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Non-adaptive phenotypic plasticity: the effects of terrestrial and aquatic herbicides on larval salamander morphology and swim speed

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Phenotypic plasticity, although ubiquitous, may not always be advantageous. Non-adaptive plasticity is likely to occur in response to novel environmental stress. Anthropogenic contaminants, such as herbicides, are novel stressors that are not present in the evolutionary history of most species. We investigated the pattern and consequences of phenotypic plasticity induced by four glyphosate-based herbicides (two terrestrial and two aquatic) in larvae of the spotted salamander, *Ambystoma maculatum*, by determining (1) whether the herbicides induced different morphologies; (2) if different morphologies translated to differences in burst swim performance; and (3) how induced individuals performed relative to non-induced controls. Different herbicide formulations led to the 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 the lateral size of tails, although the tail shapes were either similar to preliminary controls or intermediate between preliminary and final controls. These observations suggest that herbicide-induced morphology is a case of non-adaptive phenotypic plasticity, and that there is potentially a trade-off between growth and development for larvae exposed to different formulations. Understanding the functional significance of induced phenotypes is important for determining their importance in shaping an organism's ecological interactions and evolutionary trajectories. Furthermore, under different conditions, the morphological changes that we observed in response to exposure to herbicides might affect salamander fitness and influence population dynamics. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **00**, 000–000.

ADDITIONAL KEYWORDS: adaptation – amphibian – ecotoxicology – morphometrics – pesticide.

INTRODUCTION

The capacity of a single genotype to exhibit a range of phenotypes (i.e. developmental plasticity) is often advantageous in heterogeneous environments where selection favours different phenotypes depending on the environmental conditions (Gilbert & Epel, 2009). Such developmental (or phenotypic) plasticity in response to local environmental conditions provides a mechanism through which organisms can cope with spatial or temporal heterogeneity and improve their fitness in variable environments (Whitman & Agrawal, 2009). Furthermore, plasticity can play an important role in the evolution of organisms (Pfennig *et al.*, 2010; Moczek *et al.*, 2011; Wund, 2012). For example, developmental plasticity may lead to the induction of behavioural, physiological or morphological traits that, by chance, improve an organism's fitness under stressful conditions (West-Eberhard, 2003). If there is heritable variation in the form or degree of plasticity to the stressful conditions, then selection should favour those genes that best extend, refine or stabilize them, a process dubbed genetic accommodation (West-Eberhard, 2003). In this way, plasticity may facilitate adaptation to a stressful environment.

A key caveat to this process of adaptive evolution is that some of the induced phenotypes, by coincidence, approach an adaptive peak in the stressful conditions. However, if the stressor is truly novel

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(i.e. unlike anything the species experienced in its evolutionary history), there is no reason to suspect that past selection has shaped reaction norms to allow for phenotype–environment matching. In these situations, most induced variants would likely be cases of non-adaptive plasticity where disturbance to development and/or breakdown of physiological processes results in environmentally induced phenotypes with a reduced fitness relative to the ancestral phenotype (Ghalambor *et al.*, 2007). Despite this fitness reduction, traits exhibiting non-adaptive plasticity should be under strong directional selection and thus be able to potentiate rapid adaptive evolution (Ghalambor *et al.*, 2015).

Pond-breeding amphibians exhibit a wide variety of plastic responses to overcome an array of natural environmental stressors (Pfennig, 1990; Relyea, 2001, 2002; Touchon & Warkentin, 2011). Perhaps their most striking plastic responses are morphological changes that are induced by predators (Van Buskirk & Relyea, 1998). Evidence from tadpoles of *Rana lessonae* suggests that different morphologies may be favoured in different environments (Wilson, Kraft & Van Damme, 2005). Tadpoles with low tails and narrow heads were considered to be 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'. Similarly, the fastest *Hyla versicolor* tadpoles in a study by Van Buskirk & McCollum (2000) had relatively shallow bodies and tail fins, which suggests that predator-induced high-tailed tadpoles were less vulnerable to predation for reasons other than enhanced swim performance. By contrast, deeper finned *Scaphiopus holbrookii* and *Rana sphenocephala* tadpoles with small bodies produced the fastest speeds (Dayton *et al.*, 2005). In general, these predator-induced morphologies are adaptive because they are all cases of a 'natural' stressor that the species has likely encountered at some point in its evolutionary history.

