NON-TARGET IMPACTS OF CHEMICAL MANAGEMENT FOR INVASIVE PLANTS ON *LITHOBATES PIPIENS* TADPOLES

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ABSTRACT

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Invasive plants impact amphibians by altering habitat, predator-prey interactions, and reproductive sites. Despite being costly and having serious non-target impacts to wildlife, chemical management is the most common method to reduce or eliminate invasive plants. In spite of previous studies indicating that individual effects of invasive plants or pesticides can be harmful to amphibian populations, the impact of the interaction between invasive plants and herbicide management on amphibians has not yet been evaluated. In Chapter I, a controlled laboratory experiment was performed to assess the impact of the invasive aquatic plant Eurasian watermilfoil (*Myriophyllum spicatum*), the terrestrial invasive European buckthorn (*Rhamnus cathartic*a), the herbicide triclopyr and the combination of invasive plant leachate and herbicide on the growth, morphology and survival of northern leopard frog (*Lithobates pipiens*) tadpoles. Tadpoles were raised in treatments groups for a number of weeks, after which treatment additions were stopped in order to assess for lag effects.

Multiple factors including habitat loss/modification, pollutants, invasive species, and disease have contributed to the global decline of amphibians and declines in their abundance are expected to continue due to changes in climate. Climate change is expected to cause range expansion of many invasive plants; therefore, the use of chemicals to manage invasive plants may increase. Chapter II examined the effects of the invasive plant European buckthorn (*Rhamnus cathartica*), the herbicide triclopyr, and increased temperature on the on the survival, behavior, growth and morphology of Northern leopard frog (*Lithobates pipiens*) tadpoles. Results from this study encourage further examination of the effect of chemical management, but more importantly the potential impacts of climate change on declining amphibian populations.

This work is dedicated to my family, who will never actually understand what I do as a "scientist", but fully support me regardless.

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ABSTRACT

Invasive plants impact amphibians by altering habitat, predator-prey interactions, and reproductive sites. Despite being costly and having serious non-target impacts to wildlife, chemical management is the most common method to reduce or eliminate invasive plants. In spite of previous studies indicating that individual effects of invasive plants or pesticides can be harmful to amphibian populations, the impact of the interaction between invasive plants and herbicide management on amphibians has not yet been evaluated. A controlled laboratory experiment was performed to assess the impact of the invasive aquatic plant Eurasian watermilfoil (*Myriophyllum spicatum*), the terrestrial invasive European buckthorn (*Rhamnus cathartic*a), the herbicide triclopyr and the interaction of each invasive plant leachate and the herbicide on the growth, morphology and survival of northern leopard frog (*Lithobates pipiens*) tadpoles. No effect of treatment on survival was observed. There were some significant effects of the invasive plant treatments on growth and morphological measures, such as increased tadpole growth when exposed to *R. cathartica* leachates, although earlier in the development. However, the most pronounced effects were detected in tadpoles exposed to higher concentrations of the herbicide triclopyr (0.92 mg/L), which include a reduction in tadpole weight, length, and tail muscle depth.. In addition, the simultaneous application of invasive plant leachates and the herbicide had no or minimal effects on tadpole growth or morphology. In fact, the interaction between low levels of the herbicide and *R. cathartica* leachates resulted in increased growth early in tadpole development. Results from this study may be useful to

managers, since invasive species are spreading extremely rapidly in both aquatic and terrestrial environments, and current management techniques may need to be re-evaluated to minimize negative impacts on biodiversity.

INTRODUCTION

Invasive plants impact amphibians by altering habitat, predator-prey interactions**,** and reproductive sites (Watling et al. 2001; Martin and Murray 2011; Rittenhouse 2011; Rogalski and Skelly 2012). Often, invasive plants establish in thick monocultures that disrupt native plant distribution and decrease animal diversity (Schooler et al. 2006; Cohen et al. 2012). For example, Eurasian watermilfoil (*Myriophyllum spicatum*) is an aquatic invasive submerged macrophyte that hybridizes with native watermilfoil, spreads easily via fragmentation, and outcompetes native aquatic plants (Smith and Barko 1990; LaRue et al. 2012). European buckthorn (*Rhamnus cathartica*) is a terrestrial invasive plant capable of persisting in a variety of habitats, where it shades and outcompetes native plants, alters soil chemistry, and provides habitat that enhances the success of other invasive species in a suspected "invasional meltdown" (Simberloff and Von Holle 2000; Heneghan et al. 2004; Knight et al. 2007; Heimpel et al. 2010; Klionsky et al. 2011).

The effects of invasive plants on amphibian performance are not fully understood and have only recently been examined. Secondary chemicals found in invasive plant species may be toxic to amphibians. For example, it is believed that polyphenolic compounds present in invasive plants can decrease amphibian survival and slow developmental progress (Maerz et al. 2005; Brown et al. 2006; Watling et al. 2011; Martin and Blossey 2013). Tellimagrandin II, a polyphenol found in the invasive *M. spicatum,* has been suggested to deter microbial growth and herbivory (Gross et al. 1996; Choi et al. 2002; Glomski et al. 2002); however, any potential

effects on amphibians are unknown. The secondary metabolite emodin present in *R. cathartica* helps to resist herbivores, competition, pathogens, and a variety of abiotic factors (Izhaki 2002). Previous studies suggest that amphibian responses to invasive plants may be species-specific (Maerz et al. 2005; Cohen et al. 2012; Rogalski and Skelly 2012). For instance, purple loosestrife (*Lythrum salicaria*) reduced survival and development of American toad (*Bufo americanus*) tadpoles, but gray treefrog (*Hyla versicolor*) tadpoles displayed higher survival and quicker development (Maerz et al. 2005). Current research suggests that plant chemical characteristics, rather than simply invasive potential, dictates the impacts of invasive species on amphibians (Cohen et al. 2012; Martin and Blossey 2013).

Chemical management is the most common method to reduce or eliminate invasive plants, despite being costly and having serious non-target impacts to wildlife (Blossey 1999; Mack et al. 2000). Herbicides such as 2, 4-D, fluridone or triclopyr are known to cause reduced growth or death in *M. spicatum*; however, rapid regrowth after herbicide applications is common (Getsinger et al. 1992). The most effective treatment for *R. cathartica* is a direct stump cut immediately followed by application of either glyphosate or triclopyr herbicide (Pergams and Norton 2006; Delanoy and Archibold 2007). Negative effects of pesticides on amphibians have been previously documented, including malformations, hermaphroditism, behavioral changes, decreased fertility, reduced growth, morphological changes, death, and lag effects after exposure has ceased (Hayes et al. 2002; Blaustein et al. 2003; Boone and James 2003; Relyea 2005; Chen et al. 2008; Shenoy et al. 2009; Jones et al. 2009Hayes et al. 2010; Egea-Serrano et al. 2012). In nature, pesticides rarely work alone; instead, they act in concert with various abiotic and biotic factors, such as predators, UV-B radiation, pH, competition, and invasive plants to negatively affect amphibians (Blaustein et al. 2003; Relyea et al. 2005; Chen et al. 2008; Relyea et al.

2012). Therefore, it is essential to experimentally consider the interaction of multiple factors that may influence the performance of amphibians in natural systems. While previous studies have indicated that individual effects of invasive plants or pesticides can be harmful to amphibian populations, the impact of the interaction between invasive plants and herbicide management on amphibians has not yet been evaluated.

The goal of this study was to assess whether chemical management of invasive plants could impact a non-target amphibian species, such as the northern leopard frog (*Lithobates pipiens)*. Specifically, the impact of the chemical derivatives from invasive aquatic plant *M. spicatum*, the terrestrial invasive *R. cathartica*, the herbicide triclopyr, and the interaction of invasive plant chemical derivatives and herbicide on growth, morphology, and survival of *L. pipiens* was assessed. *Lithobates pipiens* is a common frog species which inhabits a wide variety of terrestrial and aquatic habitats in the United States and Canada (Smith and Keinath 2007). However, population declines, local extinctions and decreased range have been reported since the 1960s due to habitat fragmentation, pollutants, field collection, invasive species, climate change, and disease (Smith and Keinath 2007; Johnson et al. 2011). Here, it is hypothesized that exposure of *L. pipiens* tadpoles to chemical derivatives of the invasive plants *M. spicatum, R. cathartica*, and the herbicide triclopyr would negatively impact tadpole growth and survival due to potential toxic effects of the plant leachates and/or the herbicide used. Further, we expected that the interaction of these two factors would intensify the predicted negative effects. To test these hypotheses, a controlled laboratory experiment was performed, in which individual tadpoles were randomly assigned to different treatments consisting of weekly applications of *M. spicatum* leachate, *R. cathartica* leachate, the herbicide triclopyr, or both factors combined for 3-5 weeks and then applications stopped in order to assess for lag effects. Results from this study

may be useful for land management, since invasive species are spreading rapidly in both aquatic and terrestrial environments, and current management techniques may need to be re-evaluated to minimize negative impacts on biodiversity (Simberloff and Van Holle 1999; Mack et al. 2000; Pejchar and Mooney 2009).

MATERIALS AND METHODS

Selected Herbicide Treatment

Triclopyr (3, 5, 6-trichloro-2-pyridinyloxyacetic acid) is currently marketed as either triethylamine salt (TEA) or butoxyethyl ester (TBEE) for management of woody and broadleaf plants (EPA 1998). The TBEE formation is more toxic than the TEA formation to fish and amphibians (Wan et al. 1987; Wojtaszek et al. 2005). The herbicide Renovate® 3**,** which contains TEA as its main ingredient (SePRO Corporation, Carmel, IN), was selected for this study because it is specifically marketed to treat aquatic plants (including *M. spicatum*), it contains the same main ingredient as the herbicides used to manage *R. cathartica*, and because its effects on amphibians have yet to be researched. Application rates of triclopyr have been recorded at 0.25-7.86 mg/L, with a maximum expected environmental concentration of 2.7 mg/L (Netherland and Getsinger 1992; Kreutzweiser et al. 1994; Getsinger et al. 2000; Petty et al. 2003; Wojtaszek et al. 2005). Experiments examining LC_{50} values on fish, suggest that lethal concentrations may be >200 mg/L (Wan et al. 1987; Morgan et al. 2009). In this experiment, lower and more environmentally relevant concentrations of 0.2 mg/L and 0.9 mg/L were used (Wan et al. 1987; Chen et al. 2008; Battaglin et al. 2009). Herbicide concentrations were confirmed at 0.2216 mg/L and 0.9248 mg/L by the laboratory SePRO Reasearch and Technology Campus (Whitakers, North Carolina, USA).

Study Organisms

Myriophyllum spicatum plants were collected from Resthaven Wildlife Area (Castalia, Ohio, USA) and *R. cathartica* leaves were collected from the Slippery Elm Trail (Bowling Green, Ohio, USA) in October 2012. Plants were washed in distilled water, blotted dry and freeze dried. Freeze dried plant material was ground using a mortar and pestle and stored at -20 ^{0}C until used in the experiment.

Lithobates pipiens eggs were obtained from a commercial supplier (Nasco, Fort Atkinson, Wisconsin, USA) and held at room temperature in aged tap water until they hatched on 30 January 2013. On 4 February 2013, tadpoles were individually housed in plastic deli cups (500 mL) with plastic plants to mimic more natural conditions and randomly placed in the different treatment groups. Containers were randomly placed in growth chambers (21°C day: 18°C night) with a L12:D12 photoperiod and were randomly rotated daily to prevent position effects. Tadpoles were fed Sera® Micron growth food (Heinsberg, Germany) *ad libitum*.

