larval salamander species and moderately to highly toxic to larval anuran species (Relyea and Jones 2009). Pesticides sublethal effects

are also very dangerous to amphibian species however, due to both how common place low concentrations of pesticides are in numerous bodies of water and due to their effect when paired with other environmental stressors (Relyea and Edwards 2010). In studies of amphibian species, it has been determined that some individuals exposed to pesticides at certain life stages suffer from size deficiency and even developmental deformity which could have an indirect effect on mortality (Bridges 2000). For example, a bent tail deformity in a tadpole could cause a decrease in swim speed making it more susceptible to predators or deformity in limbs would most likely lead to less efficiency in terms of escaping predators, migrating, or foraging for food (Bridges 2000). Another study showed that when bullfrogs (*Rana catesbeiana*) were exposed to both pesticides and higher amounts of competition, that the pesticide was overall far more lethal to the bullfrogs (Jones et al. 2010 A). Pesticides have also been found to alter larval amphibian behavior and activity patterns. One such example demonstrated that tadpoles exposed to pesticides and under the co-occurring pressure of predation showed overall less activity and feeding; this could lead longer larval periods or smaller size at metamorphosis, both of which would harm the individual's fitness (Bridges 1999).

However, not all of the effects of pesticides on declining amphibian species are a direct effect on the species. For example, the effects of pesticides on aquatic systems found that pesticides caused a major decline in zooplankton numbers in the aquatic system. This led to a decline in mass of the larval salamander which has been shown to have a negative impact on adult salamander fitness (Hua and Relyea 2012). Pesticides may also be contributing towards amphibian decline not just through direct or indirect mortality, but also by affecting rate of recruitment. Certain pesticides have been noted to

negatively affect amphibian recruitment rate through sexual abnormalities, interfering with reproductive hormones, and through indirect effects on reproductive function (Hayes et al. 2010).

This present study will attempt to examine the effects of different Glyphosate-based herbicides (GBH) on a common larval species of salamander, the spotted salamander (*Ambystoma maculatum*). Since GBH is the most common form of pesticide used in the world, it is possible they are a leading cause in amphibian decline and therefore worth studying. A current hypothesis states that exposure to GBH causes retardation in "escape" swim speed which would make amphibians more susceptible to predation (Relyea and Edwards 2010). This study investigates whether there is a link between GBH exposure, morphology, and escapes speed to determine if the hypothesis is valid.

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CHAPTER 3

NON-ADAPTIVE PHENOTYPIC PLASTICITY: MORPHOLOGY, BUT NOT SWIM SPEED, OF SPOTTED SALAMANDER LARVAE IS AFFECTED BY "TERRESTRIAL" AND "AQUATIC" HERBICIDES

Introduction

Phenotypic plasticity—the capacity of a single genotype to exhibit a range of phenotypes—is advantageous in heterogeneous environments where selection favors different phenotypes depending on the conditions (Gilbert and Epel 2009) and provides a mechanism by which organisms can cope with environmental variability (Whitman and Agrawal 2009). Through plasticity, individuals are able to improve their fitness by altering their phenotype to match environmental conditions. In addition, by promoting population persistence, plasticity can act as an evolutionary "bridge" that buys time for genetically encoded adaptations to accumulate (Baldwin 1896; Waddington 1953; Reale et al 2003; Yeh and Price 2004). Specifically, those individuals that can facultatively adjust their behavior, physiology, or morphology in response to stressful conditions, are more likely to survive than those that cannot.

If there is variation in the form and degree of plasticity to a novel stimulus, then selection can act on this variation to stabilize or refine the optimal phenotype (West-Eberhard 2003). Over time, a phenotype that was initially only stress-induced might become genetically controlled such that it is constitutively expressed (i.e. canalized) as

demonstrated in a classic study by Waddington using Drosophila melanogaster (1953,1959). After several generations of natural selection through exposure to elevated levels of sodium chloride in their larval growth medium, he found that these flies had constitutively smaller anal papillae (Te Velde et al. 1988) and greater survival than non-selected controls (Waddington 1959). Thus, the adaptive, induced reduction of anal papillae in high osmotic conditions became developmentally canalized and is said to have undergone genetic assimilation (Waddington 1953, 1959). In addition, a more recent study has shown that plasticity can mediate the development of a stress-induced polyphenism in the tobacco hornworm (Manduca sexta; Suzuki and Nijhout 2006). Therefore, plasticity has the potential to significantly affect evolution and population persistence in the face of environmental stress.

Pond-breeding amphibians exhibit a wide variety of plastic responses in order to overcome an array of environmental stressors (Relyea 2001; Relyea 2002; Pfennig 1990; Whiteman 1994; Touchon 2014). For example, larval amphibians can increase their developmental rate in response to pond drying, which allows them to metamorphose and move to a terrestrial habitat instead of succumbing to desiccation (Semlitsch and Wilbur 1988; Gomez-Mestre et al. 2013). In addition, they may adopt an alternate feeding morphology to escape competition for shared resources (Pfennig and Murphy 2000, 2002, 2003). Thus, it appears that phenotypic plasticity plays an important role in helping amphibian populations persist in environments that vary with respect to both biotic and abiotic factors.

Glyphosate-based herbicides (GBHs) are the most widely applied herbicide in the world (Jones et al. 2011) and their use has increased 10-fold in the last 20 years (USGS

2011). Glyphosate is a synthetic compound developed in the 1970s by the biotechnology corporation Monsanto and marketed as an herbicide—glyphosate disrupts the plant-specific enzyme EPSP synthase and kills plants by preventing aromatic amino acid synthesis—under the name "Roundup". The use of GBHs continues to increase, as Monsanto has genetically engineered crop plants that are resistant to glyphosate, to accommodate large-scale application of Roundup to agricultural fields to control weeds while leaving crop plants unaffected. Additionally, the patent on glyphosate expired in 2000, leading to development of many generic versions of the product. All of the new formulations use glyphosate as the active ingredient, but the other ingredients (the adjuvants) vary. It is important to understand the effects of these new formulations of herbicide on amphibian populations because of the potential for toxic adjuvants. Indeed, various lab, mesocosm, and natural studies have found that GBHs negatively affect amphibians and aquatic systems (Baylis 2000; Chen et al 2004; Edginton et al 2004; Howe et al 2004; Wojtaszek et al 2004; Relyea et al 2005; Bernal et al 2009; Jones et al 2011).

Glyphosate-based herbicides can be placed into two broad categories—terrestrial and aquatic depending on the presence or absence of surfactant compounds aimed at helping the glyphosate active ingredient to "stick" to the plant long enough to be absorbed.. Terrestrial GBHs are formulations containing a surfactant (often polyethoxylated tallowamine [POEA]) that are typically restricted to terrestrial use—the most common location for application (USGS 2011). POEA has been found to negatively affect aquatic systems (Mann and Bidwell 2001; Tsui and Chu 2003; Howe et al 2004; Brausch et al 2007; Relyea and Jones 2009). In contrast, aquatic GBHs lack a surfactant

that may reduce potential toxicity to non-target organisms, and are supposed to be safe for aquatic systems if POEA is not added (Giesy 2000). Nevertheless, amphibians may be exposed to either of these herbicide classes through aerial drift or runoff. Sometimes exposure leads to direct mortality, but more often sublethal effects, such as altered physiology, morphology, or food web interactions, occur (Paganelli et al. 2010; Relyea et al. 2005; Chen et al. 2004; Cauble and Wagner 2005; Ortiz-Santaliestra et al. 2011).