Glyphosate is a synthetic compound developed in the 1970s by the biotechnology corporation Monsanto and is marketed as an herbicide (glyphosate disrupts the plant-specific enzyme 5-enolpyruvylshikimate-3-phosphate synthase and kills plants by preventing aromatic amino acid synthesis) under the name 'Roundup'. Glyphosate-based herbicides (GBHs) are the most widely applied herbicide in the world (Jones, Hammond & Relyea, 2011) and their use has increased 10-fold in the last 20 years (USGS, 2012). Additionally, the patent on glyphosate expired in 2000, leading to the development of many generic versions of the product that still use glyphosate as the active ingredient but contain various other ingredients (e.g. the adjuvants). This growing number of formulations, coupled with increased rates of use, increases the likelihood of exposure on nontarget organisms such as amphibians. Because glyphosate is a synthetic compound developed within the last 50 years, many species may be evolutionarily naïve in their response to its effects.

Indeed, various laboratory, mesocosm, and natural studies have found that GBHs negatively affect amphibians and aquatic systems through direct mortality or, more commonly, by sublethal effects such as altered physiology, morphology or food web interactions (Baylis, 2000; Wojtaszek *et al.*, 2004; Chen, Hathaway & Folt, 2004; Edginton *et al.*, 2004; Howe *et al.*, 2004; Cauble & Wagner, 2005; Relyea, Schoeppner & Hoverman, 2005; Bernal, Solomon & Carrasquilla, 2009; Paganelli *et al.*, 2010; Jones *et al.*, 2011; Ortiz-Santaliestra *et al.*, 2011; Levis & Johnson, 2015). Most of these studies have primarily been interested in lethality and the conservation implications of amphibian exposure to GBHs because amphibians are experiencing global declines (Houlahan *et al.*, 2000; Stuart *et al.*, 2004).

GBHs have also been shown to induce morphological changes in larval amphibians that can resemble predator-induced morphologies in some species (Relyea, 2012). Specifically, GBH exposure increased tail depth in tadpoles of *Rana pipiens* and *Rana sylvatica* to the same extent as exposure to caged dragonfly nymphs. This GBH-induced morphology is likely non-adaptive because (1) it occurs in the absence of dragonfly predators and (2) deep-tailed tadpoles grow more slowly and have a reduced survival relative to 'normal' tadpoles when dragonflies are absent (Van Buskirk & Relyea, 1998). Because non-adaptive plasticity can lead to population extinction (Ghalambor *et al.*, 2007; Morris & Rogers, 2013; Morris *et al.*, 2014), amphibians are already experiencing global population declines (Houlahan *et al.*, 2000; Stuart *et al.*, 2004), and GBH use is so widespread, an understanding of the implications of this novel stressor on induced phenotypes in amphibians is relevant for conservation efforts, as well as our general understanding of the role of plasticity in evolution.

To this end, we exposed spotted salamander larvae (*Ambystoma maculatum*; Shaw, 1802) to different GBH formulations, characterized the head and tail morphologies that these herbicides induced, and evaluated the swim performance of the induced morphologies. Using this approach, we aimed to answer three questions: (1) does exposure to different formulations result in different salamander morphologies and/or survival; (2) do morphological changes as a result of GBH exposure translate to differences in

functional swimming performance; and (3) do control individuals (i.e. individuals possessing the ancestral phenotype) outperform their induced counterparts? Swimming performance is a useful proxy for fitness for at least three reasons. First, amphibians with indirect development are fully aquatic and any perturbation to their primary form of locomotion would likely have fitness consequences under natural conditions. Second, other environmental stressors (e.g. predators) induce adaptive changes that affect swim performance (see above). Third, environmental stress can lead to early metamorphosis, which involves dramatic morphological changes in amphibian larvae. Because other larval amphibians have produced similar tail morphologies in response to GBH and dragonflies (Relyea, 2012) and because spotted salamander larvae respond to dragonflies by developing shorter tails (Shaffery & Relyea, 2015), we expected this same outcome as a result of GBH exposure. However, an alternative morphology, such as larger tail fins, could also be expected because it represents the response of this species to a gape-limited 'chase' predator (Urban, 2010). We also predicted that individuals possessing induced morphologies would be functionally inferior to controls (i.e. less fit or non-adapted) in terms of swim speed because GBHs are an evolutionarily novel stress resulting in poor environment–phenotype matching.

MATERIAL AND METHODS

Animal collection

Four egg masses of *A. maculatum* were collected from a pond in Warren County, KY (latitude: 36.87 N, longitude: −86.25 W) on 16 April 2014. Egg masses were held separately in plastic containers with 5 L of a 1 : 1 ratio of dechlorinated/deaminated tap water and natal pond water until hatching. For 2 weeks, larvae were held in their hatching containers and fed brine shrimp *ad libitum* daily. Water in hatching boxes was partially changed once per week, and dead animals were removed. After 14 days post-hatching, five individuals from each egg mass were used to establish initial control morphological and swim performance measurements ($N = 20$) and then 25 other individuals were haphazardly divided among four treatments and a final control ($N = 100$). Thus, each group (described below) contained five replicate individuals per egg mass from four replicate egg masses.