Experimental Design

To test for the effects of the selected invasive plant species, the herbicide and their interaction, we used a factorial design. The treatment groups consisted of the following: i) control (250 mL aged tap water only), ii) *M. spicatum* leachate (100 mg of freeze dried *M. spicatum* placed in a tea bag and soaked in 250 mL aged tap water for 24 hours), iii) *R.cathartica* leachate (100 mg of freeze dried *R. cathartica* placed in a tea bag and soaked in 250 mL of aged tap water for 24 hours), iv) low triclopyr herbicide concentration (0.2 mg active ingredient [ai] Renovate® 3 in 250 mL of aged tap water), v) high triclopyr herbicide concentration (0.9 mg ai Renovate® 3 in 250 mL of aged tap water), vi) combined application of *M. spicatum* and low concentration of triclopyr (milfoil leachate processed as previously explained plus 0.2 mg ai

Renovate® 3 in 250mL of aged tap water), and vii) combined application of *R. cathartica* and low concentration of triclopyr (*R.cathartica* leachate as explained above plus 0.2 mg ai Renovate® 3 in 250 mL of aged tap water).

A total of 125 individually housed tadpoles were used in this experiment, with each treatment group being replicated 15 times. Weekly water changes were performed, in which 125 mL of water in each container was removed and replenished with 125 mL of freshly prepared treatment water, with half the volume, but the same concentration (i.e., 50 mg of *M. spicatum* or *R. cathartica* soaked in 125 mL of aged tap water, 0.2 mg/L or 0.9 mg/L in 125 mL of aged tap water, or 50 mg of *M. spicatum* or *R. cathartica* plus the full concentration of herbicide). The experiment lasted 9 weeks (4 February – 8 April 2013). Weekly additions of milfoil leachate and both herbicide concentrations were conducted for the first 5 weeks of the experiment and buckthorn leachate was only added for the first 3 weeks due to an insufficient quantity of plant material. When treatments were stopped, water changes consisted of removing 125 mL of water from each tadpole container and replacing with 125 mL of aged tap water only, in order to assess for potential lag effects. On the 9th week, all tadpoles were euthanized via submersion in MS-222.

Survivorship was recorded at the end of the experiment. Tadpoles were removed from their containers and weight and total body length were measured on the 1st, 3rd, 7th, and 9th weeks of the experiment. Following the methodology of Relyea (2000), final morphological measurements of body width, body depth, body length, tail length, tail depth, and muscle depth were made using a digital caliper (±0.01 mm) post-euthanasia.

Data Analyses

All data were analyzed using SAS (version 9.2). Survival across treatments was analyzed using Fisher's exact tests. Analyses of variance (ANOVA) were performed to assess the effects of the invasive plant leachates, herbicide concentrations and their interaction on tadpole growth (i.e., weight and length) and morphology (i.e., body width, depth, and length, tail length and depth, and muscle depth).

When assumptions of normality and heterogeneity were not met, data were transformed using a logarithmic or inverse transformation. Variables that despite previous transformations still showed deviations to normality or heteroscedasticity were rank-transformed (Potvin and Roff 1993; Brunner et al. 2002). Pairwise comparisons among treatment means were performed using Tukey's honest significant difference (HSD) test. A repeated-measures multivariate analysis of variance (rmMANOVA) was use to analyze whether body weight and length changed over time. Using a mixed model repeated-measures analysis of variance (rmANOVA), the effects of treatment, time (linear term), treatment × time, time × time (quadratic term), and time × time × treatment on weight and length were analyzed. Principal components analyses (PCA) were performed to graphically evaluate patterns of integration among traits across treatments. Significance of principal components (PCs) was based on a benchmark of 0.55 (i.e., absolute values of PC loadings) suggested by Tabachnick & Fidell (1989).

RESULTS

Results from this study revealed no significant effects of treatment on survival ($\chi^2 = 6.01$, df= 6; P = 0.42). However, significant differences in weight, length, and morphological measurements were observed across treatment groups (Table 1, and Figures 1 and 2). Repeated measures MANOVA showed that weight and total body length significantly changed over time

(both P<0.0001; Table 1). The mixed model rmANOVA revealed significant effects of treatment, time, time^2, and the interaction of treatment × time on both weight and length (see Table 1). However, the time^2 × treatment interaction was significant only for weight (Table 1). Although tadpole weight did not differ among treatments during the 1st week of the experiment ($F_{6, 97} = 1.41$, P = 0.2178), significant differences were observed in the other weeks (Fig. 1a). In the 3rd week, significant differences in weight were observed between tadpoles exposed to either *R. cathartica* or *R. cathartica* + low herbicide concentrations and tadpoles in the control group ($F_{6, 93} = 16.72$, P < 0.0001). Specifically, tadpoles exposed to either *R. cathartica* or *R. cathartica* + low herbicide were 44% and 47% heavier, respectively, than tadpoles exposed to control conditions. During week 7, treatment means were not significantly different from those of the control group, but there was a significant effect of treatment on tadpole weight when comparing the means of the other treatment groups ($F_{6, 92} = 2.81$, P = 0.0148). . Specifically, tadpoles raised in *R. cathartica* leachates were 21% heavier than those reared in the high herbicide concentration group. Lastly, the effect of treatment on final weight (week 9) was significant ($F_{6, 92} = 2.65$, P = 0.0203). On the final week of the experiment, tadpoles that were exposed to high herbicide concentrations were 25% lighter than those from the control group.

Significant treatment effects on total length were found (Fig. 1b). During week 1, treatment means did not differ from those of the control group ($F_{6, 97} = 3.34$, P = 0.0049), but tadpoles in *R. cathartica* were 9% longer than tadpoles in *M. spicatum,* and 11% longer than tadpoles in the high herbicide concentration treatment. Treatment effects on tadpole length during the 3rd week of the experiment were significant ($F_{6, 93} = 15.09$, P < 0.0001). On this 3rd week, tadpoles exposed to either *R. cathartica* leachates or the combination of *R. cathartica* + low herbicide concentrations were 13% and 15% longer, respectively, than tadpoles in the

control group. On the other hand, treatments had no significant effects on length in the 7th week of this experiment ($F_{6, 92} = 2.06$, $P = 0.0651$). During the final week of the experiment, treatments significantly influenced length ($F_{6, 92} = 2.97$, $P = 0.0107$); however, in this week, the outcome was apparently associated with some lag effects of the high herbicide concentration treatment, which caused a significant decrease in tadpole length (8% shorter than tadpoles in the control group). Similar to the trends observed with weight, *R. cathartica* and *R. cathartica* + low herbicide concentration treatments significantly increased tadpole lengths on the 3rd week, and the high herbicide concentration significantly decreased tadpole length during the 9th week of the experiment.

Regarding morphological measurements, treatment effects were significant only for body width (Fig. 2a) and muscle depth (Fig. 2f). No significant effects of treatments on body length (Fig. 2b), body depth (Fig. 2c), tail length (Fig. 2d), or tail depth (Fig. 2e) were found. Specifically, the *M. spicatum* treatment induced a 14% decrease in body width compared to tadpoles in the control group (Fig. 2a). Likewise, tadpoles exposed to the high herbicide concentration treatment exhibited a 14% reduction in muscle depth compared to those of the control group (Fig. 2f).

The PCA graphically revealed differences in associations among morphological traits across treatment groups (Fig. 3). The PCA graphs showed a higher number of associations between traits in the treatment groups compared to the control treatment. These differences were especially pronounced in both the *M. spicatum* and the high herbicide concentration treatments, which had 7-8 significant PC loadings compared to only 3 in the control group. In addition, the presence of negative loadings in the *M. spicatum*, high herbicide concentration, and *R. cathartica* + low herbicide concentration groups suggests potential tradeoffs among traits induced by these

treatments. For example, the combination of *R. cathartica* and low herbicide concentration could have caused a tradeoff between muscle depth and tail length. Additionally, in the high herbicide concentration treatment, body width was inversely related to body depth and tail depth, but the latter was directly related to muscle depth, tail length, and body length.

DISCUSSION

Previous studies have evaluated the individual effects of invasive plants or herbicides on amphibians (Maerz et al. 2005; Relyea 2005; Egea-Serrano et al. 2012; Martin and Blossey 2013; Sacerdote and King 2014); however, the combined effects of both factors on these organisms are not yet known. This study is the first to simultaneously assess the impact of two invasive plant species (i.e., *R. cathartica and M. spicatum*), and an herbicide (i.e., triclopyr) commonly used to control these plants on the survival, growth and morphology of the Northern leopard frog *Lithobates pipiens*. The specific invasive plant species and the type of herbicide (triclopyr) selected for this experiment have not yet been evaluated in previous studies using amphibians, with the exception of one that assessed the role of a secondary chemical present in *R. cathartica* on the survival and development of embryos of two frog species (Sacerdote and King 2014). Results from this study suggest that the use of the herbicide triclopyr to chemically control the selected invasive plants may negatively affect tadpole growth and morphology (muscle depth), but only at higher concentrations (0.92 mg/L or higher levels) and with a lag effect (i.e., after applications were stopped for four weeks). On the other hand, the herbicide triclopyr did not appear to affect tadpole growth or survival at the lower concentrations used in this study. Other researchers have also found that higher concentrations of herbicides can be more detrimental to tadpoles than lower concentrations (Diana et al. 2000; Shenoy et al. 2009). In contrast to what was expected, individual applications of either *M. spicatum* or *R. cathartica*

leachates did not have any significant negative effects on tadpoles. In fact, *R. cathartica* appeared to enhance tadpole growth during some developmental stages (both when applied alone or in combination with the herbicide), which could have been due to nutritional components present in the plant leachate. These results have direct implications for invasive plant management and suggest that alternatives to chemical control need to be explored, especially when high concentrations of herbicides have to be used to control persistent invasive plants..

It was hypothesized that because of the potential inhibitory properties of polyphenols in *M. spicatum* and anthraquinones in *R. cathartica*, tadpoles would exhibit reduced survival and growth. Sacerdote and King (2014) found that emodin extracted from *R. cathartica* caused malformations and increased mortality in African clawed frog (*Xenopus laevis*) and Western chorus frog (*Pseudacris triseriata*) embryos. However, no negative effects of *R. cathartica* on tadpole survival and growth were detected in this study. In fact, as previously mentioned, *R. cathartica* appeared to have some beneficial effects on tadpole weight and length while weekly additions of plant leachates were replenished, although this outcome disappeared after leachate applications ceased. Since this potentially beneficial effect was found only during the active development of tadpoles (weeks 3-7) and only while the plant leachates were applied, the enhanced growth could have resulted from the presence of some nutritional component(s) present in *R. cathartica* leachates. Results from plant tissue analysis of *R. cathartica* leaves and litter from a second experiment (not included here) revealed that these were high in nitrogen content, with green leaves containing 2.27% weight and litter containing 2.37% weight of nitrogen (Unpublished data), which confirm previous reports of 2.2% N in buckthorn litter (Heneghan et al. 2002). Previous studies have shown that the carbon-to-nitrogen ratio (C:N) of plant tissues could influence tadpole larval period and mass at metamorphosis (Maerz et al.

2010; Cohen et al. 2012). In addition, other studies have shown that *R. cathartica* leaves had high nitrogen content and a low C:N ratio (Heneghan et al. 2002; Heneghan et al. 2004; Heneghan et al. 2006). This previous knowledge about nitrogen content of *R. cathartica* leaves and its potential effects on tadpoles may provide a possible explanation for the apparent growth enhancing effects of this plant species' leachate on *L. pipiens* tadpoles.