Often, larval amphibians exhibit morphological changes in response to predator chemical cues, the presence of competitors, or different food sources (Relyea 2001; Relyea 2002; Pfennig 1990, 1992). In these cases, the induced morphology is adaptive and allows an individual to gain some fitness advantage it would not have without the induced morphology. For example, some tadpoles exhibit a larger and/or brighter tail when exposed to predator chemical cues (McCollum and Van Buskirk 1996; Fitzpatrick et al. 2003; Teplitsky et al. 2005). These morphological changes improve escape swimming performance and provide a non-vital target for predators to attack. That is, the morphological responses to predator cues are adaptive because they confer a fitness advantage when predators are present.

Recently, GBHs have been shown to induce morphological changes in larval amphibians that can resemble the predator-induced morphology (Relyea 2012; Levis and Johnson in press). Unlike predator-induced plasticity that improves swimming speeds and presumably enhances survival, GBH-induced morphology changes may be a maladaptive side effect of exposure to the herbicides (Ghalambor 2007). Indeed, in the absence of the normal inducing stimulus (i.e. predators), costs of plasticity (such as reduced growth and/or slower development) may cause an induced phenotype to be maladaptive (Relyea

2002; Auld et al. 2010; McCollum and Van Buskirk 1996; DeWitt 1998; DeWitt et al. 1998; Murren et al. 2015; but see Auld et al. 2009; Van Buskirk and Steiner 2009). Thus, three alternatives exist for an induced phenotype: 1) it is adaptive, 2) it is maladaptive, or 3) it is non-adaptive (i.e. neutral). If an induced phenotype is adaptive, then induced individuals should perform better than non-induced ones. If an induced phenotype is maladaptive, then non-induced individuals should outperform induced individuals because induced individuals have moved away from their phenotypic optimum (Ghalambor et al 2007). Lastly, if an induced phenotype is simply non-adaptive, then there would be no difference in performance between induced and non-induced individuals.

We evaluated these predictions for adaptive versus maladaptive plasticity by measuring larval spotted salamander (Ambystoma maculatum) morphology and swim speed in response to name-brand and generic terrestrial and aquatic GBH formulations. We sought to answer three questions: 1) Does exposure to different formulations result in different salamander morphologies? 2) Do morphological changes due to GBH exposure translate to differences in functional swimming performance? 3) Do non-induced individuals outperform their induced counterparts? We predicted that terrestrial formulations of herbicide would induce the greatest morphological changes and result in the greatest reduction in swim speed because they contain surfactant that negatively affects amphibians.

Materials and Methods

Animal collection

Four egg masses of *A. maculatum* were collected from a pond in Warren County, KY (Lat: 36.87N, Long: -86.25 W) on 16 April 2014. Egg masses were held separately in plastic containers with 5L of a 1:1 ratio of dechlorinated/deaminated tap water and natal pond water until hatching. After 14 days post-hatching, five individuals from each family were haphazardly assigned to one of five treatments (described below).

Experimental design

Our five treatments included two "aquatic" herbicides, two "terrestrial" herbicides, and dechlorinated/deaminated water as a control. For each herbicide class, one formulation was the Monsanto name brand, and the other was a generic formulation. The specific herbicides were AquaMaster (Monsanto), AquaNeat (Nufarm), Roundup Pro Concentrate (Monsanto), and Helosate Plus Advanced (HELM). The key differences among these herbicides is that the terrestrial formulations each contain a proprietary surfactant, the aquatic herbicides do not, and the name brand and generic formulations potentially contain different amounts and compositions of other "inactive", proprietary ingredients (Table 3.1). Approximately five larvae from each egg mass family were placed individually into 500 mL glass jars containing 200 mL of 3 mg a.e./L of each herbicide. This concentration is within the range of actual worse case scenarios seen in nature (Edwards et al 1980), and does not lead to significant mortality in this species (Relyea and Jones 2009). Larvae were fed a 2 mL aliquot of highly concentrated brine shrimp after placement into experimental jars. Because herbicides break down over time, 5 L of

3mg a.e./L of each herbicide was prepared and stored until jar water needed to be replaced (because of fouling due to brine shrimp carcasses and larvae excretion). Therefore, the replacement water should have been at a similar concentration to the experimental water and not a "fresh", higher-concentrated dose. Water was changed in all jars halfway through the experiment (7 days) and a 2ml aliquot of highly concentrated brine shrimp was again added. No larvae died during the experimental procedure.

Table 3.1. Description of the herbicides used in this study. Both of the terrestrial herbicides contained a proprietary surfactant, and the aquatic herbicides lack a surfactant. Herbicide concentrations used in this experiment were standardized to 3 mg acid equivalent/L of glyphosate. They had different amounts of "other" ingredients. Percent ingredient information came directly from the manufacturer's label.

Herbicide	Type	Surfactant	Percent active ingredient	Percent "other" ingredients
Roundup Pro Concentrate	T	proprietary	50.2	49.8 (13% surfactant)
Helosate Plus Advanced	T	proprietary	41.0	59.0
AquaMaster	A	none	53.8	46.2
AquaNeat	A	none	53.8	46.2

T = terrestrial; A = aquatic

Swim tests

Swim tests were performed by placing an individual in a clear plastic container containing 5 L of dechlorinated tap water on top of a grid and filming from above with a Nikon D700 camera at 29 frames per second. After acclimation to the container for two minutes, the larva was gently prodded with a blunt wire perpendicular to the abdomen. Each individual was tested three times, but all larvae completed their first trial before any individual's second was run. Likewise, all larvae completed their second trial before any individual was tested for a third time. Videos were analyzed using the free, open-source

Kinovea (Kinovea.org) software that allows for placement of markers and timers on a slow motion video. We determined speed as the time it took the larvae to swim three body lengths away from the point of origin because it is exceeds the distance of danger by a sit-and-wait predator (Van Buskirk and McCollum 2000). Each family had five individuals measured before exposure to any treatment (Initial), and after two weeks of exposure to each treatment (described above). After completion of all swim trials, larvae were euthanized in 0.2% MS-222, fixed in 10% formalin, and stored in 70% ethanol until morphology was analyzed.

Morphology measurements

We photographed every viable specimen (the tails of four larvae were damaged via preservation) with a Nikon Shuttlepix digital microscope mounted on a motorized stand, such that each specimen was photographed with the same field depth. We used a photo-stacking technique that merged digital images taken at equal height intervals over a range of several millimeters, ensuring visual focus despite the three dimensional surface portrayed in the images. The right lateral surface of each specimen was photographed this way. Linear measurements were made for the length of the visual field, which allowed metric units to be applied to morphometric measurements summarizing size. We used landmark-based geometric morphometrics (GM) to quantify attributes of body shape (Adams, Rohlf & Slice 2013), based on anatomical landmarks digitized on resulting photographs. The primary GM method we used was generalized Procrustes analysis (GPA) (Rohlf & Slice 1990), which describes organismal shape via the residual spatial positions of "homologous" landmarks in configurations that have been rendered invariant

in size, orientation, and position via generalized least squares superimposition. These Procrustes residuals can be projected into a Euclidean space tangent to the shape space that contains them, and used as shape variables for various statistical analyses that rely on linear models.