Experimental design

Despite the diversity of formulations available, all glyphosate-based herbicides can be placed into one of 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 for a sufficient length of time to be absorbed. Terrestrial GBHs contain a surfactant (often polyethoxylated tallowamine; POEA) and are typically restricted to terrestrial use, which is the most common location for GBH application (USGS, 2012). POEA has been found to negatively affect aquatic systems (Mann & Bidwell, 2001; Tsui & Chu, 2003; Howe *et al.*, 2004; Brausch, Beall & Smith, 2007; Relyea & Jones, 2009). By contrast, aquatic GBHs lack a surfactant that may reduce potential toxicity to nontarget organisms, and are considered to be safe for aquatic systems if POEA is not added (Giesy, Dobson & Solomon, 2000; but see Brodman *et al.*, 2010). Therefore, 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 difference among these herbicides is that the terrestrial formulations each contain a proprietary surfactant and the aquatic herbicides do not. Typically, the aquatic herbicide formulations would be combined with a surfactant before being used, although we did not add any surfactants in this experiment. Thus, the aquatic herbicides can be considered as controls because they only contain glyphosate and not surfactant. Another difference is that the name-brand and generic formulations potentially contain different amounts and compositions of other 'inactive', proprietary ingredients (Table 1). These unknown differences may have important effects on amphibians.

Five larvae from each egg mass were placed individually into 500-mL glass jars containing 200 mL of dechlorinated/deaminated tap water or 3 mg a.e. L^{-1} of each herbicide formulation. This concentration is within the range of actual observations recorded in nature (Edwards, Triplett & Kramer, 1980), but does not lead to significant mortality in this species (Relyea & 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 3 mg a.e. L^{-1} of each herbicide was prepared and stored until jar water needed to be replaced because of fouling as a result of 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 (i.e. after 7 days; total duration was 14 days) and a 2-mL aliquot of highly concentrated brine shrimp was again added. This relatively infrequent feeding regime may have influenced larval morphology/swim performance. However, because conditions were consistent across treatments, valid comparisons can still be made (see Discussion).

Swim tests

After 14 days, 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 s^{-1}. After acclimation to the container for 2 min, each larva was gently prodded with a blunt wire perpendicular to the abdomen. Each individual was tested three times, although all larvae completed their first trial before any individual was observed a second time. Similarly, all larvae completed their second trial before any individual was tested for a third time. Videos were analyzed using the free, open-source Kinovea (http://kinovea.org) software that allows for placement of markers and timers on a slow motion video. We determined speed as the time that it took the larvae to swim three body lengths away from the point of origin because this distance exceeds the zone of danger presented by a sit-and-wait predator (Van Buskirk & McCollum, 2000). We determined the fastest speed as the fastest trial per individual. The conversion of this time to a linear rate (mm s^{-1}) based on larva length altered neither interpretation, nor statistical outcomes. Each family had five individuals measured before exposure to any treatment (CI), as well as after 2 weeks of exposure to each treatment (described above), aiming to determine how morphology and swim speed changed during ontogeny when exposed to different conditions. After completion of all the swim trials, larvae were euthanized in 0.2% MS-222, fixed in 10% formalin, and stored in 70% ethanol until morphology was analyzed.

Morphological data collection

We photographed every viable specimen (some larvae were damaged during preservation) with a Shuttlepix digital microscope (Nikon) mounted on a motorized stand, such that each specimen was photographed with the same field depth. We used a photo-stacking technique that merged 12 digital images taken at equal height intervals over a range of 10 mm, ensuring visual focus despite the three-dimensional surface portrayed in the images. The right lateral surface of each specimen was photographed in this manner. We digitized photos from 114 of the original 120 larval salamanders used in the experiment. Samples were 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 (TG) and Monsanto terrestrial treatments, respectively. For each specimen, we estimated tail shape using six fixed- and 58 semi-landmarks and head shape using one fixed- and 25 semi-landmarks (Fig. 1).