Few effects of *M. spicatum* leachates on tadpole growth and morphology were detected in this study, and these were mainly related to a significant decrease in the final body width of tadpoles. Some negative effects of *M. spicatum* have been observed in other aquatic organisms in lakes invaded by this plant species. For example, the presence of *M. spicatum* decreased fish size and abundance (Lyons 1989; Valley and Bremigan 2002), as well as slowed development of the aquatic moth larva *Acentria ephemerella* and inhibited its gut bacteria *A. ephemerella* (Choi et al. 2002; Walenciak et al. 2002). Previous studies using other invasive plant species, however, have found positive or no effects of invasive plants on amphibians (Rittenhouse 2011; Rogalski and Skelly 2012). For instance, Rogalski and Skelly (2012) found that bullfrog (*Lithobates catesbeianus*) tadpoles were larger and had greater survival in wetlands invaded by the common reed (*Phragmites australis*). This outcome is in agreement with the apparently positive effect of *R. cathartica* on tadpole growth found in the present study. Recent evidence indicates that some chemical constituents of plant invasive species, such as phenolics, tannins or saponins, may have a significant impact on amphibians; although different species may respond in different ways to these chemicals (Maerz et al. 2005; Cohen et al. 2012; Martin and Blossey 2013). Therefore, it may be possible that although *R. cathartica* and *M. spicatum* have little effect on *L. pipiens*, they could have more pronounced effects on other amphibian species. The limited response of tadpoles to the invasive plant species in this study could also be due to a relatively low

concentration of the plant leachates used. Unfortunately, there is no information about the actual amount of *M. spicatum* or *R. cathartica* that amphibians are likely to encounter in nature or whether tadpoles would be more sensitive to higher amounts of *M. spicatum* or *R. cathartica* plant extracts. Moreover, the polyphenolic chemicals present in these plant species, which were hypothesized to negatively impact tadpoles, are believe to degrade quickly, at least in *M. spicatum* (Glomski et al. 2002). Therefore, they may not necessarily be present in a concentration that could be toxic to amphibians in nature. Experiments that incorporate different plant leachate concentrations or specific secondary chemicals on various amphibian species are necessary to fully elucidate potential effects of *R. cathartica* and *M. spicatum* on amphibian growth and development.

As predicted, we found detrimental effects of the herbicide triclopyr on tadpole growth; however, only when using it in a higher concentration. It is interesting to note, though, that these effects were detected towards the end of the experiment (on week 9), which suggest that there could be a lag or cumulative effect of herbicide applications on tadpole responses. These results are consistent with previous findings that short periods of pesticide exposure can cause lag effects in ranids (Jones et al. 2009; Egea-Serrano et al. 2012; Hammond et al. 2012). Jones et al. (2009) conducted LC_{50} experiments examining the effects of the pesticide endosulfan on nine different species of tadpoles and found that mortality drastically increased after tadpoles were removed from treated water and placed in clean water. Additionally, the family Ranidae (which includes *L. pipiens*) is especially vulnerable to pesticide exposure, which may not be the case for other amphibian groups (Jones et al. 2009; Egea-Serrano et al. 2012). Moreover, since smaller tadpoles often take longer to metamorphose, these tadpoles are at increased risk of desiccation, predation, and disease (Werner 1986; Downie et al. 2004; Cabrera-Guzmán et al. 2013).

Additionally, smaller tadpoles often become smaller adults that can experience decreased sexual selection and smaller clutch sizes (Berven and Gill 1983; Semlitsch et al. 1988). Since this experiment only examined effects over 9 weeks of the larval period of *L. pipiens*, it is not known if the potential lag effects of exposure to triclopyr would persist after metamorphosis and could be a cause of reduced adult reproduction or survival.

Multiple stressors can sometimes have additive detrimental effects on organisms. Chen et al. (2008) found that when *L. pipiens* tadpoles were exposed to low pH and low food availability along with triclopyr (TBEE formulation), the negative effects of triclopyr increased. In this experiment, the TEA formulation of triclopyr was used, which is less toxic to fish and amphibians (Wan et al. 1987; Wojtaszek et al. 2005). Perhaps the effects of the TBEE formulation of triclopyr may be more harmful to non-target organisms and should be avoided. Based on results from this study, it is possible that the higher concentration (0.92 mg/L) of triclopyr used may be near the threshold concentration to where detrimental effects on amphibians become evident, since the lower concentration of this herbicide (0.22 mg/L) did not produce any negative effects. Additionally, since triclopyr is reported to have a rapid breakdown in water, the actual concentration that tadpoles could encounter in the wild may be lower than the concentration used in this experiment (Petty et al. 2003). Undoubtedly, more information on the effects of triclopyr on aquatic organisms for short-and long-term exposure is necessary.

When individually evaluated, the morphological variables appeared to be largely unaffected by exposure to the different treatment groups. However, when the relationship among these variables was graphically evaluated using PCA, a treatment-induced reorganization of tadpole morphological traits was observed. These results may indicate plasticity in the integration (reorganization) of the tadpole morphology when exposed to novel or potentially

harmful environments. A higher number of associations among traits (both inverse and direct) were found in the treatment groups compared to the control group. In addition, a higher number of inverse associations among traits were observed in some of the treatment groups, especially in the high herbicide concentration versus the control group. Overall, tail depth, body depth, and muscle depth seemed to have strong direct associations across almost all the treatment groups. Even though there were more direct than inverse associations among variables, some inverse associations were detected, which may be indicative of environmentally induced trade-offs among variables. For example, the *R. cathartica* + low herbicide concentration treatment showed an inverse relationship between muscle depth and tail length, suggesting that tadpoles may invest in deeper tail muscles, which may at the cost of a shorter tail in this environment. However, tadpoles exposed to *M. spicatum* showed an inverse relationship between muscle depth and body length, which suggests that increasing muscle depth maybe more efficient in these environments. In the high herbicide treatment, body width was inversely associated with body depth and tail depth; again, suggesting that different phenotypes that may maximize growth and survival can be induced under stressful environments (Relyea 2001; Bidart-Bouzat et al. 2004). From previous studies, it is known that tadpole morphology is plastic, and that different phenotypes and trait associations are induced by different factors, such as predation, competition or exposure to the herbicide glyphosate (Relyea 2001; Relyea 2004; Relyea 2012). Results from this experiment are in accordance with these previous studies and indicate that the presence of plant invasive species or the herbicide triclopyr in the environment can also influence tadpole plasticity and integration of morphological traits.

This experiment suggests that using chemical control methods to manage invasive plants may pose a greater threat to tadpoles than the presence of these invasive plants themselves. It

also highlights that higher concentrations of herbicides can produce lasting effects after exposure halted, which can harm not only amphibians but also other aquatic organisms. Although *M. spicatum* and *R. cathartica* did not appear to negatively affect tadpoles in this study, the effects on habitat and native species displacement should not be discounted and the impacts on other amphibian and aquatic species cannot be extrapolated from these results. Additionally, this study provides evidence that different environments can influence the plasticity of integration of traits, and that this may induce a change in tadpole morphology, which may be essential to maximize survival under stressful conditions. It is still unknown how multiple stressors, such as competition, predation, disease, climate change, invasive plants, and herbicides (among many others) interact to affect amphibians as well as many other aquatic organisms. In addition to controlled laboratory experiments, more field studies are needed to further assess the effect of using chemical management to control invasive plant species in the wild and to elucidate its impacts on amphibians. The range of habitat occupied by invasive plants will likely continue to expand; therefore, alternative control measures for invasive plants should be explored to prevent potential negative effects of herbicides on non-target organisms. A more complete understanding of the evolutionary ecology of invasive plants is also needed to re-evaluate current management practices and to use a more integrative approach to eradicate them while minimizing effects on the surrounding ecosystem.

LITERATURE CITED

Battaglin WA, Rice KC, Focazio MJ, Salmons S, Barry RX (2009) The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005-2006. Environmental Monitoring Assessment 155: 281-307

Berven KA ,Gill DE (1983) Interpreting geographic variation in life-history traits. Integrative and Comparative Biology 23: 85-97

Bidart-Bouzat MG, Portnoy S, DeLucia EH, Paige KN (2004) Elevated CO_2 and herbivory influence trait integration in *Arabidopsis thaliana*. Ecology Letters 7: 837-847

Blaustein A R, Romansic JM, Kiersecker JM, Hatch AC (2003) Ultraviolet radiation, toxic chemicals and amphibian population declines. Diversity and Distributions 9: 123-140

Blossey B (1999) Before, during, and after: the need for long-term monitoring in invasive plant species management. Biological Invasions 1: 301-311

Boone MD, James SM (2003) Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. Ecological Applications 13: 829-841

Brown CJ, Blossey B, Maerz JC, Joule SJ (2006) Invasive plant and experimental venue affect tadpole performance. Biological Invasions 8: 327-338

Brunner, E, Domhof, S, Langer, F (2002) Nonparametric Analysis of Longitudinal Data in Factorial Experiments. John Wiley & Sons, New York, 288 pp

Cabrera-Guzmán E, Crossland MR, Brown GP, Shine R (2013) Larger body size at metamorphosis enhances survival, growth and performance of young cane toads (*Rhinella marina*). PLoS ONE 8: e70121. doi:10.1371/journal.pone.0070121

Chen CY, Hathaway KM, Thompson DG, Folt CL (2008) Multiple stressor effects of herbicide, pH, and food on wetland zooplankton and a larval amphibian. Ecotoxicology and Environmental Safety 71: 209-218

Choi C, Bareiss C, Walenciak O, Gross EM (2002) Impact of polyphenols on growth of the aquatic herbivore *Acentria ephemerella*. Journal of Chemical Ecology 28: 2223-2235

Cohen JS, Maerz JC and Blossey B (2012) Traits, not origin explain impacts of plants on larval amphibians. Ecological Applications 22: 218-228

Delanoy, L. and O.W. Archibold. 2007. Efficacy of control measures for European buckthorn (*Rhamnus cathartica*) in Saskatchewan. Environmental Management. 40: 709-718

Downie JR, Bryce R and Smith J (2004) Metamorphic duration: an under-studied variable in frog life histories. Biological Journal of the Linnean Society 83:261-272

Egea-Serrano A, Relyea RA, Tejedo M and Torralva M (2012) Understanding of the impact of chemicals on amphibians: a meta-analytic review. Ecology and Evolution 2: 1382-1397

Getsinger KD, Turner EG and Madsen JD (1992) Field evaluation of the herbicide triclopyr for managing Eurasian watermilfoil. U.S. Army Corp of Engineers Waterways Experiment Station, Vickburg, MS A-92-3: 1-6

Getsinger KD, Petty DG, Madsen JD, Skogerboe JG, Houtman BA, Haller WT, Fox AM (2000) Aquatic dissipation of the herbicide triclopyr in Lake Minnetonka, Minnesota. Pest Management Science 56: 388-400

Glomski LM, Wood KV, Nicholson RL, Lembi CA (2002) The search for exudates from Eurasian watermilfoil and Hydrilla. Journal of Aquatic Plant Management 40: 17-22

Gross EM, Meyer H, Schilling G (1996) Release and ecological impact of algicidal hydrolysable polyphenols in *Myriophyllum spicatum*. Phytochemistry 41: 133-138

Hammond JI, Jones DK, Stephens PR, Relyea RA (2012) Phylogeny meets ecotoxicology: evolutionary patterns of sensitivity to a common insecticide. Evolutionary Applications 5: 593-606

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A (2002) Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences of the United States of America. 99: 5476-5480

Hayes TB, Khoury V, Narayan A, Nazir M, Park A, Brown T, Adame L, Chan E, Buchholz D, Stueve T, Gallipeau S (2010) Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proceeding of the National Academy of Sciences of the United States of America 107: 4612-4617

Heimpel GE, Frelich LE, Landis DA, Hopper KR, Hoelmer KA, Sezen Z, Asplen MK, Wu K (2010) European buckthorn and Asian soybean aphid as components of an extensive invasional meltdown in North America. BiologicalInvasions, 12: 2913-2931

Heneghan L, Clay C, Brundage, C (2002) Rapid decomposition of buckthorn litter may change soil nutrient levels. Ecological Restoration 20: 108-111

Heneghan L, Rauschenberg C, Fatemi F, Workman M (2004) European buckthorn (Rhamnus cathartica) and its effect on some ecosystem properties in urban woodland. Ecological Restoration. 22: 275-280

Izhaki, I (2002) Emodin-a secondary metabolite with multiple ecological functions in higher plants. New Phytologist 155: 205-217

Johnson PTJ, McKenzie VJ, Peterson AC, Kerby JL, Brown JB, Blaustein AR, Jackson T (2011) Regional decline of an iconic amphibian associated with elevation, land-use change, and invasive species. Conservation Biology 25: 556-566

Jones DK, Hammond JI, Relyea RA (2009) Very highly toxic effects of endosulfan across nine species of tadpoles: lag effects and family-level sensitivity. Environmental Toxicology and Chemistry 28: 1939-1945