We were able to digitize photos from 114 of the original 118 larval salamanders used in the experiment, comprised of 18 specimens from the initial control treatment (CI); 19 specimens from the final control treatment (CF); 20 specimens each from the aquatic generic and Monsanto treatments (AG and AM, respectively); and 18 and 19 specimens from the generic and Monsanto terrestrial treatments (TG and TM, respectively). For each specimen, we digitized two configurations: one represented only tail shape and one representing only head shape. For tail shape we used 6 landmarks and 58 semi-landmarks (sliding landmarks) to quantify tail shape; for head shape we used 1 fixed and 25 semi-landmarks (Fig. 3.1). Whereas landmarks are fixed in position, representing the Cartesian coordinates of discrete anatomical features, semi-landmarks are used to estimate curves and are free to "slide" along tangency vectors during GPA, such that homologous curves or surfaces can be quantified by the resulting Cartesian points (Bookstein 1997; Gunz & Mitteroecker 2013). We used the method of minimized Procrustes distances among specimens, where Procrustes distance is the square root of summed squared distances between corresponding landmarks. Procrustes distance calculated with resulting Procrustes residuals is also commonly used as a metric of shape difference between specimens.

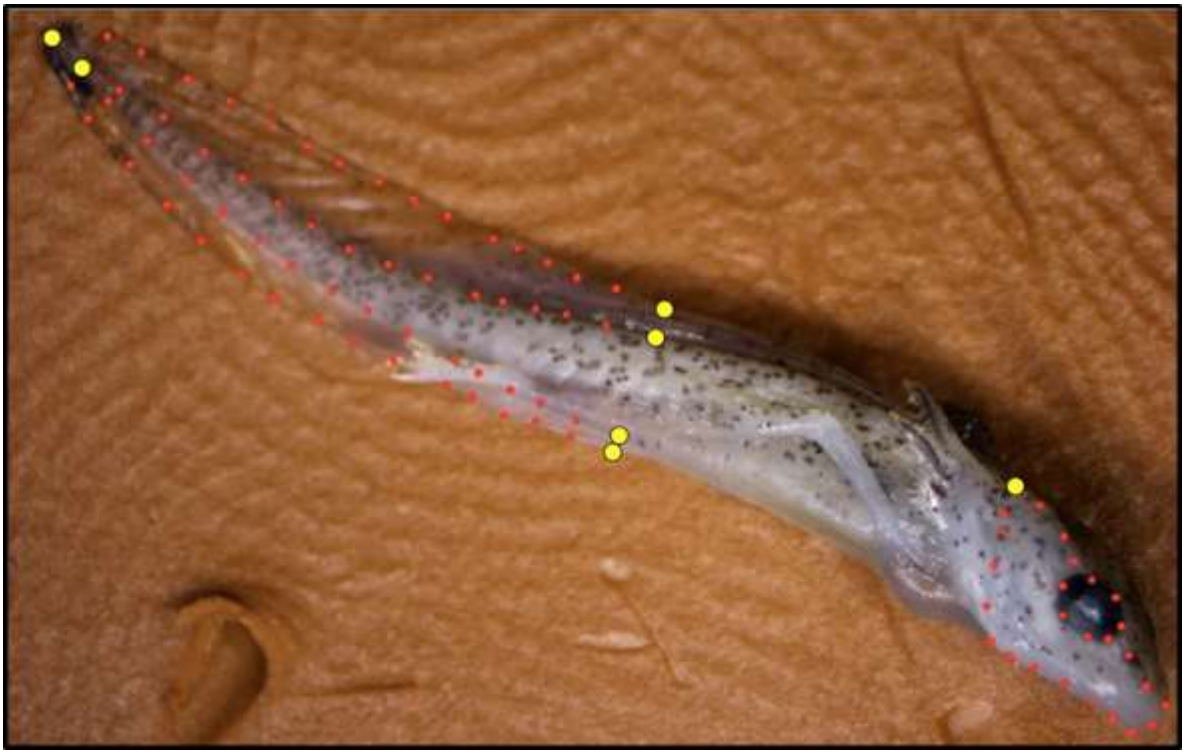

Figure 3.1. Anatomical landmarks used in this study. Yellow points are fixed landmarks; red points are semilandmarks.

Resulting Procrustes residuals were used as shape variables in subsequent statistical analyses. Digitization of landmarks on specimens was performed with the software, tpsDig2 (Rohlf 2014). GPA, was performed with the package geomorph, version 2.1.3 within R, version 3.1.3 (R Core Team 2015).

Statistical analysis

At the individual level, correlations between shape, size, and swim speed were performed with two-block partial least squares (PLS) analyses. PLS is a matrix association (correlation) test that performs a singular-value decomposition (SVD) on the cross-covarianaces between variables of two matrices. A Pearson product-moment correlation

coefficient is calculated between scores of values projected on the "left" and "right" singular vectors obtained via SVD. The correlation coefficient is recalculated many times after randomizing the vectors of values within one of the matrices, to create random cross-covariances. The percentile of the observed correlation is used as an estimated *P*-value for inferential tests. When matrices are comprised of single variables, the correlation coefficient is the same as a univariate Pearson product-moment coefficient, and the test is a randomization test rather than a test that relies on parametric distributions, such as the *t*-distribution, with associated stringent assumptions. We performed PLS on all logical associations between shape and size, swim speed and size, and swim speed and shape. We considered swim speed as the matrix of all three swim trials, or univariate responses of maximum speed or mean speed. Head and tail sizes were measured as the centroid sizes of their landmark configurations. Centroid size (CS) is calculated as the square root of summed squared distances of landmarks from their center of gravity (centroid), based on the configurations of landmarks that defined their shape, prior to GPA. CS values were log-transformed prior to analysis. PLS performed on head shape and tail shape is a test of their morphological integration (Bookstein *et al.* 2003).

We subsequently performed several analyses using a non-parametric (np) method of (multivariate) analysis of variance (ANOVA) for high-dimensional data (Collyer, Sekora & Adams 2015). High dimensional data are data comprised of variables that exceed the number of subjects analyzed. The np-ANOVA uses traces of sum of squares and cross-products matrices to calculate sums of squares (*SS*) and evaluate model effect sizes via a randomized residual permutation procedure (RRPP). These statistics are not

dependent on degrees of freedom, and it has been shown that using more landmarks rather than less can increase effect sizes and result in better resolution to detect subtle effects (Collyer, Sekora & Adams 2015). As such, we were able to analyze treatment effects for the different representations of shape, size, and swim speed described above, with the same analytical method. Initially, mixed linear models that included family effects, plus family nested in treatment effects, were used to determine if family effects were significant or varied with treatments. The results of preliminary tests are provided in the supplemental information but the following two conclusions were pervasive: 1) although there were significant family effects in our analyses, the effects sizes for interactions between family and specimen size, or between family and treatment were exceedingly small as to be inconsequential; and 2) although there was significant allometric scaling in our analyses – where shape allometry is the covariation of shape and size – any interaction between specimen size and a model factor (treatment, family) was exceedingly small as to be inconsequential. We therefore removed interactions from the linear models, retained size as a covariate, and accounted for family as a "random" effect by adjusting Procrustes residuals, as

$$\mathbf{y}'_j = \hat{\boldsymbol{\mu}} + \mathbf{y}_{ij} - \bar{\mathbf{y}}_i,$$

where $\mathbf{y}_{ij}$ is the vector of Procrustes residuals for the *j*th individual from family *i*, , $\bar{\mathbf{y}}_i$ is the vector of Procrustes residuals for the mean of family *i*, and $\hat{\boldsymbol{\mu}}$ is the overall mean. Thus, $\mathbf{y}\text{¢}_j$ is the vector of Procrustes residuals independent of the effect of family. Subsequent analyses used these Procrustes residuals as shape variables, treatment as a fixed effect, and the log of specimen CS as a covariate.