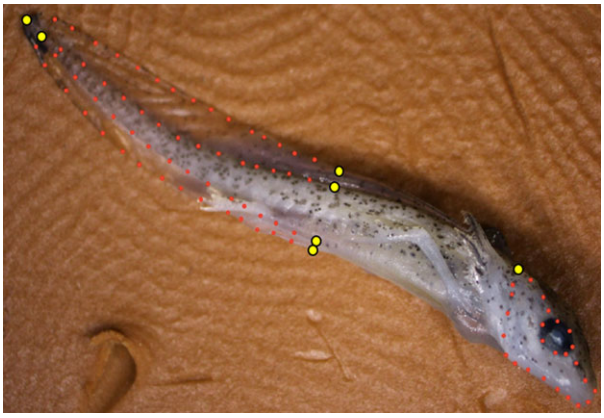

Figure 1. Anatomical landmarks used in the present study. Large yellow points are fixed landmarks; small red points are semi-landmarks. Photograph by M. Schooler.

Table 1. Description of the herbicides used in the present study

Herbicide	Type	Surfactant	Percentage active ingredient	Percentage unlisted 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

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 glyphosate)$^{-1}$. They had different amounts of unlisted ingredients. Percentage ingredient information came directly from the manufacturer's label.
T, terrestrial; A, aquatic.

We used landmark-based geometric morphometrics to quantify attributes of body shape (Adams, Rohlf & Slice, 2013), based on anatomical landmarks digitized on resulting photographs. A generalized Procrustes analysis (GPA) (Rohlf & Slice, 1990) was used to render landmark configurations invariant in size, orientation, and position via generalized least squares superimposition. These 'Procrustes' residuals were 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. During GPA, semi-landmarks (Bookstein, 1997; Gunz & Mitteroecker, 2013) were used to estimate curves based on a minimized squared Procrustes distances criterion (Procrustes distance is the square root of the summed squared distances between corresponding landmarks). Resulting Procrustes residuals (projected into tangent space) were used as shape variables in subsequent statistical analyses. Digitization of landmarks on specimens was performed with TPSDIG2 (Rohlf, 2014). GPA was performed with GEOMORPH, version 2.1.3 (Adams & Otarola-Castillo, 2013) within R, version 3.1.3 (R-Core-Team, 2015).

Statistical analysis

At the individual level, correlations between shape, size, and swim speed were investigated by two-block partial least squares (PLS) analyses (Rohlf & Corti, 2000). PLS is a matrix association (correlation) test that performs a singular-value decomposition on the cross-covariances between variables of two matrices. A randomization test is used to infer the significance of matrix association. For univariate data, PLS is the same as a randomization test on a Pearson product-moment correlation coefficient. We performed PLS on associations between shape and size, fastest swim speed and size, and fastest swim speed and shape. Head and tail sizes were measured as the centroid sizes of their landmark configurations. Centroid size (CS) is calculated as the square root of the summed squared distances of landmarks from their centre 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). A significant result in this case would indicate that these separate aspects of shape are not independent.

We subsequently performed several analyses using a nonparametric method of (multivariate) analysis of variance (ANOVA) for high-dimensional data (Collyer, Sekora & Adams, 2015). High-dimensional data are comprised of variables that exceed the number of subjects analyzed. The nonparametric ANOVA uses traces of sum of squares and cross-products matrices to calculate sums of squares 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 a better resolution for detecting subtle effects (Collyer *et al.*, 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 whether family effects were significant or varied with treatments. The results of preliminary tests are provided in the Supporting information (Table S1). Two conclusions were pervasive: (1) although there were significant family effects in our analyses, the effect sizes for interactions between family and specimen size, or between family and treatment, were not significant 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 not significant. 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

$$\mathrm{y}\prime_j = \hat{\mu} + \mathrm{y}_{ij} - \bar{\mathrm{y}_i},$$

where $\boldsymbol{y}_{\mathrm{ij}}$ is the vector of Procrustes residuals for the j_{th} individual from family i, $\bar{y}_i$ is the vector of Procrustes residuals for the mean of family i, and $\hat{\mu}$ is the overall mean. Thus, y'_{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.

We performed a nonparametric ANOVA with RRPP for 1000 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 *et al.*, 2015), which facilitated comparisons within and across analyses. An additional benefit of the nonparametric 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 nonparametric 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 GEOMORPH, version 2.1.3 (Adams & Otarola-Castillo, 2013) 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 (PC) analysis (performed on the covariance matrix estimated from allometry-free Procrustes residuals) and projection of Procrustes residuals onto the PCs. 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 carried out 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 Supporting information, Table S2 and above).

In addition to visualizing shape variation among specimens using allometry-free Procrustes residuals, a thin-plate spline (Bookstein, 1991) was used to generate transformation grids of different locations of means in the morphospace, providing a mechanistic interpretation of shape change among treatments.