Klionsky SM, Amatangelo KL, Waller DM (2011) Above-and belowground impacts of European buckthorn (*Rhamnus cathartica*) on four native forbs. Restoration Ecology 19: 728-737

Knight KS, Kurylo JS, Endress AG, Stewart JR, Reich PB (2007) Ecology and ecosystem impacts of common buckthorn (*Rhamnus cathartica*): a review. Biological Invasions. 9: 925-937

LaRue EA, Zuellig MP, Netherland MD, Heilman MA, Thum RA (2012) Hybrid watermilfoil lineages are more invasive and less sensitive to a commonly used herbicide than their exotic parent (Eurasian watermilfoil). Evolutionary Applications 6: 462-471

Lyons J (1989) Changes in the abundance of small littoral-zone fishes in Lake Mendota, Wisconsin. Canadian Journal of Zoology 67: 2910-2916

Mack RN, Simberloff D, Lonsdale WM, Evans H, Clout M, Bazzaz FA (2000) Biotic invasions: Causes, epidemiology, global consequences, and control. Ecological Applications 10: 689-719

Maerz JC, Brown CJ, Chapin CT, Blossey B (2005) Can secondary compounds of an invasive plants affect larval amphibians? Functional Ecology 19: 970-975

Martin LJ, Murray BR (2011) A predictive framework and review of the ecological impacts of exotic plant invasions on reptiles and amphibians. Biological Reviews 86: 407-419

Martin LJ, Blossey B (2013) Intraspecific variation overrides origin effects in impacts of litter-derived secondary compounds on larval amphibians. Oecologia 173: 449-459

Potvin C, Roff D (1993) Distribution-free methods: Viable alternatives to parametric statistics. Ecology: 74: 17-28

Pergams, O. R.W. J.E. Norton (2006) Treating a single stem can kill the whole shrub: a scientific assessment of buckthorn control methods. Natural Areas Journal 26: 300-309

Pejchar L, Mooney HA (2009) Invasive species, ecosystem services and human well-being. Trends inEcology and Evolution 24: 497-504

Relyea, RA (2000) Trait-mediated indirect effects in larval anurans: reversing competition with the threat of predation. Ecology 81: 2278-2289

Relyea RA (2001) Morphological and behavioral plasticity of larval anurans in response to different predators. Ecology 82: 523-540

Relyea RA (2004) Fine-tuned phenotypes: tadpole plasticity under 16 combinations of predators and competitors. Ecology 85: 172-179

Relyea RA (2005) The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecological Applications 15: 1118-1124

Relyea RA (2012) New effects of Roundup® on amphibians: predators reduce herbicide mortality while herbicides induce anti-predator morphology. Ecological Applications 22: 634-647

Rittenhouse TAG (2011) Anuran larval habitat quality when reed canary grass is present in wetlands. Journal of Herpetology 45: 491-496

Rogalski MA, Skelly DK (2012) Positive effects of nonnative invasive *Phragmites australis* on larval bullfrogs. PLoS ONE 7

Sacerdote AB, King RB (2009) Direct effects of an invasive European buckthorn metabolite on embryo survival and development in *Xenopus laevis* and *Pseudacris triseriata*. Journal of Herpetology 48: 51-58

Schooler SS, McEvoy PB, Coombs EM (2006) Negative per capita effects of purple loosestrife and reed canary grass on plant diversity of wetland communities. Diversity and Distributions 12: 351-363

Semlitsch RD, Scott DE, Pechmann JHK (1988) Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology 69: 184-192

Shenoy K, Cunningham BT, Renfroe JW, Crowley PH (2009) Growth and survival of northern leopard frog (*Rana pipiens*) tadpoles exposed to two common pesticides. Environmental Toxicology and Chemistry 28: 1469-1474

Simberloff D, Von Holle B (1999) Positive interactions of nonindigenous species: invasional meltdown. Biological Invasions 1: 21-32

Smith CS, Barko JW (1990) Ecology of Eurasian watermilfoil. Journal of Aquatic Plant Management 28: 55-64

Smith BE, Keinath DA (2007). Northern Leopard Frog (*Rana pipiens*): a technical conservation assessment. [Online]. USDA Forest Service, Rocky Mountain Region. Available: http://www.fs.fed.us/r2/projects/scp/assessments/northernleopardfrog.pdf

Valley RD, Bremigan MT (2002) Effects of selective removal of Eurasian watermilfoil on age-0 largemouth bass piscivory and growth in southern Michigan lakes. Journal of Aquatic Plant Management 40: 79-87

Walenciak O, Zwisler W, Gross EM (2002) Influence of *Myriophyllum spicatum*-derived tannins on gut microbiota of its herbivore *Acentria ephemerella*. Journal of Chemical Ecology 28: 2045-2056

Wan MT, Moul DJ, Watts RG (1987) Acute toxicity to juvenile Pacific salmonids of Garlon 3A™, Garlon 4™, triclopyr, triclopyr ester and their transformation products: 3,5,6-trichloro-2-pyridinol and 2-methoxy-3,5,6-trichloropyridine. Bulletin Environmental Contamination and Toxicology 39: 721-728

Watling JI, Hickman CR, Orrock JL (2011) Predators and invasive plants affect performance of amphibian larvae. Oikos 120: 735-739

Werner EE (1986) Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. The American Naturalist 128: 319-341

Wojtaszek BF, Buscarini TM, Chartrand DT, Stephenson GR, Thompson DG (2005) Effect of Release® herbicide on mortality, avoidance response, and growth of amphibians larvae in two forest wetlands. Environmental Toxicology and Chemistry 24: 2533-2544

Table 1. Repeated measures MANOVA and mixed-model repeated measures ANOVA evaluating treatment and time effects on tadpole weight and length.

Source of variation	Weight		Length	
	DF	F	DF	F
Repeated measures MANOVA				
Treatment	24, 312	4.41**	24, 312	3.43**
Repeated measures ANOVA				
Time	1, 284	2438.45**	1, 284	3686.64**
$Time^2$	1, 284	100.95**	1, 284	37.99**
Treatment	6, 97	6.09**	6, 97	7.78**
Time × Treatment	6, 284	2.32*	6, 284	2.15*
$Time^2$ × Treatment	6, 284	2.63*	6, 284	0.38

* P<0.05; ** P<0.0001

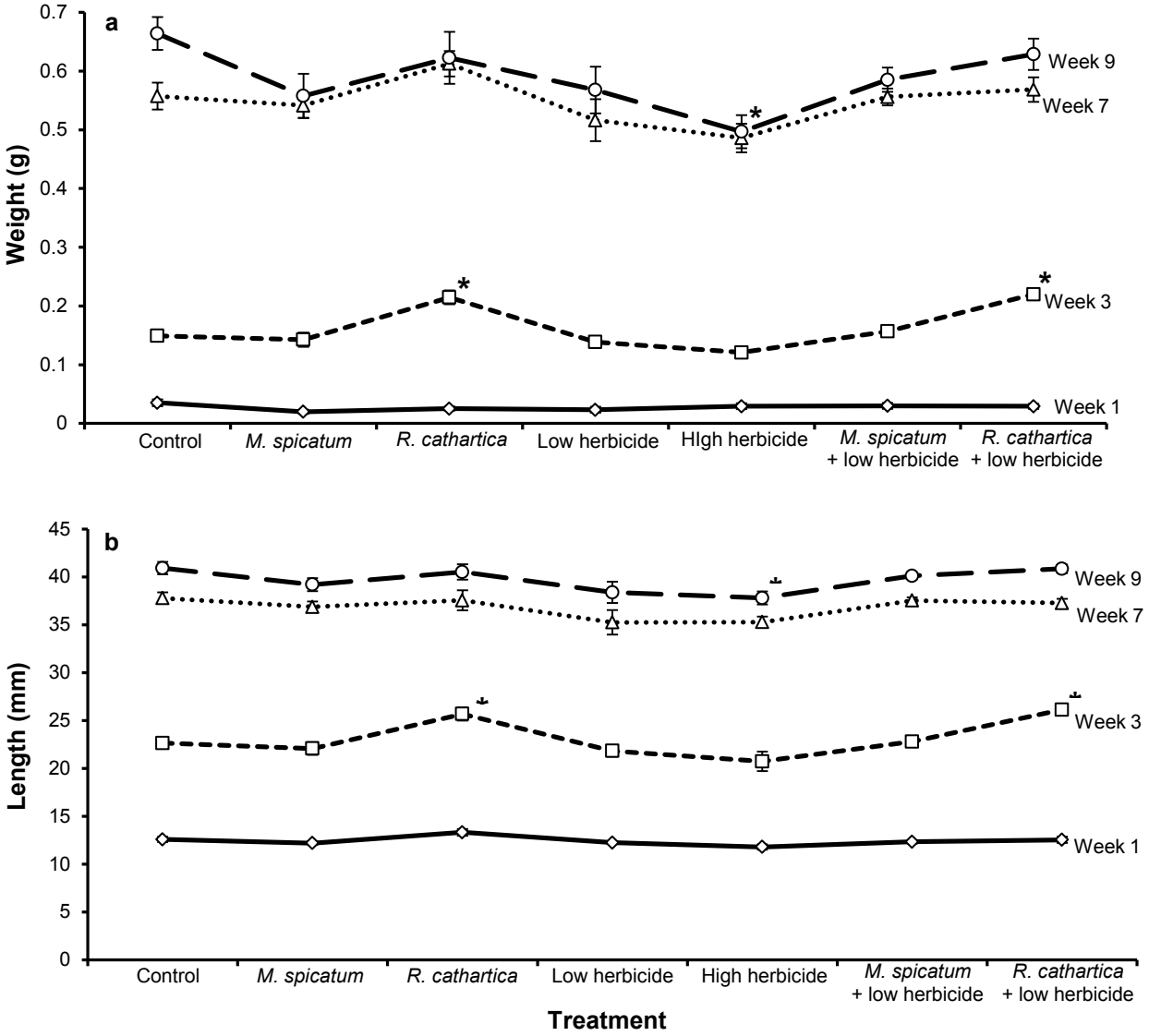

Figure 1. Effects of invasive plant leachates, the herbicide triclopyr and the combination of both factors on tadpole weight (a) and length (b) over time. Error bars represent standard errors and asterisks indicate significant difference between treatment means and those from the control group (based on Tukey's post-hoc multiple comparisons). *P<0.05