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We performed np-ANOVA with RRPP for 1,000 random permutations (including observed cases). In each test, the standard deviate of observed *SS* for model effects (Z-score) from the empirical sampling distributions of random *SS* was calculated as a measure of effect size (Collyer, Sekora & Adams 2015), which facilitated comparisons within and across analyses. An additional benefit of the np-ANOVA procedure is that appropriate pairwise comparisons between treatments could be performed simultaneously with the same random permutations used to analyze model effects. We performed all pairwise comparisons of least squares means among treatments in each np-ANOVA. The test statistic in each case was the Procrustes distance between treatment levels. Because this procedure is a simultaneous test of multiple tests statistics rather than multiple post-hoc tests, we did not adjust the family-wise acceptable type I error rate of $\alpha = 0.05$ for multiple comparisons. All statistical analyses were performed with the package geomorph, version 2.1.3 (Adams, Collyer & Sherratt 2015) within R, version 3.1.3 (R Core Team 2015)

Visualization of shape variation in the space tangent to shape space – henceforth, the morphospace – was made possible via a principal component analysis (performed on the covariance matrix estimated from allometry-free Procrustes residuals) and projection of Procrustes residuals onto the principal components. Shape allometry was held constant by first regressing Procrustes residuals against the log of specimen size and adding residuals from this regression to the consensus (overall mean) configuration, as was done with family effects previously. This procedure is analogous to finding least squares means in analyses of covariance, and was also justified by an indication that

shape allometries were consistent among treatments (see supplemental information and above).

In addition to visualizing shape variation among specimens using allometry-free Procrustes residuals, a thin-plate spline (TPS) (Bookstein 1991) function was used to visualize how body shape changed across the morphospace. TPS maps a reference configuration (in our case, the consensus configuration) onto a "target" configuration and measures the deformation of the transformation between the two forms. This transformation can be shown via a "transformation grid", which visually displays the deformation of the consensus configuration at different locations in the morphospace, providing a mechanistic interpretation of shape change among specimens.

Results

Head size and tail size were significantly (positively) correlated, head shape and tail shape were significantly correlated, and each shape was correlated with size calculated from the same configuration (i.e., significant shape allometry). However, swim speed was not significantly correlated with any morphological attribute with the lone exception of tail shape and fastest speed (Table 3.2). Transformation grids associated with PLS shape scores suggested a greater propensity for deeper finned tails, especially at the posterior of the tail, associated with faster swim speed (Figure 3.2). Even in this case, the correlation was weak and swim speeds were quite variable for deeper finned tails (Table 3.2). Because using each swim trial speed or the mean speed of the three trials showed no significant correlation with any morphological attribute, and only if we focused on the fastest speed observed did we find a weak but significant correlation, this result suggests that burst (escape) speed might be associated with tail shape.

The significant correlation between head shape and tail shape suggests that these morphological attributes were "integrated". The pattern of morphological integration indicated elongation of snouts associated with tapering of the posterior tail. These patterns were largely consistent with allometric trends, suggesting that morphology is integrated through development. Despite this integration, there was much variation in either shape with respect to the other shape, and treatment differences in head shape and tail shape were not completely consistent (see below); therefore, we chose to keep head shape and tail shape separate for analyses of inter-treatment variation in subsequent tests.

Table 3.2. Two-block partial least squares correlations for relevant comparisons.

Trait 1	Trait 2	*r*	*P*
Head shape	Tail shape	0.398	0.005
$\log(CS_{head})$	Head shape	0.530	0.001
$\log(CS_{tail})$	Tail shape	0.516	0.001
$\log(CS_{head})$	$\log(CS_{tail})$	0.438	0.001
All speeds	Tail shape	0.290	0.107
Mean speed	Tail shape	0.254	0.226
Fastest speed	Tail shape	0.318	0.031
All speeds	Head shape	0.236	0.428
Mean speed	Head shape	0.220	0.489
Fastest speed	Head shape	0.206	0.636
All speeds	$\log(CS_{tail})$	0.097	0.685
Mean speed	$\log(CS_{tail})$	0.079	0.212
Fastest speed	$\log(CS_{tail})$	0.061	0.273
All speeds	$\log(CS_{head})$	0.145	0.373
Mean speed	$\log(CS_{head})$	0.119	0.104
Fastest speed	$\log(CS_{head})$	0.087	0.183

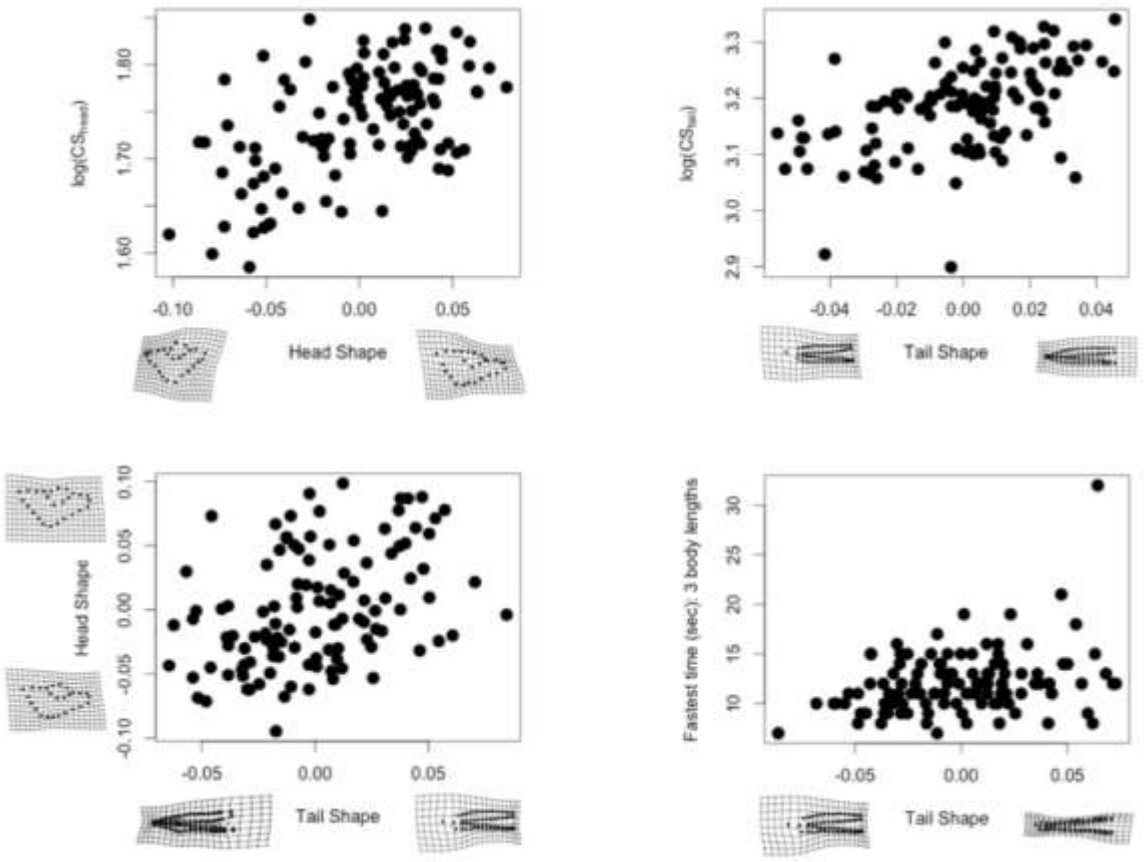

Figure 3.2. Two-block partial least squares (2B PLS) projections of shape values for correlation analyses using shape. Transformation grids emphasize extremes along the shape axis. Greater change in shape indicates greater association between shape and the alternative variable.