RESULTS

We did not observe any differences in mortality among treatments; all animals survived for the duration of the experiment. Our PLS tests revealed several significant positive correlations (Table 2). Significant allometric patterns suggested that, as the head and tail size increased, both shapes became more 'pointed' (Fig. 2A, B); furthermore, there was a significant integration of head shape and tail shape; individuals with 'broad' shaped heads tended to have tails with 'broad' tail fins (Fig. 2C). Swim speed was significantly correlated with tail shape (Table 2), but not tail size. Transformation grids associated with PLS shape scores suggested a greater propensity for deeper-finned tails, especially at the posterior of the tail, which was associated with a faster swim speed (Fig. 2D).

Table 2. Above the diagonal: two-block partial least squares correlations (r) for relevant comparisons; below the diagonal: corresponding P-values for each correlation

	Head shape	Tail shape	log (CS_{head})	log (CS_{tail})	Swim speed
Head shape	–	**0.398**	**0.53**	NA	0.206
Tail shape	**0.005**	–	NA	**0.516**	**0.318**
log(CS_{head})	**0.001**	NA	–	**0.438**	0.087
log(CS_{tail})	NA	**0.001**	**0.001**	–	0.061
Swim speed	0.636	**0.031**	0.183	0.273	–

CS, centroid size; NA, not applicable (i.e. irrelevant comparison that was not made).
Bold indicates significant correlations.

Shape variation among treatments was significant for each configuration and effect sizes were similar (Table 3). Pairwise Procrustes distances in head shape were significant in each case, except the contrast between AG and AM, as well as the contrast between CF and TM. This pattern was similar for tail shape, although Procrustes distances between CI and both aquatic treatments, and between AM and TM were also not significant (Table 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 tail shape or head shape (Fig. 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 CF 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 diverged in a direction almost 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 (reduced tail fins) for the CF treatment. All other treatments either had less reduced tail fins (TM), retained deep-finned tails (AM and AG) or developed deeper-finned tails (TG). Tail shape variation associated with the second and third PCs appeared to indicate more heterogeneity in the relative depth of dorsal and ventral fins.

Although the GBH treatments appeared to hinder morphological development in terms of head and tail shapes, growth in head size was largely consistent with the control for all treatments (Fig. 4A) and growth in tail size generally exceeded the control for GBH treatments (Fig. 4B). All GBH treatments except the TG treatment, which had the relatively