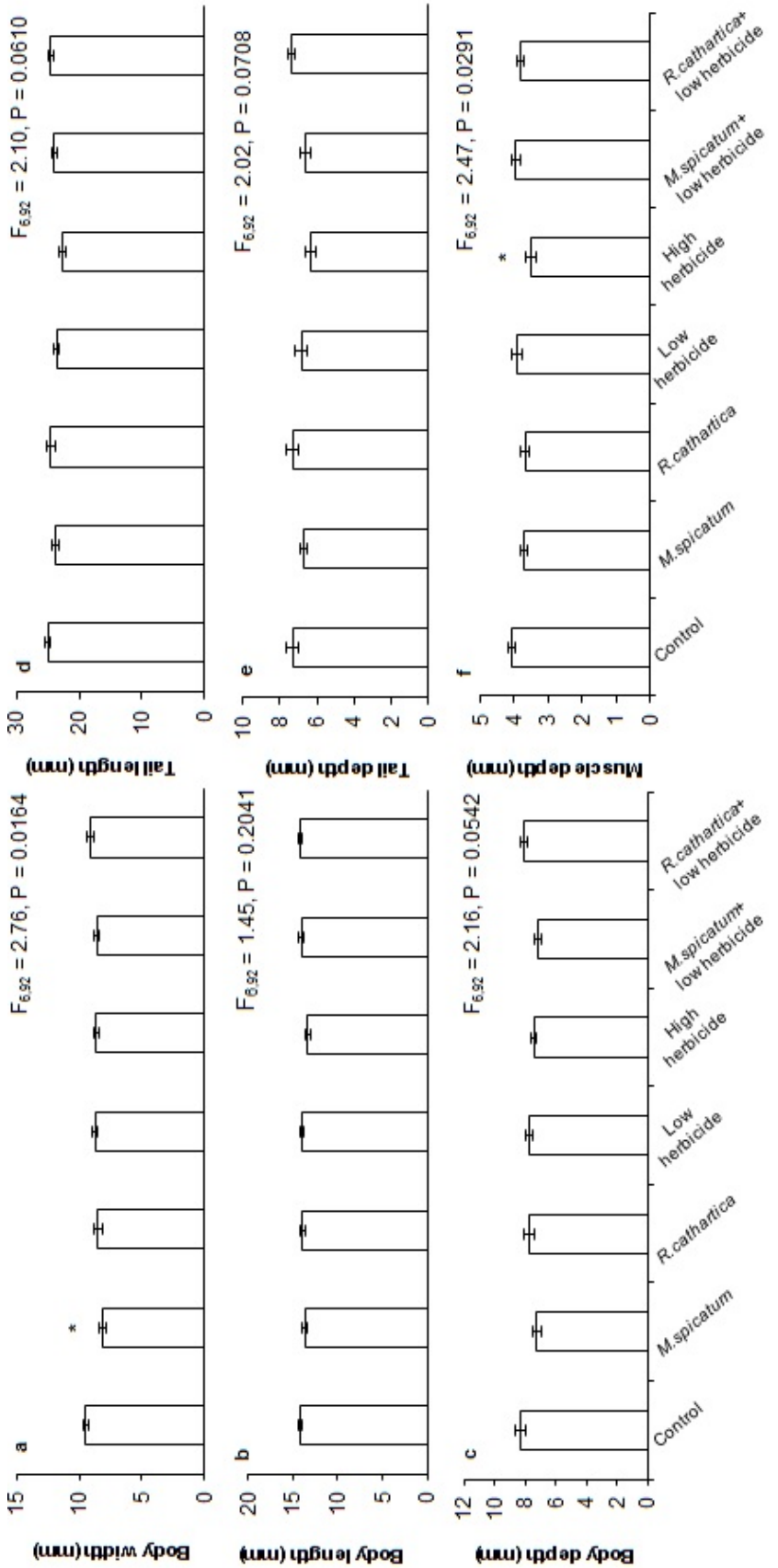

Figure 2. Effects of invasive plant extracts, the herbicide triclopyr and the combination of both factors on post-mortem tadpole morphological variables: mean body width (a), mean body length (b), mean body depth (c), mean tail length (d), mean tail depth (e), and mean muscle depth (f). Error bars represent standard errors and asterisks indicate significant difference between treatment means and those from the control group (based on Tukey's post-hoc multiple comparisons). * $P<0.05$

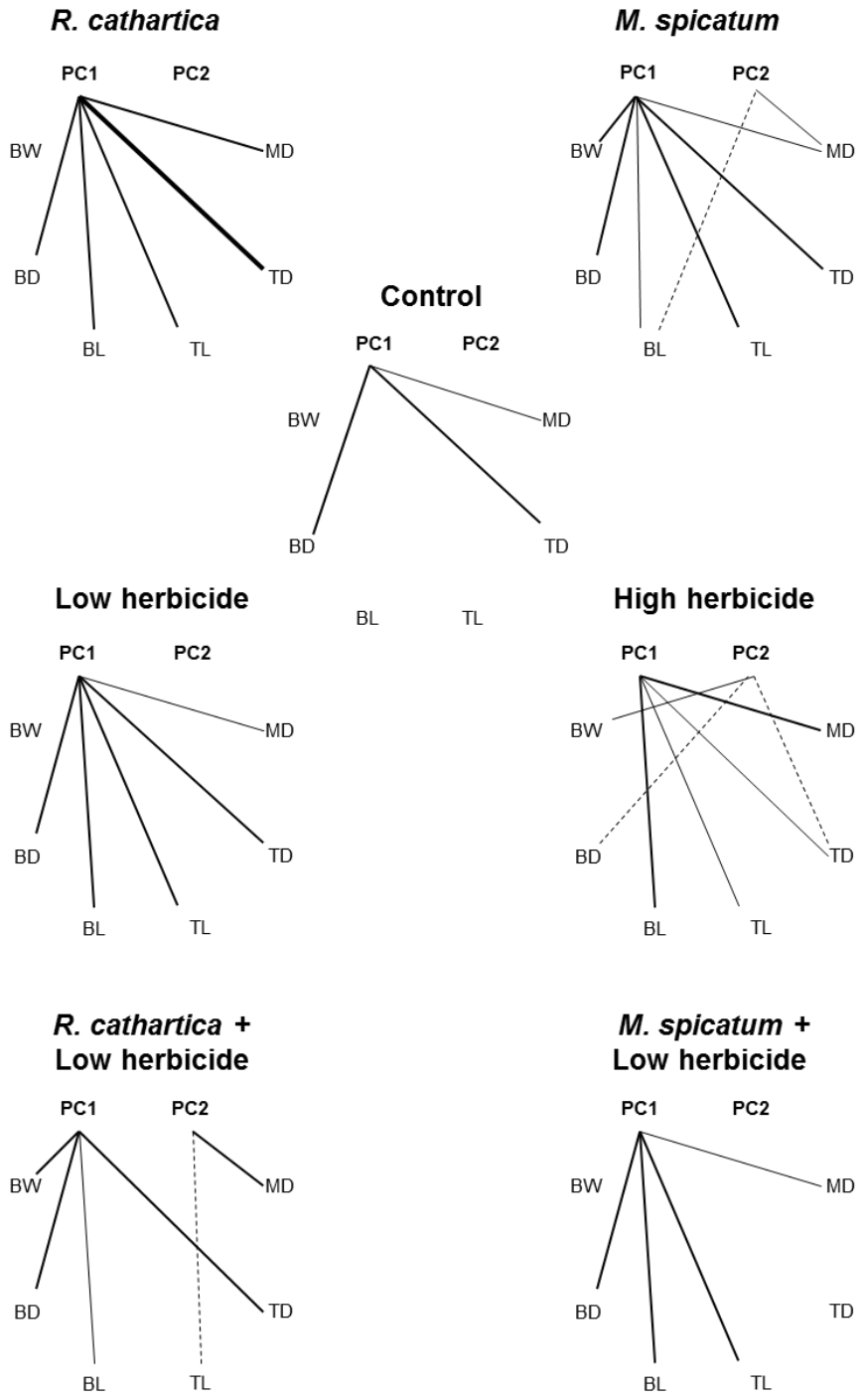

Figure 3. Principal component analysis by treatment including the following variables: body width (BW), body depth (BD), body length (BL), tail length (TL), tail depth (TD), and muscle depth (MD). Lines denote variables with significant component loadings, sij > 0.55 or < 0.55 (Tabachnick & Fidell 1989; McGarigal et al. 2000). Line thickness indicate three different ranges of absolute values of loadings: 0.55–0.73 (thinnest), 0.73–0.91 (intermediate) and > 0.91(thickest). Solid lines represent positive loadings and dashed lines indicate negative loadings

CHAPTER II:

IMPACTS OF ELEVATED TEMPERATURE, EUROPEAN BUCKTHORN, AND THE HERBICIDE TRICLOPYR ON *LITHOBATES PIPIENS* GROWTH, MORPHOLOGY, AND BEHAVIOR

ABSTRACT

Multiple factors including habitat loss/modification, pollutants, invasive species, and disease have contributed to the global decline of amphibians and declines in their abundance are expected to continue due to changes in climate. Climate change will likely allow for greater proliferation and range expansion of invasive plants. Since use of chemical management is currently the most common method to control invasive plants, chemical use may increase. A controlled laboratory experiment was performed to examine the individual and interactive effect of several environmental factors on the survival, behavior, growth and morphology of Northern leopard frog (*Lithobates pipiens*) tadpoles. Specifically, the impact of the invasive plant European buckthorn (*Rhamnus cathartica*), the herbicide triclopyr, and increased temperature were evaluated on the tadpoles for eight weeks. Results from this study showed that the presence of *R. cathartica* extracts benefited tadpole growth (i.e., increased weight and length). However, *R. cathartica* litter caused a significant reduction in dissolved oxygen, which likely contributed to significantly higher tadpole mortality. Furthermore, elevated temperatures significantly enhanced tadpole growth (i.e., increased weight and length) during the first four weeks, but this variable reached a plateau afterwards. At the conclusion of eight weeks, tadpoles in the warmer temperature were smaller in size, but more developmentally advanced. The use of triclopyr to chemically manage invasive plants appears to have minimal negative effects on tadpole growth or survival at the concentration used in this experiment. Interaction treatments designed to

simulate active chemical management using triclopyr to manage *R. cathartica* resulted in slight negative effects to tadpoles. Results from this study encourage further examination of the effect of chemical management, but more importantly the full impacts of climate change on declining amphibian populations.

INTRODUCTION

Multiple factors including habitat loss/modification, pollutants, invasive species, disease, and climate change have contributed to global declines of amphibians (Alford and Richards 1999; Bridges and Semlitsch 2000; Sparling et al. 2001; Blaustein and Kiesecker 2002; Stuart et al. 2004; Beebee and Griffiths 2005; Pounds et al. 2006; Hayes et al. 2006; Lips et al. 2008; Wake and Vredenburg 2008; Hayes et al. 2010; Hof et al. 2011; Johnson et al. 2011; Adams et al. 2013; Greenburg and Green 2013). Furthermore, amphibians are considered one of the groups most affected by climate change (Parmesan 2006). Population declines, extinctions, earlier breeding, reduced fecundity, decreased body size, and alterations in trophic interactions in amphibians have already been associated as byproducts of climate change (Blaustein et al. 2001; Walther et al. 2002; Carey and Alexander 2003; Corn 2005; Parmesan 2006; Reading 2007; McMenamin et al. 2008; Blaustein et al. 2010). While it is believed that climate change will likely exacerbate amphibian declines, either through direct environmental changes or by interaction between current stressors, experimental examinations on how increased temperatures or how the interaction of increased temperature and other stressors will affect amphibians is lacking (Donnelly 1998; Corn 2005; Blaustein et al. 2010; Hayes et al. 2010; Rohr et al. 2011; Rohr and Palmer 2012; Li et al. 2013; O'Regan et al. 2014; Zhao et al. 2014).

Increases in temperature and precipitation caused by climate change will potentially increase rates of chemical degradation, run-off, and the amount of pesticides used, thereby

increasing the toxicity of pollutants (Chen and McCarl 2001; Bloomfield et al. 2006; Noyes et al. 2009; Kattwinkel et al. 2011). Higher temperature also increases metabolic rate, therefore, may increase pesticide uptake and toxicity, decrease immune response, and increase bioaccumulation (Noyes et al. 2009). Rohr et al. (2011) found that salamander larvae developed faster when exposed to both atrazine and increased temperatures suggesting that the rapid development may offset some of the detrimental effects of atrazine exposure. However, other studies have found reduced survival of tadpoles when simultaneously exposed to increased temperature and an insecticide (Boone and Bridges 1999). A plethora of evidence has shown that pesticides can have detrimental effects on amphibians resulting in mortality, reduced size, altered morphology, behavioral changes, and endocrine disruption (Bridges 2000; Blaustein et al. 2003; Boone and James 2003; Hayes et al. 2006; Relyea and Diecks 2008; Brunelli et al. 2009; Jones et al. 2009; Relyea 2009; Relyea and Edwards 2010; Egea-Serrano et al. 2012; Relyea 2012).

Despite evidence showing negative effects of pesticides, chemical management is currently the most common method to control invasive plants in the field. In fact, since climate change will likely allow for greater proliferation and range expansion of invasive species, chemical use will presumably increase (Blossey 1999; Dukes and Mooney 1999; Mack et al. 2000; Hellman et al. 2008; Walther et al. 2009; Bradley et al. 2010). European buckthorn (*Rhamnus cathartica*) is a terrestrial invader of a variety of habitats, which shades native plants, alters soil chemistry, enhances the success of other invasive species in an "invasional meltdown", and produces the secondary chemicals emodin and physicon which are suspected to aid in outcompeting native plants (Izhaki 2002; Heneghan et al. 2004; Knight et al. 2007; Heimpel et al. 2010; Klionsky et al. 2011). Furthermore, emodin extracted from *R. cathartica* is known to cause mortality and malformations in frog embryos (Sacerdote and King 2014).