Shape variation among treatments was significant for each configuration and effects sizes were similar (Table 3.3). Pairwise Procrustes distances in head shape were nearly significant in each case, except the contrast between AG and AM, and the contrast between CF and TM. This pattern was similar for tail shape, but Procrustes distances between CI and both aquatic treatments, and between AM and TM were also not significant (Table 3.4). When viewed in terms of the contrast between CI and CF treatments – representing an expected shape change in the absence of GBH – no treatment diverged morphologically from pre-treatment conditions as much as CF, for either head shape or tail shape (Fig. 3.3). For head shape, the TG mean did not diverge significantly from the CI mean; all other treatment means diverged significantly in the same general direction as the CM mean, but not to the same extent. As such, the first PC (42.8% of overall variation) largely reflected a divergence axis associated with CI-CF shape differences, which was principally indicative of snout elongation. For tail shape, the pattern of shape change was more complex. The AM and AG treatment means did not diverge significantly from the CI mean. The TM treatment mean diverged in a direction consistent with the CF treatment, but not to the same extent; the TG mean diverges in a direction nearly opposite the CF mean along the first PC (38.0% of the overall variation explained). The first PC was again largely aligned with the shape change between CI and CF, and indicated tapering of the posterior tail (loss of tail fins) for the CF treatment. All other treatments either lost tail fins at a slower rate (TM), retained deep-finned tails (AM and AG), or developed deeper fined tails (TG). Tail shape variation associated with the 2nd and 3rd PCs appeared to indicate more so heterogeneity in the relative depth of dorsal and ventral fins.

Table 3.3 Non-parametric (np) analysis of variance (ANOVA) statistics for inter-treatment variation. Because variables significantly covaried with specimen size, effects are also presented for the log of centroid size (*CS*), unless the response variable is a measurement of size, itself. Effect sizes (*Z*-scores) indicate the size of the effect as a standard deviate of the sums of squares (*SS*) from its sampling distribution.

	log(*CS*)*				Treatment			
	SS	R^2	*Z*	*P*	*SS*	R^2	*Z*	*P*
Head shape	0.052	0.094	8.374	0.001	0.108	0.195	4.661	0.001
Tail shape	0.015	0.042	3.918	0.004	0.076	0.215	4.831	0.001
Head size	--	--	--	--	0.161	0.242	4.576	0.001
Tail size	--	--	--	--	0.142	0.271	5.382	0.001
All speeds	74.100	0.009	0.649	0.337	1116.000	0.131	2.723	0.002
Mean speed	15.910	0.008	0.599	0.314	330.500	0.170	3.334	0.002
Fastest speed	7.900	0.007	0.424	0.393	220.030	0.189	3.686	0.001

* *CS* of tail shape used for swim trial analyses; otherwise *CS* matched configuration used to estimate shape

Table 3.4. Pairwise Procrustes distances in shape. Distances for head shape are above the diagonal and distances for tail shape are below. Distances that are significantly greater than 0 ($P < 0.05$) are bolded.

	AG	AM	CF	CI	TG	TM
AG		0.030	**0.042**	**0.041**	**0.044**	**0.039**
AM	0.024		**0.040**	**0.046**	**0.049**	**0.041**
CF	**0.049**	**0.043**		**0.072**	**0.073**	0.024
CI	0.028	0.021	**0.052**		0.028	**0.068**
TG	**0.045**	**0.041**	**0.072**	**0.032**		**0.069**
TM	**0.036**	0.024	0.026	**0.034**	**0.051**	

Whereas the GBH treatments appeared to retard morphological development in terms of head and tail shapes, growth in head size was largely consistent with the control for all treatments, and growth in tail size generally exceeded the control for GBH treatments (Fig. 3.4). All GBH treatments except the TG treatment – which had the largest and deepest posterior tailfin – grew significantly larger tail sizes (measured as the log of centroid size) than the CF treatment, which was also not significantly larger than the CI treatment mean. The TG treatment mean size was intermediate between CF and all other GBH means, and significantly larger than the CI mean. Taken with the shape results, the general trend was that GBH treatments (1) retarded snout elongation but had no affect on the increase in head size and (2) increase the size of the tail while maintaining a deep profile. As deeper tails were associated with fastest swim speed, it would appear that GBH herbicides induce morphologies that increase burst speeds. Such results were not found for inter-treatment comparisons. Regardless of variables used for swim speed, significant inter-treatment variation was found, but only because the CI treatment was faster than the others. Removal of the CI treatment rendered non-significant variation in each case (results not shown; but see Fig. 3.4). Our results could misinform actual swim speeds, as we measured the amount of time to travel three body lengths. Larger salamanders would have to swim farther in the same amount of time to produce the same speeds as smaller salamanders. However, because the tail size was used as a covariate in the analysis of swim speed, a spurious result seems unlikely unless the experiment did not adequately measure burst speed.

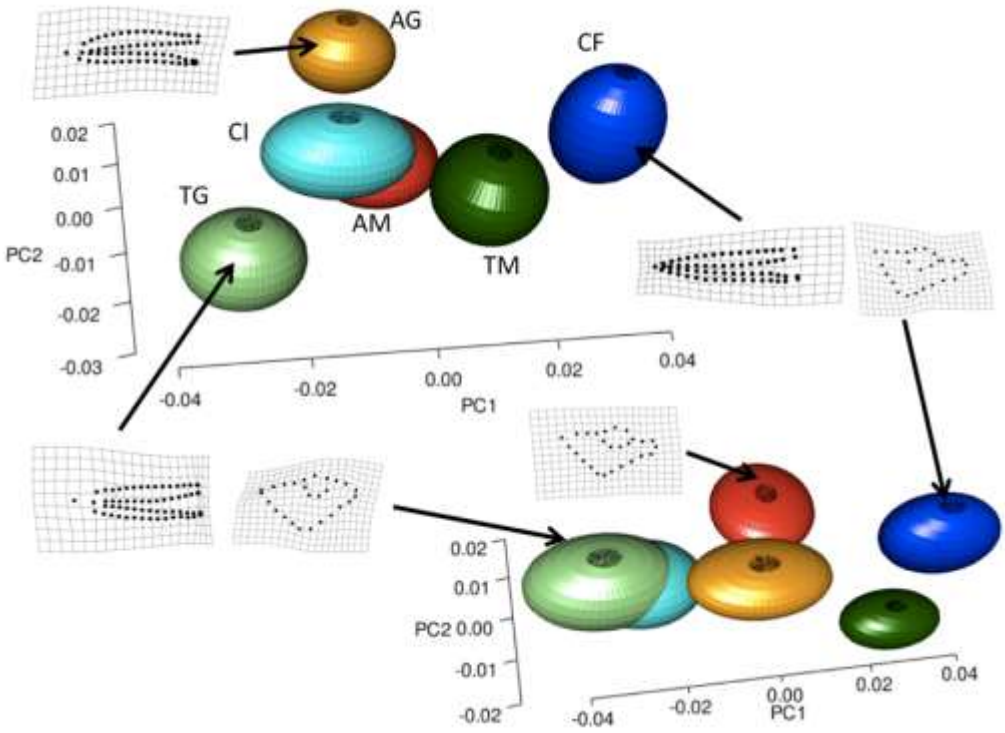

Figure 3.3. Principal component (PC) plots of shape variation. Plots are shown for the first three PCs are shown accounting for 63.2% and 73.2% of the overall shape variation in all dimensions for head shape and tail shape, respectively. Each treatment is represented by a 95% confidence ellipsoid. (Non-overlapping ellipsoids are generally but not necessarily significantly different, as not all dimensions are shown.) Treatments are labeled in the tail shape plot and colors correspond between the two plots. Transformation grids (scaled 2x) are shown to help visualize shape change. (These grids were estimated using all dimensions of shape).