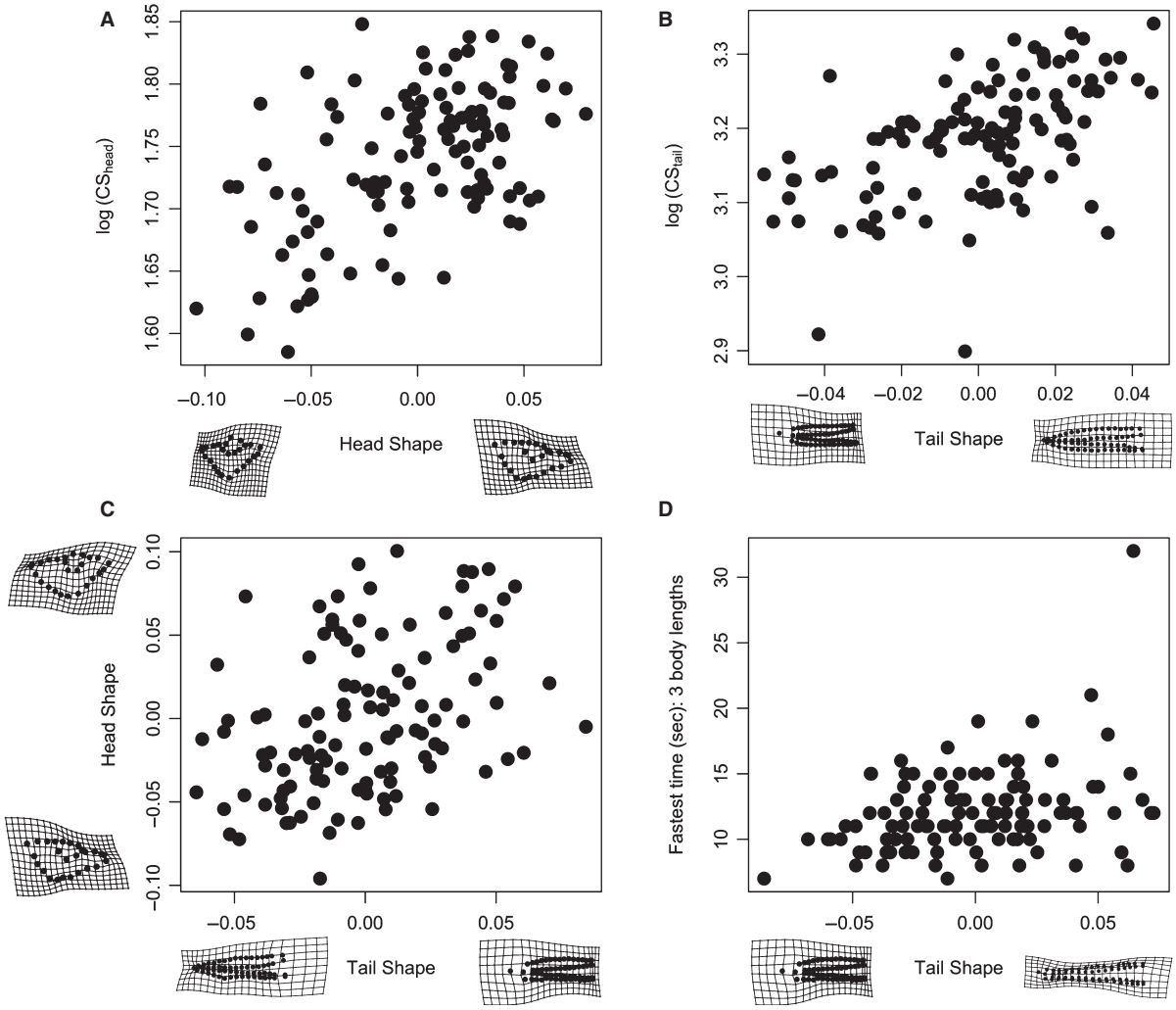

Figure 2. Two-block partial least squares (2B PLS) projections of shape values for correlation analyses using shape. Transformation grids emphasize extremes along the shape axis. Greater change in shape indicates greater association between shape and the alternative variable. Individuals with larger heads (A) and tails (B) tended to have more pointed snouts and tails, respectively. Similarly, individuals with 'broader' heads typically had 'broader' tails (i.e. larger tail fin relative to tail muscle; C). In (D), burst swim time increased as tails became more pointed. Points in (D) are scaled relative to tail size to help facilitate allometric scaling of shape.

largest and deepest posterior tail fin, grew significantly larger tail sizes (measured as the log of centroid size) than the CF treatment, which did not significantly differ from 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. The general trend was that GBH treatments (1) retarded snout elongation but had no effect on the increase in head size and (2) increased the size of the tail at the same time as maintaining a deep profile. 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 variation nonsignificant in each case (results not shown; but see Fig. 4C). Our results could misinform actual swim speeds because 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. We also converted the time to swim three body lengths into a rate, using body length to estimate distance. This conversion produced an almost identical correlation between swim speed and tail size, which was also not significant; results not shown. Furthermore, because the tail size was used as a covariate in the analysis of swim speed, a spurious result is unlikely unless the experiment did not

Table 3. Nonparametric analysis of variance statistics for inter-treatment variation

	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
Fastest speed	7.900	0.007	0.424	0.393	220.030	0.189	3.686	0.001

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

Table 4. Pairwise Procrustes distances in shape

	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**	

AG, aquatic generic; AM, aquatic Monsanto; CF, final control; CI, initial control; TG, terrestrial generic; TM, terrestrial Monsanto.
Distances for head shape are above the diagonal and distances for tail shape are shown below. Distances that are significantly > 0 ($P < 0.05$) are shown in bold.

adequately measure burst speed. Finally, it is odd that tail size and tail shape were correlated, and tail shape and fastest speed were correlated, although tail size and fastest speed were not. This is a possible outcome with multivariate data because correlations can manifest in different dimensions of the morphospace. This appears to be the case in the present study, as indicated by the transformation grids in these separate relationships (Fig. 2).

DISCUSSION

Our results indicate that these herbicide formulations are nontoxic to salamanders at the concentrations used, although they differentially alter both head and tail morphology. In addition, tail morphology was significantly correlated with the fastest escape swim speed (Fig. 2D and Table 2), although there was no difference in any swim speed measurement among treatments. Therefore, our data suggest that herbicide-induced morphological change in spotted salamanders has no negative consequences on swim speed under controlled conditions. However, the observed plasticity may represent a trade-off between growth and development that could be adaptive in other environmental contexts.

The significant correlation between head shape and tail shape (Table 2) suggests that these morphological attributes were 'integrated'. The pattern of morphological integration indicated that elongation of snouts is 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.