Amphibian responses to invasive plants appear to be species-specific; however, decreased survival, delayed development, population declines, deformities, and altered behavior have been observed (Maerz et al. 2005; Brown et al. 2006; Watling et al. 2011; Cohen et al. 2012; Cotten et al. 2012; Greenburg and Green 2013; Hickman and Watling 2014; Sacerdote and King 2014). Precisely how climate change may interact with invasive plant to impact amphibian species is unknown. But, Saenz et al. (2013) examined how climate change could alter exposure time of tadpoles to invasive plant extracts and found reduced survival when tadpoles were exposed to the invasive plant Chinese tallow (*Triadica sebifera*) extract earlier in the season. Therefore, it is probable that earlier amphibian breeding may expose tadpoles to invasive plant extracts earlier and have detrimental impacts on population survival (Saenz et al. 2013).

The most effective strategy to control *R. cathartica* consists of cutting a stump followed by a direct application of glyphosate or triclopyr herbicides, but resprouting and persistent seed banks can require repeated herbicide treatments (Pergams and Norton 2006; Delanoy and Archibold 2007). Glyphosate herbicides have been shown to cause morphological changes, mortality, reduced size, delayed development, and gonadal irregularities in amphibians (Howe et al. 2004; Relyea 2005; Relyea 2012). Similarly, amphibian exposure to triclopyr resulted in mortality and behavioral changes (Wojtaszek et al. 2005; Chen et al. 2008). For example, Wojtaszek et al. (2005) found no significant effect of triclopyr on tadpole growth, but mortality and altered behavior were observed. While individual effects of some invasive plants or herbicides used to control these plants have been studies, the combined effects of these two factors on amphibians are largely unknown.

This study sought to examine if chemical management of an invasive plant species in a changing climate would impact northern leopard frog (*Lithobates pipiens*) tadpoles. Northern

leopard frogs inhabit a variety of habitats, but have been experiencing population declines, local extinction, and range reductions (Smith and Keinath 2007; Johnson et al. 2010). Specifically, a controlled laboratory experiment was performed in which individually housed tadpoles were exposed to *R. cathartica* leachates, the herbicide triclopyr, and the interaction of *R. cathartica* leachates and triclopyr in both ambient and increased temperature conditions. Tadpole responses to these treatments were evaluated in terms of growth, survival, development, abnormalities, behavior, morphology, and intestine length.

The effect of water quality on tadpoles was also investigated. It was hypothesized that tadpoles would be negatively affected by the herbicide triclopyr but not by *R. cathartica* or the interaction of these two factors, as nutritional components in these extracts may enhance tadpole growth, and thus, ameliorate potential detrimental effects of herbicides. In addition, the effect of temperature was expected to modify responses of tadpoles to herbicides and plant extracts. A previous study has shown that tadpoles grown under increased temperature regimes had increased growth rates and completed their development faster (Rohr et al. 2011). This study is the first to simultaneously address the potential effects of invasive plants, herbicides and chemical management for invasive plants under projected temperature increases.

MATERIALS AND METHODS

Triclopyr Herbicide

Triclopyr is used for management of woody, broadleaf, or aquatic plants. There are two current formulations of triclopyr (3, 5, 6-trichloro-2-pyridinyloxyacetic acid) available, a triethylamine salt (TEA) or butoxyethyl ester (TBEE) (EPA 1998; Petty et al. 2003). In this study we used the herbicide Renovate® 3 (SePRO Corporation, Carmel, Indiana, USA) with TEA as the main ingredient. A Triethylamine salt break down rapidly into oxamic acid,

trichloropyridnol and/or trichloromethoxypyridine, and has an average half-life of about 6.5 days in a field-setting (Petty et al. 2003). The TBEE formulation has been shown to be more toxic than the TEA formulation to fish and amphibians (Wan et al. 1987; Perkins et al. 1999; Wojtaszek et al. 2005). Maximum triclopyr application is not believed to exceed 2.7 mg/L, but recorded application rates ranged from 0.25-7.86 mg/L (Netherland and Getsinger 1992; Kreutzweiser et al. 1994; Getsinger et al. 2000; Petty et al. 2003; Wojtaszek et al. 2005). In this study, a lower and likely more environmentally relevant concentration of 0.2 mg active ingredient (ai)/L was used (Wan et al. 1987; Perkins et al. 1999; Battaglin et al. 2009). The herbicide concentration was confirmed at 0.2057 mg/L (SePro Research and Technology Campus, Whitakers, North Carolina, USA).

Study Organisms

Fresh, green European buckthorn leaves were collected from the Slippery Elm Trail in Bowling Green, Ohio during the spring, summer and fall of 2013. Half of the collected leaves were washed with distilled water and frozen at -20°C until use. The remainder of the leaves were placed in mesh bags and placed at the Bowling Green State University (BGSU) Ecological Research Station (Bowling Green, Ohio) in an open field to experience natural drying conditions for 8-10 days to create buckthorn leaf litter. Afterwards, the leaves were placed under constant fluorescent lights in a laboratory setting for 35 days to completely dry. In this experiment, we chose to use concentrations of 1g fresh buckthorn or buckthorn litter to 1 L of aged tap water which conforms to the range of 0.5-5g of invasive plants used in current publications (Maerz et al. 2005; Watling et al. 2011).

Northern leopard frog eggs were ordered from Carolina Biological (Burlington, North Carolina, USA). Eggs were delivered to BGSU on 17 January 2014, hatched on 20 January, and

were randomly placed into treatment groups on 27 January for experiment I and 1 February for experiment II at Gosner stage 25 (Gosner 1960). Both experiments concluded on 23 March, when tadpoles were euthanized by submersion in MS-222 and preserved in a formalin solution for post-mortem analyses.

Experimental Design

Adopting the methodology of Maerz et al. (2005), plant leachate was achieved by soaking weighed plant material placed in a tea bag in 1 L of aged tap water for 48 hours. The tea bag was then removed, squeezed, and a randomly selected tadpole was added. Interaction treatments consisted of leachate along with the addition of Renovate® 3 immediately before the random addition of tadpoles. Water changes were conducted weekly, in which half of the existing water (0.5 L) was removed and replenished with freshly prepared treatment solution consisting of half the volume, but with the same concentration (e.g., 0.5 g of fresh buckthorn leaves soaked in 0.5 L of aged tap water). The herbicide concentration of water removed during water change was confirmed at 0.2357 mg/L (SePro Research and Technology Campus, Whitakers, North Carolina, USA). Fresh buckthorn treatment water, buckthorn litter treatment water, and aged tap water were analyzed for nutrient analysis by Michigan State University Soil and Plant Nutrient Laboratory (East Lansing, Michigan, USA).

Tadpoles were held individually in plastic deli cups (1.4 L) with plastic plants to mimic natural refugia sites, and randomly placed into four growth chambers. Two chambers were set at an ambient temperature of 20°C and the other two at an increased temperature of 25°C. These temperatures were chosen because the 5°C range conforms with the predicted 1.4-5.8°C increase in temperature by 2100, and they fall within the range used in similar previous studies (Gitay et al. 2002; Rohr et al. 2011; Rohr and Palmer 2012). Containers were randomly rotated within

chambers three times a week and among chambers two times a week to prevent position and chamber effects. Light conditions were held constant at 12 h light: 12 h dark. Each container received the same measured amount of Sera Micron growth food (Heinsberg, Germany) *ad libitum*. Tadpoles were checked daily for mortality. Conductivity and pH were measured for six weeks during the experiment and dissolved oxygen was measured for four weeks (Hach sensION 156 probe, Loveland, Colorado, USA).

Before tadpoles were randomly assigned to the treatment groups, their initial weights and total length were measured using an analytical balance (± 0.01g) and a digital caliper (± 0.01mm), respectively. These variables were measured weekly until tadpoles were euthanized. At the conclusion of the experiment, a stereomicroscope was used to identify developmental stage of each tadpole (Gosner 1960). Abnormalities witnessed during the experiment were confirmed with post-mortem photographs. Final morphological measurements (body width, body depth, body length, tail length, tail depth, muscle width, and muscle depth) were measured post-mortem using a digital caliper (± 0.01 mm) following the methodology of Relyea (2000). Using a stereomicroscope, pictures were taken of each tadpole's denticle and were analyzed using ImageJ (version 1.47; National Institute of Health). In order to measure intestine length, the intestines were removed from dissected tadpoles, uncoiled, and measured using a digital caliper (±0.01mm).

Tadpole behavior modified from Lawler (1989) and Bridges (2002) was evaluated by observing each tadpole for 5 seconds and then documenting the activity and position where each tadpole spent the majority of that time in its respective container. Activity of tadpoles was recorded as swimming (tail movement visible) or stationary (not moving). Position was recorded as bottom or not-bottom (middle or top) of the container. Behavior was recorded each Saturday

from 10 AM-4 PM. Although tadpoles were fed *ad lib* during the course of the experiment, food was not added until after behavior observations, so as to not be an incentive for movement.

Experiment I: Effects of European buckthorn and herbicide on tadpoles grown at two different temperatures

Tadpoles were randomly placed in one of the following treatment groups : (i) control (1L of aged tap water), (ii) fresh buckthorn leaves (1g of fresh *R. cathartica* soaked in 1L of aged tap water), (iii) buckthorn leaf litter (1g of *R. cathartica* leaf litter soaked in 1 L of aged tap water), (iv) herbicide (0.2 mg ai Renovate® 3 in 1 L of aged tap water), (v) fresh buckthorn and herbicide (1g of fresh *R. cathartica* leaves soaked in 1L of aged tap water + 0.2 mg ai Renovate® 3), and (vi) buckthorn leaf litter and herbicide (1 g of *R. cathartica* litter soaked in 1L of aged tap water + 0.2 mg ai Renovate® 3). Each treatment group was replicated 20 times in two temperature regimes consisting of 20°C and 25^0C (total of 240 tadpoles). Due to the unexpected mortality of tadpoles in treatments containing buckthorn litter (65-100% mortality) in this experiment (probably due to low oxygen concentrations), tadpoles in these treatments were eliminated from statistical tests and a separate experiment (II) was conducted to assess the effects of buckthorn litter, herbicide and temperature on tadpole growth.

Experiment II: Effects of European buckthorn litter and herbicide on tadpoles grown at two different temperatures

In order to further assess the effect of *R. cathartica* litter on tadpoles, tadpoles were randomly placed into one of the following treatments: (i) control (1 L aged tap water), (ii) buckthorn litter (0.5 g of *R. cathartica* leaf litter soaked in 1 L of aged tap water), and (iii) the interaction of buckthorn litter and herbicide (0.5 g *R. cathartica* soaked in 1 L aged tap water +

0.2 mg ai Renovate® 3). Each treatment group was replicated 15 times in both 20°C and 25^{0}C for a total of 90 tadpoles.

Statistical Analyses

All data were analyzed using SAS (version 9.2). When assumptions of normality and heterogeneity were not met, data were logarithmically or rank-transformed (Potvin and Roff 1993; Brunner et al. 2002). Post-hoc pairwise comparisons among treatment means were performed using Tukey's honest significant difference (HSD). Tadpole survival and abnormalities across treatments were analyzed using Fisher's exact tests. A repeated-measures multivariate analysis of variance (rmMANOVA) was used to analyze whether different treatment levels (i.e., control, buckthorn leachate, herbicide, and buckthorn leachate + herbicide) and temperature affected tadpole weight and total length over time. Specifically, the effects of treatment, temperature, time, treatment × temperature, treatment × time, temperature × time, and treatment × temperature × time were evaluated on tadpole weight and total length, water pH, dissolved oxygen, and conductivity. Analyses of variance (ANOVA) were performed to assess the effects of treatment, temperature, and treatment × temperature on tadpole weight, total length, conductivity, pH, dissolved oxygen, intestine length, developmental stage, and tadpole morphology (i.e., body width, body depth, body length, tail length, tail depth, muscle width, muscle depth, denticle width, and denticle length).