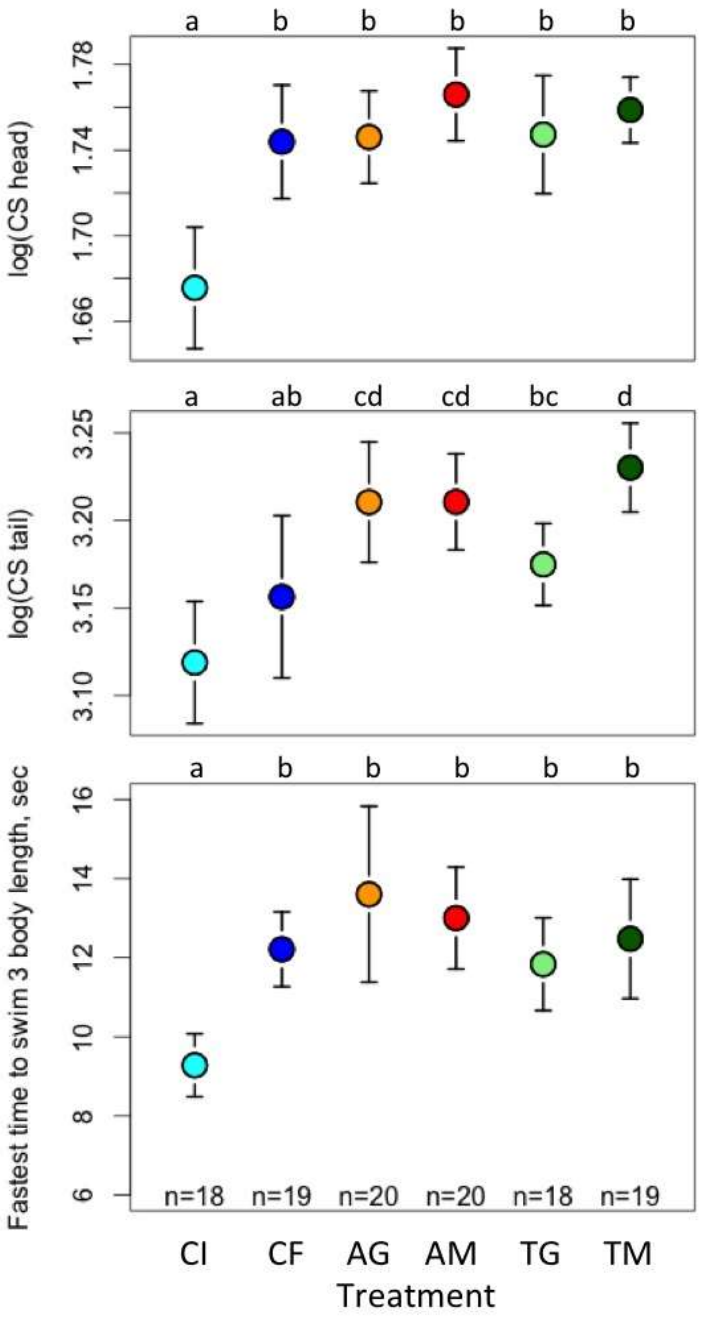

Figure 3.4. Treatment means and 95% confidence intervals for head size, tail size, and fastest swim speed. Colors match ellipsoid color in Fig. 3.3. Sample sizes are shown at the bottom of all three plots. Letters above plots correspond to results of pairwise tests. Letters above means that are shared mean that the treatment means are not significantly different ($P > 0.05$).

Discussion

Amphibians are able to cope with environmental novelty (e.g. stress) through expression of phenotypic plasticity. To evaluate whether glyphosate-based herbicides (GBHs) induce adaptive, maladaptive, or non-adaptive plasticity in spotted salamander larvae, we sought to answer three questions: 1) Does exposure to different formulations result in different salamander morphologies? 2) Do morphological changes due to GBH exposure translate to differences in functional swimming performance? 3) Do non-induced individuals outperform their induced counterparts? Our results indicate that both head and tail morphology varied among treatments and tail morphology was significantly correlated with fastest escape swim speed (Table 3.2; Fig. 3.3), but there was no difference in swim speed among treatments. Therefore, our results suggest that herbicide-induced morphology changes in spotted salamanders is a case of non-adaptive, rather than adaptive or maladaptive, plasticity in regard to swim speed. However, the observed plasticity may represent a trade-off between growth and development.

Evidence for such a trade-off was revealed by the contrasting results between analyses of size and shape. In terms of head shape and tail shape, the CF treatment had the most divergent mean shapes that also tended more so toward the typical head and tail shapes of terrestrial adult salamanders (similar to the expected changes preceding metamorphosis). GBH-treated salamanders had head shapes that remained like initial larval head shapes or were intermediate between CI and CF head shapes, but were the same size as the final untreated salamanders. These results suggest an arrest or slowing of morphological developmental change but a continuation of growth. Accelerated growth rates, in terms of lateral size of tails, were observed for salamanders in three of

the four GBH treatments, but tail shapes for these three treatments (AG, AM, and TM) were either similar to CI tail shape (AM or AG) or intermediate between CI and CF tail shapes (TM). The tail shape of salamanders in the TG treatment is a bit difficult to reconcile. One hand the little changed or intermediate tails shapes of salamanders in the other three GBH treatments with larger tail sizes could suggest either a slowing of the developmental process or its cessation early in the experiment, followed by recovery after growing larger tails. However, the divergent tail shape of the TG salamanders to have a deeper-finned tail, especially in the posterior of the tail, and an intermediate tail size that was more consistent with the tail size of salamanders in the CF treatment, suggests that larval salamanders can either grow larger tails or change the shape of their tails when confronted with herbicides. This result also suggests that different GBHs might induce different size-shape trade-offs. Future studies aimed at collecting detailed longitudinal data of larvae in GBH treatments that also vary the concentration of herbicides might elucidate more precisely whether developmental trade-offs are pulsed or continuous during development.

Despite the significant differences in morphology among treatments, swim speed did not seem to be compromised. Consistent with previous studies (Landberg & Azizi 2010), fastest swim speeds correlated with deeper tail fins (i.e. tail area). However, all treatments, regardless of morphology, had similar swim speeds (excluding CI). This suggests that the plasticity we observed had little functional significance for swim speed and/or no costs associated with morphology change. Indeed, recent investigations suggest that the costs of plasticity are low or non-existent (Auld et al. 2009), but the apparent lack of costs in our study may not hold under more realistic conditions. Other environmental

factors, such as predators, may influence the adaptive value of the morphologies/speed we observed. Evidence from tadpoles of *Rana lessonae* suggests that different morphologies may be favored in different environments (Wilson, Kraft & Van Damme 2005). Low tails and narrow heads (similar to our CI treatment), were considered good swimmers and were induced by a "chase" predator, Pumpkinseed Sunfish (Lepomis gibossus). Conversely, an ambush predator, dragonfly larvae (Aeshna cyanea), induced high tails and wide heads, and tadpoles with this morphology were typically "bad swimmers". It could be that burst speed combined with maneuverability is important for avoiding ambush predators; thus, morphology (tail especially) might be a better indicator than the swim trials if the latter do not simulate predator avoidance well. If a salamander larva was attacked by an ambush predator, such as a dragonfly larva, it is reasonable to imagine that it would turn away from the predator while simultaneously accelerating. In fact, this is the typical escape response of this species (Landberg & Azizi 2010). Future studies should investigate the relationship between tail morphology, maneuverability, predator avoidance.