In terms of head shape and tail shape, the CF treatment had the most divergent mean shapes that also qualitatively tended more 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 similar to initial larval head shapes or were intermediate between CI and CF head shapes, although they 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 (i.e. heterochrony). We observed an increased lateral size of tails relative to controls for salamanders in three of the four GBH treatments, whereas 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 slightly difficult to reconcile. On the one hand, the small change or intermediate tail shapes of salamanders in the other three GBH treatments with larger tail

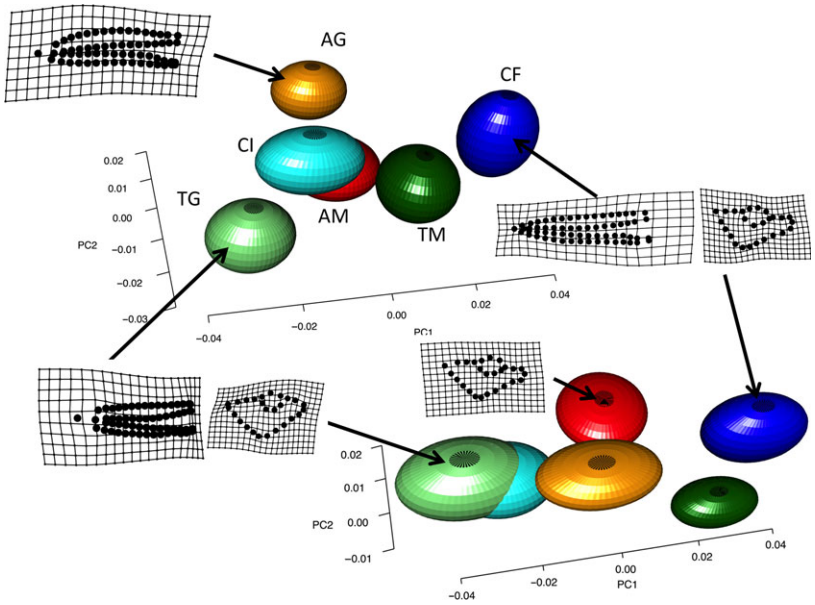

Figure 3. Principal component (PC) plots of shape variation in tail (A) and head (B). Plots are shown for the first three PCs, accounting for 63.2% and 73.2% of the overall shape variation in all dimensions for head shape and tail shape, respectively. Each treatment is represented by a 95% confidence ellipsoid (non-overlapping ellipsoids are generally but not necessarily significantly different because not all dimensions are shown). Transformation grids (deformation scaled × 2) are shown (for some treatment means) to help visualize shape change (these grids were estimated using all dimensions of shape). Treatment labels and colours correspond between this plot and those in Fig. 4. AG, aquatic generic; AM, aquatic Monsanto; CF, final control; CI, initial control; TG, terrestrial generic; TM, terrestrial Monsanto.

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 deeper-finned tail shape of the TG salamanders, 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 exposed to herbicides. This result also suggests that different GBHs might induce different size–shape trade-offs, presumably as a result of the different chemical formulations of adjuvants. Despite the possibility of herbicide-induced heterochrony, we cannot say for certain whether a delay in development occurred because we terminated the experiment prior to metamorphosis to obtain morphometric data. Future studies aimed at collecting detailed longitudinal data of larvae in GBH treatments that also vary the concentration of herbicides might clarify more precisely whether developmental trade-offs are pulsed or continuous during development.

The exact mechanism leading to our observed morphological changes is unknown. The mechanism may involve disruption of the hypothalamic-pituitary-thyroid axis because of its role in development and metamorphosis (Fort *et al.*, 2007). It may also be related to perturbation of the immune system because previous studies have shown an increase in the number neutrophils, a decrease in the number of lymphocytes, and a reduction in T-lymphocyte recruitment in larval amphibians exposed to GBHs (Burraco, Duarte & Gomez-Mestre, 2013; Levis & Johnson, 2015). Another possibility is that elevated levels of corticosterone also played a role in the developmental plasticity that we observed. However, this is unlikely because the corticosterone level did not change in response to 1 mg L^{-1} GBH in other species (Burraco *et al.*, 2013). Although the mechanisms underlying developmental changes leading to metamorphosis are fairly well understood, much less is known about the mechanisms leading to the production of non-adaptive morphologies. Thus, further investigation of these possible pathways should yield valuable insights.