Behavioral data were divided into the first four weeks (or in the case of experiment II, the first three weeks) and the last four weeks. The proportion of weeks spent swimming to stationary and the proportion of weeks at the bottom to not bottom was calculated for each tadpole. Lastly, the difference between the weeks for activity and position was calculated. An ANOVA was conducted on this difference to assess the effects of treatment, temperature, and

treatment x temperature on tadpole behavior. Tukey's HSD was used to identify significant differences between treatment groups.

RESULTS

Experiment I: Effects of European buckthorn and herbicide on tadpoles grown at two different temperatures

Survival was high in all treatments (95-100%); therefore, there were no significant treatment effects on survival (P = 0.5845; Fig.4a). Repeated-measures multivariate analysis of variance revealed significant effects of treatment, and time on tadpole weight and length over the course of the experiment (Table 2). The repeated-measures analysis of variance (rmANOVA) showed that treatment, time, temperature, treatment x time, treatment x temperature, and time x temperature significantly influenced tadpole weight (Table 2). In addition, a rmANOVA for tadpole total length revealed that treatment, time, treatment x time, treatment x temperature, time x temperature, and treatment x time x temperature significantly impacted this response variable (Table 2).

Gosner stage was significantly affected by treatment ($F_{3, 149} = 5.47$, P = 0.0014; Fig. 4c), temperature ($F_{1, 149} = 34.26$, $P < 0.0001$), and temperature × treatment ($F_{3, 149} = 10.58$, $P < 0.0001$). Tadpoles in elevated fresh buckthorn, elevated fresh buckthorn + herbicide, elevated control, and ambient herbicide had significantly more developed limb buds than ambient control tadpoles. Thus, temperature had a clear impact on tadpole development across all treatments, with the exception of tadpoles exposed to the herbicide treatment and elevated temperature. Further, when examining development within the ambient temperature, tadpoles exposed to herbicides and ambient temperature had a significantly higher Gosner stage than those growing under control conditions or exposed to fresh buckthorn.

No body or tail abnormalities were observed in tadpoles growing under control conditions and there was no significant difference among treatment groups ($P = 0.0828$). However, abnormalities were observed in tadpoles exposed to all the other treatments, with 1-5 abnormalities per 15-19 tadpoles in each treatment (Fig. 5). More body and tail abnormalities (2-5 per treatment) were found in tadpoles grown under elevated temperatures, especially in the fresh buckthorn + herbicide treatment. On the other hand, only one abnormal tadpole was found in the fresh buckthorn, herbicide, and fresh buckthorn + herbicide treatments exposed to ambient temperatures.

Initial tadpole weight was not significantly different before placement in treatment groups ($F_{3, 152} = 2.38$, $P = 0.0721$; Fig. 6a), but all subsequent tadpole weights differed significantly. By the second measurement of weight at the end of week 1, all tadpoles in the elevated temperature displayed significantly greater weights than those in the ambient temperature ($F_{3, 151} = 2.00$, $P = 0.1165$). Additionally, tadpoles in elevated fresh buckthorn and elevated fresh buckthorn + herbicide were found to be significantly larger during the second and third week of the experiment (2^{nd} Week: $F_{3, 151} = 12.55$, $P < 0.0001$; 3^{rd} Week: $F_{3, 151} = 14.34$, $P < 0.0001$). During week 4, tadpoles growing under ambient temperature caught up in growth to match the increased weights found under elevated temperatures (in the buckthorn and buckthorn + herbicide treatments). Furthermore, tadpoles growing under elevated temperatures in the control and herbicide treatments were significantly smaller than control tadpoles raised at ambient temperatures at weeks 4 and 5 (Week 4: $F_{3, 151} = 8.51$, $P < 0.0001$; Week 5: $F_{3, 151} = 4.23$, $P = 0.0067$). Weights of tadpoles exposed to elevated temperature in the control, herbicide, and fresh buckthorn + herbicide treatments were significantly lower than those growing under ambient temperature and control conditions on weeks 6 and 7 (Week 6: $F_{3, 151} = 6.59$, $P =$

0.0003; Week 7:$F_{3, 151} = 9.48$, $P < 0.0001$). Reduced final weight was observed in tadpoles exposed to all treatments when growing under elevated temperature ($F_{3, 150} = 11.58$, $P < 0.0001$). Within the elevated temperature group, tadpoles exposed to fresh buckthorn and fresh buckthorn + herbicide were generally significantly heavier than those exposed to control or herbicide treatments on weeks 1, 2, 3, 5, 6, 7, and 8.

Initial tadpole total length was not significantly different among treatment groups ($F_{3, 151} = 0.22$, $P = 0.8819$; Fig. 6b). However, similar to the trend observed with tadpole weight, tadpoles in all the treatments under elevated temperatures were significantly longer on week 1 and 2 (Week 1: $F_{3, 151} = 4.97$, $P = 0.0026$; Week 2: $F_{3, 151} = 12.50$, $P < 0.0001$). Tadpoles growing in the fresh buckthorn and fresh buckthorn + herbicide treatments under elevated temperatures were significantly longer than those reared all other treatments on weeks 3 and 4 (Week 3: $F_{3, 151} = 7.00$, $P = 0.0002$; Week 4: $F_{3, 151} = 3.60$, $P = 0.0151$). On the 5th week, length of tadpoles growing under ambient temperature increased to match the length of those growing under elevated temperature conditions. This trend was maintained until the conclusion of the experiment. Tadpoles in the herbicide treatment under elevated temperature were significantly shorter than those exposed to control conditions and ambient temperature on weeks 5 and 7 (Week 5: $F_{3, 151} = 1.19$, $P = 0.3161$; Week 7: $F_{3, 151} = 2.81$, $P = 0.0416$). Tadpoles exposed to control conditions and elevated temperatures displayed significantly reduced length compared to ambient control tadpoles during weeks 6 and 8 (Week 6: $F_{3, 151} = 2.65$, $P = 0.0511$; Week 8: $F_{3, 150} = 6.47$, $P = 0.0004$). Comparable to previous trends observed for tadpole weights, tadpoles exposed to elevated temperatures were significantly larger than those growing at ambient temperature, especially in the control and herbicide treatments throughout the experiment.

Almost all the morphological measures exhibited significant differences that were predominantly influenced by temperature and treatment, with few temperature× treatment interaction effects (Table 4, Fig. 7a-j). Tail depth was significantly reduced in the control, herbicide and fresh buckthorn + herbicide treatments under elevated compared to the ambient temperature and control treatment ($F_{3, 149} = 11.53$, $P < 0.0001$; Fig. 7a). Under elevated temperatures, tadpoles exposed to fresh buckthorn produced significantly thicker tail depth versus those from tadpoles reared in the control and herbicide treatments. Tail length of tadpoles exposed to all treatments was not significantly different from those of the control under ambient temperature ($F_{3, 149} = 3.36$, $P = 0.0204$; Fig. 7b). However, within the elevated temperature, tadpoles in fresh buckthorn and fresh buckthorn + herbicide displayed significantly longer tails versus those in the control treatment under elevated temperature.

Tadpoles grown in control, herbicide and fresh buckthorn + herbicide treatments under elevated temperature had significantly shorter bodies compared to tadpoles in the control treatment under ambient temperature ($F_{3, 149} = 7.85$, $P < 0.0001$; Fig. 7c). Body depth of tadpoles in fresh buckthorn and fresh buckthorn + herbicide was significantly larger than tadpoles in the control treatment group under the ambient temperature ($F_{3, 149} = 14.89$, $P < 0.0001$; Fig. 7d). Tadpole body depth of fresh buckthorn tadpoles was significantly larger than tadpoles in herbicide and control treatment under the elevated temperature, while the fresh buckthorn + herbicide tadpoles were significantly larger than the control tadpoles exposed to the elevated temperature. Under the elevated temperature, significantly thinner body width was observed in tadpoles fresh buckthorn + herbicide, herbicide, and control tadpoles compared to control tadpoles raised under the ambient temperature ($F_{3, 149} = 9.47$, $P < 0.0001$; Fig 7e). Yet, when body width of tadpoles exposed to the different treatments within the elevated temperature was

compared, tadpoles in fresh buckthorn and fresh buckthorn + herbicide treatments exhibited significantly wider bodies than the control tadpoles.

All treatments in the elevated temperature had significantly reduced muscle width, with the herbicide treatment having the thinnest tail muscles ($F_{3, 149} = 5.89$, $P = 0.0008$; Fig. 7f). Muscle depth was significantly reduced in tadpoles exposed to herbicide under the elevated temperature and fresh buckthorn tadpoles reared under the ambient temperature were significantly larger compared to tadpoles grown in the control treatment under the ambient temperature ($F_{3, 149} = 3.49$, $P = 0.0123$; Fig. 7g).

Under the elevated temperature, denticle width was significantly reduced in the herbicide and control treatments ($F_{3, 149} = 1.94$, $P = 0.1254$; Fig. 7h). However, denticle length was not significantly different ($F_{3, 149} = 3.07$, $P = 0.0299$; Fig. 7i). Intestine length was reduced in control, herbicide, and fresh buckthorn + herbicide treatments in the elevated temperature ($F_{3, 149} = 4.28$, $P = 0.0062$; Fig. 7j). Additionally, within the elevated temperature tadpoles exposed to fresh buckthorn had significantly longer intestines than control and herbicide tadpoles.

During behavioral observations, tadpoles were generally stationary and remained at the bottom of the container. Therefore, tadpole activity (swimming or stationary) was not significantly influenced by treatment ($F_{3, 151} = 0.48$, $P = 0.6958$), temperature ($F_{1, 151} = 0.13$, $P = 0.7151$), or temperature ×treatment ($F_{3, 151} = 0.03$, $P = 0.9920$). Treatment ($F_{3, 151} = 3.69$, $P = 0.0129$) and temperature ($F_{1, 151} = 16.51$, $P < 0.0001$) significantly impacted tadpole position. Tadpoles raised in the herbicide treatment under the ambient temperature spent more time in the middle or top of the container in the first four weeks of the experiment, but during the last four weeks spent more time at the bottom of the container. Whereas, fresh buckthorn, herbicide, and fresh buckthorn + herbicide tadpoles under the elevated temperature and fresh buckthorn

tadpoles in the ambient temperature were almost always found at the bottom of the container during the whole experiment.

Significant effects of treatment, time, temperature, treatment ×time, temperature ×time, treatment ×temperature, and treatment ×time ×temperature on dissolved oxygen, pH and conductivity were found (Table 6). Despite the significant differences, water quality ranges in this experiment conform to values that tadpoles would likely experience in nature (Noland and Ultsch 1981; Hechar and M'Cluskey 1996). For instance, the pH range recorded in nature and in published studies is 6.00-10.20, while the range of pH for all treatments in this experiment was 6.78-8.08 (Schlichter et al. 1981; Hecnar and M'Cluskey 1996; Ouellet et al. 1997; Harris et al. 1998; King et al. 2007). Control treatment groups, particularly the ambient control group tended to have pH values closer to neutral, while other treatments generally had more alkaline pH values. Dissolved oxygen levels in this experiment ranged from 2.96- 5.09 mg/L, which conform to levels of 1-19 mg/L that amphibians may experience in nature (Noland and Ultsch 1981; Harris et al. 1998). Generally, dissolved oxygen was significantly lower in all elevated temperature treatments, but also in fresh buckthorn and fresh buckthorn + herbicide treatments in the ambient temperature. Conductivity was 476-618 μS/cm for all treatments, consistent with the expected natural range of 124-3100 μS/cm (Hecnar and M'Cluskey 1996; Harris et al. 1998; King et al. 2007). All treatments had higher conductivity than the control treatment in the ambient temperature.