The type of growth/development trade-off we witnessed is not uncommon (Werner 1986; Miaud, Guyetant & Faber 2000; Morrison & Hero 2003). In addition, previous studies have found that exposure to GBHs can increase body size in salamanders (Ortiz-Santaliestra et al. 2011; Levis & Johnson 2015) and the exact mechanism remains unknown, but may involve disruption of the hypothalamic-pituitary-thyroid axis because of its role in development and metamorphosis (Fort et al. 2007). Interestingly, the terrestrial generic group experienced reduced growth and divergent tail morphology as well. However, the tail morphology of this group was on the other end of

the spectrum from the final control group (Fig. 3). In this case, it appears that this herbicide may have deleterious effects on both growth and morphology/development. In the present study, we did not observe any major side effects of these differences in growth and development, but in nature, they may have significant long-term effects. For example, whereas the larger, less developed individuals may have a reduced likelihood of predation by gape-limited predators, the smaller, more developed larvae may be more likely to escape a rapidly drying pond.

Understanding the role of this morphological plasticity is important because amphibians are experiencing global declines (Houlahan et al. 2000; Stuart et al. 2004) and maladaptive plasticity can lead to population extinction (Ghalambor et al. 2007; Morris & Rogers 2013; Morris et al. 2014). Fortunately, we found no evidence of maladaptive plasticity in this study in regard to swim speed. In addition, recent studies that have looked for an interaction between GBH exposure and predator cues have found either no effect (Burraco, Duarte & Gomez-Mestre 2013) or a beneficial effect (Relyea 2012; but see Relyea 2005). Furthermore, GBHs do not seem to have negative effects on amphibian survival under natural pond conditions (Edge et al. 2012, 2013); other environmental factors (e.g. UV) can mitigate the negative effects of GBHs (Levis & Johnson 2015); amphibians are able to adapt to contaminant exposure (Hua, Morehouse & Relyea 2013b); and amphibians are even able to develop cross-tolerance to formulations that have similar modes of action (Hua et al. 2013a). Yet with that said, it can still be difficult to make generalizations about responses to pesticide exposure because even exposure to similar formulations (e.g. our terrestrial Monsanto and terrestrial generic) can result in different outcomes (likely due to their “inactive”

adjuvants). Thus, under more natural conditions, the herbicide-induced morphology and body size changes we observed may alter salamander interactions with various biotic and abiotic factors and have considerable long-term consequences for survival and/or reproduction.

Conclusion

In sum, we found that both salamander head and tail morphology were significantly affected by herbicide exposure, and there were no differences in our measure of swim speed among treatments. This suggests that our observations of herbicide-induced morphology changes is a case of non-adaptive plasticity: there were differences, but no apparent advantage or cost because these differences. However, under more realistic conditions, these observations may change and morphological differences could become more important. We did find evidence for a possible trade-off between growth and development. The largest individuals had a morphology closely resembling the initial control (i.e. larval) morphology and were the most distinct from the final control (pre-metamorph) morphology. Finally, these results may indicate a glimmer of hope for amphibian populations exposed to these herbicide formulations, because, when taken together with previous studies, they indicate that although GBHs are widely used, their deleterious effects on amphibians may be mitigated under certain environmental conditions. Understanding patterns of plasticity develops our understanding of how organisms interact with their environment and how these interactions shape their ecological and evolutionary trajectrories.

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CHAPTER 4

SUPPLEMENTAL MATERIAL

Analytical details for family effects

An ideal analysis of "fixed" treatment effects takes into consideration the "random" effects of groups, like families, that were randomly sampled for the experiment. Such an analysis identifies the inherent similarity (non-independence) in observations because of the relatedness of subjects. For parametric tests, this can be critically important, as test statistics (e.g., F or χ^2 statistics) measure the difference in error between models containing and lacking certain effects. Failing to account for random effects can inflate estimates of error, thus reducing statistical power. Although certain non-parametric tests, such as randomization tests, avoid statistical power pitfalls often associated with sample size (and concomitant degrees of freedom) the issue of properly estimating model error persists. The appropriateness of hypothesis tests depends on properly identifying the exchangeable units under the null hypothesis. Exchangeable units are typically the residuals of a null model (Collyer, Sekora & Adams 2015). If residuals are calculated ignoring random effects, they are not correct exchangeable units because they confound non-independent error and random effects. (Randomization tests that fail to account for random effects randomly assign such effects to different treatments in random permutations).

Accounting for random effects is fairly straightforward for univariate data but poses some challenges for multivariate data. Namely, coefficients for fixed effects tend to be estimated with maximum likelihood (ML) or restricted (RE) ML rather than least squares estimation. Thus, there is not a common linear algebra that can be applied to either univariate or multivariate data, and estimating coefficients is more so an algorithm of applying REML to each dependent variable. Applying a permutation procedure that randomizes data and performs REML on many variables within each permutation can result in severely long computation time for analyses. A simpler procedure is to adjust dependent variables by removing random effects; i.e., if a mixed model can be represented as $Y \sim Fixed + Random + Error$, then $Y¢ \sim Fixed + Error$ is a model where the adjusted values, $Y¢$ are found by estimating fixed effects with random effects in the model, but subsequently subtracting random effects,
$Y¢ = (Fixed + Random + Error) - Random$, such that fixed effects are still estimated with respect to random effects. This is possible provided the model,
$Y \sim Fixed + Random + Fixed \mid Random + Error$ is not a viably better model, where $Fixed \mid Random$ is the term for random effects nested within fixed effects. Such a model would indicate that not only do the groups that comprise random effects possibly vary in terms of the dependent variable, but the patterns of within-group changes among levels of fixed effects also vary.

It is possible to evaluate if (1) random effects are important and (2) if random effects nested within fixed effects are important by performing likelihood ratio tests between $Y \sim Fixed + Error$ and $Y \sim Fixed + Random + Error$, and between

$Y \sim Fixed + Random + Error$ and $Y \sim Fixed + Random + Fixed \mid Random + Error$, respectively. The log-likelihood ratio between models can be estimated as

$$LR = -2\log\left(\frac{\frac{trace\left(\mathbf{E}_r^t\mathbf{E}_r\right)}{n}}{\frac{trace\left(\mathbf{E}_f^t\mathbf{E}_f\right)}{n}}\right) = -2\log\left(\frac{\frac{SS_r}{n}}{\frac{SS_f}{n}}\right) = -2\log\left(\frac{SS_r}{SS_f}\right) = -2\left[\log\left(SS_r\right) - \log\left(SS_f\right)\right],$$

where *r* and *f* refer reduced and full models compared, and $\mathbf{E}^t\mathbf{E}$ is a sums of squares and cross-products matrix calculated from the $n \times p$ matrix of residuals, **E**, for the *n* observations of *p* variables. The subscript, ^{t}, indicates matrix transposition for calculating sums of squares and cross-products, and the *trace* is the sum of diagonal elements, which happen to be variable sums of squares. Thus, $trace\left(\mathbf{E}^t\mathbf{E}\right)$ is the sum of each variable's sum of squared error. When using a permutation procedure, the constant, -2, is inconsequential as it is a consistent scalar in each permutation, and as can be seen by the equation, difference in model parameters (degrees of freedom) are unnecessary for inferential tests.