Although morphology significantly differed among treatments, swim speed did not appear to be affected. Consistent with previous studies (Landberg & Azizi, 2010), the fastest swim speeds were correlated with deeper tail fin area. However, all treatments, regardless of morphology, had similar swim speeds. Differences in morphology and swim speed between CI and all other treatments may be, at least partially, a result of differences in growth or feeding regime. Specifically, the experimental larvae may have been under more food stress than CI because the brine shrimp in

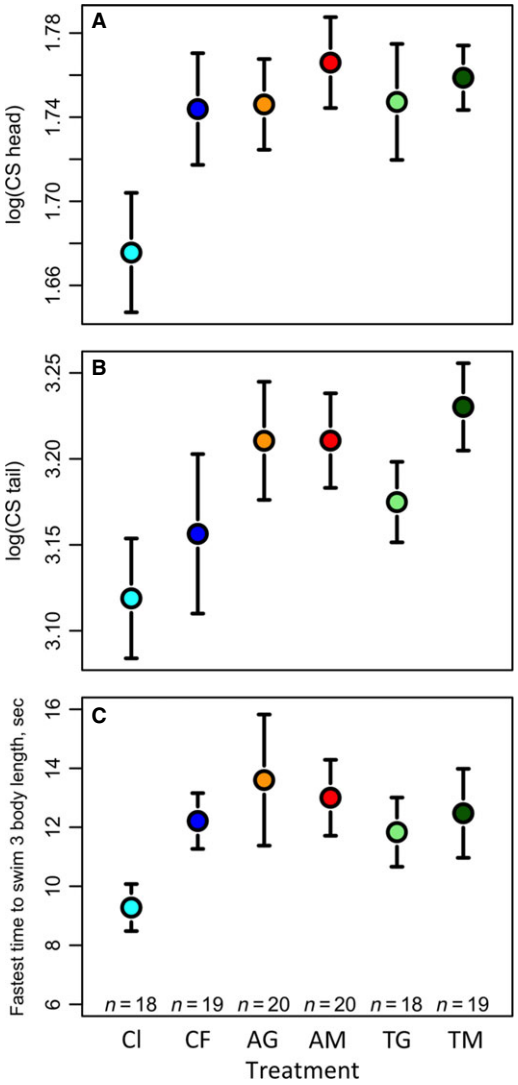

Figure 4. Treatment means and parametric 95% confidence intervals for head size (A), tail size (B), and fastest swim speed (C). Colours match ellipsoid colour in Fig. 3. Sample sizes are shown at the bottom of all three plots. Letters above plots correspond to the results of pairwise tests and similar letters denote that the treatment means are not significantly different ($P > 0.05$).

each jar died after approximately 3 days. However, because the non-CI treatments all experienced similar feeding conditions, the differences in morphology, as well as similarities in swim speed, among them were likely a result of herbicide formulation differences or different interactions between herbicide exposure and food stress. In the case of morphology, herbicide differences must be important because at least some of the treatment groups differed from CF, which is the group that only experienced food stress. Conversely, for swim speed, the potential food stress may be more important because all non-CI groups (including CF) were similar. The similarity in fastest swim speed among non-CI groups suggests that the plasticity observed in the present study had little functional significance for swim speed and/or no costs associated with morphology change under our experimental conditions. Indeed, recent investigations suggest that the costs of plasticity are low or non-existent (Auld, Agrawal & Relyea, 2009), although the apparent lack of costs in the present study may not hold under more realistic conditions.

For *A. maculatum* larvae, it may be that burst speed combined with manoeuverability is important for avoiding predators; thus, shape (the tail especially) might be a better indicator than burst speed if the latter does not simulate predator avoidance well. In contrast to a previous study using larval anurans (Relyea, 2012), our GBH-induced morphology, specifically increased tail size, is more consistent with the morphology induced by a gape limited predator of *A. maculatum* (Urban, 2010) and not the morphology induced by dragonfly larvae (Shaffery & Relyea, 2015), an ambush predator. This suggests the possibility that the morphological developmental pathways activated (or inhibited) by GBH exposure are different for larval anurans and caudates. Because the typical escape response of this species is to turn away from the predator at the same time as accelerating, and deeper tail fins result in quicker bursts (Landberg & Azizi, 2010), the non-adaptive GBH-induced morphology that we observed might be advantageous in the presence of certain predators. More generally, under natural ecological conditions, the morphological changes that we observed may have considerable long-term fitness consequences.

CONCLUSIONS

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 morphological changes had no apparent cost or disadvantages. However, under different conditions, these observations may change and morphological variation could become more important. We did find evidence for a possible trade-off between growth and development. The largest individuals (as measured by tail size) had a morphology closely resembling the initial control (i.e. larval) morphology and were the most distinct from the final control morphology. Determining the patterns and consequences of plasticity develops our understanding of how organisms interact with their environment and how these interactions shape their ecological and evolutionary trajectories.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Summary statistics for likelihood ratio tests for family effects.
Table S2. Analysis of variance statistics for a test of homogeneity of slopes between log(CS) and treatment.