A randomized residual permutation procedure (RRPP) randomizes the vectors of residuals (error) in each reduced model – the exchangeable units under the null hypothesis that two models produce the same error (i.e., the variance of the additional effect in the full model is 0) – to produce random pseudovalues. For example, to test the *Random* effect, pseudovalues of the reduced model are found as $Y^* \sim Fixed + Error^*$ and the SS of the full model is calculated using Y^* in every random permutation (Collyer, Sekora & Adams 2015). Doing this many times produces sampling distributions of $\log\left(SS_r\right) - \log\left(SS_f\right)$. The percentile of the observed value in this distribution can be used to estimate the *P*-value for the test.

We performed likelihood ratio tests using RRPP with 1,000 random permutations to test for random effects and nested random effects using R 3.1.3 (R core team 2015). The lmer function of the lme4 package (Bates *et al.* 2014) was used to estimate fixed and random effects with REML. (R script is provided below.) We performed these tests on every dependent variable of interest (see main article). The "null model" included Treatment as a fixed effect and log(*CS*) as a covariate (where *CS* was the *CS* measured for either head or tail shape configurations); however, when log(*CS*) was the dependent variable, only *Treatment* was included. We calculated sequential sums of squares by adding first Family and then *Treatment|Family* to the null model. In the case of shape data, we used the principal components of shape variation that explained ~95% of the overall variation to avoid issues with non-convergence with REML. We also calculated effect size as the standard deviate (*Z*-score) of observed likelihood ratios in the empirical sampling distributions (*sensu* Collyer, Sekora & Adams 2015).

In no case was the *Treatment|Family* effect significant, indicating any Family effects were consistent across treatments (Table 4.1). Although family effects were also small (and could probably be ignored), we chose to adjust dependent variables by subtracting family effects (as described above), meaning all subsequent analyses (presented in the main article) were sure to not confound family and treatment effects.

We subsequently performed homogeneity of slopes tests for all *CS* × Treatment interactions to determine if shape or swim speed allometries were consistent among treatments. For these tests, np-ANOVA (Collyer, Sekora & Adams 2015) was performed strictly for the model comparison between $Y \sim \log(CS) + Treatment + Error$ and $Y \sim \log(CS) + Treatment + \log(CS)' Treatment + Error$ for either shape or swim speed

(*Y*). These tests were performed with the family-adjusted values described above, using the advanced.procD.lm function of the package, geomorph, version 2.1.3 for R (Adams, Collyer & Sherratt 2015).

We found in no case a significant interaction between log(*CS*) and Treatment (Table 4.2). However, in each case significant allometric scaling was evident (results not shown). We, therefore, performed all subsequent inferential tests with the log of specimen size as a covariate. (For swim speed analyses, we used tail *CS* as a measure of specimen size, simply because it was most correlated with swim speed; see main article).

Table 4.1. Summary statistics for likelihood ratio tests for family effects. The likelihood ratio statistic (*LR*) is the log of the ratio of residual sums of squares between models containing and lacking the effect listed in the header. Standard deviates of this statistic (*Z*) and *P*-values are derived from empirical sampling distributions with 1,000 random permutations of a randomized residual permutation procedure.

	Family			Family|Treatment		
Veraibles	*LR*	*Z*	*P*	*LR*	*Z*	*P*
Head shape	0.016	0.370	0.685	0.145	0.605	0.984
Tail shape	0.045	0.664	0.687	0.111	0.542	0.988
CS_{head}	0.087	0.771	0.642	0.373	0.775	0.902
CS_{tail}	0.118	0.820	0.628	0.307	0.743	0.924
All speeds	<0.001	<0.001	0.600	0.097	0.492	0.907
Mean speed	<0.001	<0.001	0.564	0.129	0.537	0.831
Fastest speed	<0.001	<0.001	0.583	0.136	0.544	0.828

Table 4.2. ANOVA statistics for a test of homogeneity of slopes between log(*CS*) and treatment. Standard deviates of observed sums of squares (*Z*) and *P*-values are derived from empirical sampling distributions with 1,000 random permutations of a randomized residual permutation procedure.

Variables	*F*	*Z*	*P*
Head shape	1.309	1.300	0.094
Tail shape	0.856	0.877	0.607
All speeds	0.576	0.553	0.753
Mean speed	0.418	0.380	0.774

Fastest speed	0.392	0.346	0.782

References for Supplemental Information

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R-script for Mixed Model analyses

```
### Family effects (Replace Y with variable of interest.
Adjust effects as needed; i.e., if CS is used as a
dependent variable, remove it as a covariate)

# Needed functions

pval = function(s){# s = sampling distribution
  p = length(s)
  r = rank(s)[1]-1
  pv = 1-r/p
  pv}

effect.size <- function(x, center = FALSE) {
  z = scale(x, center=center)
  z[1]}

sse = function(R) sum(diag(t(R)%*%R))

# Define variable
Y = prcomp(shape)$x[,1:p] # p is desired number of
dimensions (number of positive eigenvalues = ~95%)

# LS fit for fixed effects
fit1 = lm(Y ~ log(CS)*Treatment) # CS from same landmark
configuration
Rlm = as.matrix(resid(fit1)) # Residuals from linear model
Plm = as.matrix(predict(fit1)) # Predicted values from
linear model

# Matrices of predicted values and residuals for mixed
models
Rmix1 = Rmix2 = Pmix1 = Pmix2 = array(, dim(Rlm))

# Mixed model parts (fill in matrices)
for(i in 1:ncol(Rlm)) {
  fit2 = lmer(Y[,i] ~ log(CS)*Treatment + (1|Family))
  fit3 = lmer(Y[,i] ~ log(CS)*Treatment + (1|Family) +
(0+Family|Treatment))
  Rmix1[,i] = resid(fit2)
  Rmix2[,i] = resid(fit3)
  Pmix1[,i] = predict(fit2)
  Pmix2[,i] = predict(fit3)    }

# Model errors
sse(Rlm)
```

```
sse(Rmix1)
sse(Rmix2)

result = c(sse(Rlm),sse(Rmix1),sse(Rmix2))

# Random permutations
iter = 999 # can be changed to desired number of
permutations - 1
for(i in 1:iter){
  print(noquote(paste("perm", i))) # counter
  Yr1 = Plm + Rlm[sample(nrow(Rlm)),] # random pseudovalues
  Yr2 = Pmix1 + Rmix1[sample(nrow(Rmix1)),] # random
pseudovalues
  Yr3 = Pmix2 + Rmix2[sample(nrow(Rmix2)),] # random
pseudovalues

  Rlm.r = resid(lm(Yr1 ~ log(totcs)*Treatment))
  Rmix1.r = Rmix2.r = array(,dim(Rlm))
  for(ii in 1:ncol(Rlm)) {
    fit2 = lmer(Yr2[,ii] ~ log(totcs)*Treatment +
(1|Family))
    fit3 = lmer(Yr3[,ii] ~ log(totcs)*Treatment +
(1|Family) + (0+Family|Treatment))
    Rmix1.r[,ii] = resid(fit2)
    Rmix2.r[,ii] = resid(fit3)  }
  result = rbind(result, c(sse(Rlm.r), sse(Rmix1.r),
sse(Rmix2.r)))
}

# Likelihood ratios
lrt = cbind(log(result[,1]/result[,2]),
log(result[,2]/result[,3]))

lrt[1,] # observed LRs (multiply by -2 for Chi-square type
stat)
apply(lrt, 2, effect.size) # effect sizes
apply(lrt, 2, pval) # P-values
```