Experiment II: Effects of European buckthorn litter and herbicide on tadpoles grown at two different temperatures

Tadpole survival was effected by treatment ($P = 0.0004$, Fig. 4b). Buckthorn litter (47% for 20^0C and 73% in 25^0C) and buckthorn litter +herbicide (60% in 20^0C and 87% in 25^0C)

treatments had mortality in both temperatures, while tadpoles in control treatments at ambient or elevated temperatures experienced no mortality. The repeated-measures multivariate analysis of variance found that treatment and temperature significantly affected tadpole weight and length over time (Table 3). Similarly, rmANOVAs found that group, treatment, time, temperature, treatment ×time, and temperature ×time significantly influenced tadpole weight and length over the course of the experiment (Table 3).

Gosner stage at the conclusion of the experiment was influenced by treatment ($F_{2, 64}$ = 5.92, P = 0.0044), temperature ($F_{1, 64}$ = 65.88, P < 0.0001), and temperature × treatment ($F_{2, 64}$ = 3.32, P = 0.0424; Fig. 4d). At the end of the experiment, tadpoles reared under the elevated temperature were significantly more developed. Moreover, within the elevated temperature, tadpoles in the buckthorn litter + herbicide treatment were significantly more developed than tadpoles in the control treatment. The occurrence of body or tail abnormalities was not significantly different among treatments (P = 0.1437). Further, abnormalities (1 tadpole/treatment) were only observed in the buckthorn litter treatments in the ambient temperature and elevated temperature (Fig. 8).

Tadpole weight did not differ before addition to treatment groups ($F_{2, 84}$ = 5.13, P = 0.0079; Fig. 9a). By the end of week 1, tadpoles in the control and buckthorn litter under the elevated temperature were significantly heavier than other tadpoles ($F_{2, 64}$ = 14.91, P < 0.0001). On week 2, tadpoles in buckthorn litter in the elevated temperature were significantly heavier than control tadpoles in the ambient temperature ($F_{2, 64}$ = 1.85, P = 0.1648). Similar to the trend observed in Experiment I, tadpoles in ambient temperature caught up to weight of tadpoles grown under the elevated temperature. At weeks 3 ($F_{2, 64}$ = 11.35, P < 0.0001) and 4 ($F_{2, 64}$ = 8.72, P = 0.0004) tadpoles in the ambient buckthorn litter were significantly heavier than control

tadpoles grown in the ambient temperature. Tadpoles in the buckthorn litter and buckthorn litter + herbicide treatments in the ambient temperature were significantly heavier on weeks 5 ($F_{2, 64}$ = 12.85, $P < 0.0001$), 6 ($F_{2, 64}$ = 13.58, $P < 0.0001$), and 7 ($F_{2, 64}$ = 23.39, $P < 0.0001$) than the control treatment in the ambient temperature control tadpoles. For weeks 4, 5, 6, and 7, tadpoles grown in the control treatment under elevated temperature had significantly reduced weights.

Initial total length of tadpoles was not significantly different prior to random placement into treatment groups ($F_{2, 84}$ = 2.23, P = 0.1135; Fig. 9b). By the end of the 1st week, tadpoles in the control and buckthorn treatment in the elevated temperature were significantly longer ($F_{2, 64}$ = 43.29, $P < 0.0001$) and on the 2nd week, all treatments in the elevated temperature were significantly larger ($F_{2, 64}$ = 6.53, P = 0.0061). On week 3, total length of tadpoles was not significantly different ($F_{2, 64}$ = 5.94, P = 0.0043). Buckthorn litter tadpoles under the ambient temperature were significantly longer than control tadpoles under the ambient or elevated temperatures on week 4 ($F_{2, 64}$ = 5.84, P = 0.0047) and on week 5, buckthorn litter tadpoles were significantly longer than control tadpoles in the elevated temperature ($F_{2, 64}$ = 5.53, P = 0.0061). Tadpoles in the buckthorn litter grown in the ambient temperature were significantly longer on weeks 6 and 7 (Week 6: $F_{5, 64}$ = 7.88, P = 0.0009; Week 7: $F_{2, 64}$ = 12.66, $P < 0.0001$) than the control treatment raised under the same temperature.

Morphology was most affected by temperature, treatment, and seldom by temperature × treatment (Table 5). Treatments in the elevated temperature exhibited significantly reduced tail depth ($F_{2, 64}$ = 12.46, $P < 0.0001$; Fig. 10a). The buckthorn litter tadpoles under the ambient temperature had deeper and significantly longer tails than control tadpoles in the ambient temperature ($F_{2, 64}$ = 12.48, $P < 0.0001$; Fig. 10b). Under the elevated temperature, buckthorn litter + herbicide tadpoles had longer and deeper tails than the control tadpoles.

Body length ($F_{2, 64} = 9.94$, $P = 0.0002$; Fig. 10c), body depth ($F_{2, 64} = 7.48$, $P = 0.0012$; Fig. 10d), and body width ($F_{2, 64} = 8.99$, $P = 0.0004$; Fig. 10e) of control tadpoles in the elevated temperature were significantly reduced compared to control treatment tadpoles in the ambient temperature. Additionally, within the elevated temperature, tadpoles in the buckthorn litter + herbicide treatment had significantly larger and longer bodies than tadpoles in the control treatment. Tail muscle width ($F_{2, 64} = 8.09$, $P = 0.0007$; Fig. 10f) was reduced in all treatments in the elevated temperature and tail muscle depth was reduced only in the control treatment ($F_{2, 64} = 12.80$, $P < 0.0001$; Fig. 10g). Buckthorn litter and buckthorn litter + herbicide treatments in the ambient temperature had significantly greater tail muscle depth.

Denticle width ($F_{2, 64} = 1.52$, $P = 0.2263$; Fig. 10h) and denticle length ($F_{2, 64} = 1.02$, $P = 0.3671$; Fig. 10i) were not significantly different from the control treatment under the ambient temperature. However, in the elevated temperature denticle length of buckthorn litter + herbicide treatment was significantly longer than control treatment. A temperature influence on denticle length was observed between the buckthorn litter + herbicide in the elevated temperature, which exhibited long mouths, and the buckthorn litter + herbicide tadpoles in the ambient temperature, which exhibited shorter mouths. Tadpoles in the buckthorn litter and buckthorn litter + herbicide treatments under the ambient temperature had significantly longer intestines ($F_{2, 64} = 20.78$, $P < 0.0001$; Fig. 10j). Conversely, ambient buckthorn litter tadpoles had longer intestines than tadpoles in the elevated buckthorn litter treatment.

Treatment, time, temperature, treatment × time, treatment × temperature, and treatment × temperature × time significantly affected dissolved oxygen, pH, and conductivity (Table 7). Ranges of dissolved oxygen (3.03-4.92 mg/L), pH (6.97-8.10), and conductivity (503-632 μS/cm) measured in this experiment are consistent with ranges that amphibians would likely

experience in nature (Noland and Ultsch 1981; Schlitcher et al. 1981; Hechar and M'Cluskey 1996; Ouellet et al. 1997; Harris et al. 1998; King et al. 2007). Similar to the findings of experiment I, neutral pH values were observed in the control treatment under the ambient temperature, while other treatments groups exhibited more alkaline values. Dissolved oxygen was generally lower under the elevated temperatures, especially in the buckthorn litter and buckthorn litter + herbicide treatments. Conductivity tended to be significantly higher in all treatments compared to the control treatment in the ambient temperature.

As with experiment I, tadpoles were generally stationary and spent most of the time at the bottom of the container. Treatment ($F_{2, 64} = 0.13$, $P = 0.8749$), temperature ($F_{1, 64} = 0.58$, $P = 0.4494$) or temperature × treatment ($F_{2, 64} = 0.00$, $P = 0.9985$) did not influence tadpole activity. Likewise, tadpole behavior was not affected by treatment ($F_{2, 64} = 0.35$, $P = 0.7040$), temperature ($F_{1, 64} = 0.63$, $P = 0.4294$), or temperature × treatment ($F_{2, 64} = 0.37$, $P = 0.6955$).

DISCUSSION

In this experiment, the potential non-target effects of chemical management to control *R. cathatrica* on *L. pipiens* tadpoles under an ambient (normal temperatures for *L. pipiens* development) and elevated temperature (a temperature that falls within predicted climate change projections) were examined. At the concentration used in this experiment (0.2057 mg ai /L), the use of triclopyr to chemically manage invasive plants appeared to have no effect on tadpole survival, but did negatively influence tadpole growth. The presence of *R. cathartica* seemed to have a beneficial impact on tadpole growth. Minimal negative effects on tadpole growth or morphology were found in the interaction treatments (*R. cathartica* + triclopyr). Elevated temperature appeared to strongly influence early growth and final developmental stage.

Elevated temperatures appeared to increase tadpole weight and length for approximately the first four weeks, but plateaued until the conclusion of the experiments. While some studies have found that warmer temperatures can increase tadpole growth and decrease developmental time, others have found a reduction in size likely associated with temperature associated increases in metabolism (Rohr et al. 2011; Caruso et al. 2014; O'Regan et al. 2014; Zhao et al. 2014). Metabolism is largely determined by body size and temperature and the trend observed in these experiments may be largely explained by an increased metabolism in treatment groups grown under elevated temperature (Gillooly et al. 2001; Dillon et al. 2010). Therefore, in the elevated temperature, the increased growth during the first four weeks of the experiments may have been caused by an increased metabolism which allowed for faster growth. However, it can be speculated that between week 4 and 5 (or week 3 and 4 for experiment II), the larger tadpole weight coupled with the warmer temperature resulted in metabolic rates that might have been so high that continued growth of tadpoles nearly plateaued. Personal observations revealed that limb buds were visible by week 2 in almost all tadpoles for experiment I. In experiment II, some tadpoles in the elevated temperature had limb buds at week 1, but nearly all had limb buds visible by week 2, regardless of temperature or treatment. Therefore, tadpoles in the elevated temperature may have devoted more energy to a quicker development and stopped allocating energy toward increasing size.

Increased development observed under increased temperature for both experiments corresponds with previous findings that under warmer temperatures amphibians larvae appear to develop faster (Rohr et al. 2011; Rohr and Palmer 2012; O'Regan et al. 2014). However, the tadpoles exposed to herbicide were more developed in the ambient temperature than in the elevated temperature. Rohr et al. (2011) found that salamander embryos and larvae exposed to

the herbicide atrazine and warmer temperature exhibited larger size and accelerated development. Moreover, O'Regan et al. (2014) found evidence that warming induced earlier metamorphosis, but no impact on tadpole size. In these experiments, we found the opposite: elevated temperatures clearly decreased tadpole size, but accelerated development. Since increased temperature allows for greater evaporation of water and likely contributes to pool drying, tadpoles may associate a warmer temperature with oncoming pool drying and therefore develop quicker (Newman 1992; O'Regan et al. 2014). It may be possible that at an elevated temperature tadpoles face a trade-off between developing faster at a smaller size, but risk their pool drying up and either desiccating or drying before they have completed metamorphosis.

Additionally, significant morphological differences between the ambient and elevated temperatures were observed. The elevated temperature reduced tail depth, body length, body width, muscle width, denticle width, and intestine length which could result in reduced survival, increased predation and reduced fitness (Werner 1986; Touchon et al. 2013). Tadpoles with longer tails and more muscular tails tend to be faster swimmers capable of avoiding predation better (Teplitsky et al. 2005; Arendt 2010). Therefore, it may be possible that tadpoles exposed to fresh buckthorn in the ambient temperature had longer, deeper tails with deeper tail muscles, that they are faster swimmers that would face reduced predation (Touchon et al. 2013). While it was not addressed in the study, it may be possible that these morphological induced changes could affect predator avoidance, probability of surviving to adulthood and adult fitness.

Tadpole behavior was not very different across different treatments and temperatures, despite personal observations of tadpoles in elevated temperatures quickly darting wildly in their containers. Overall, tadpoles remained relatively stationary at the bottom of the container, which may be a predator-avoidance strategy (Lawler et al. 1989). Significant differences were

observed in the herbicide treatment in the ambient temperature, where tadpoles spent more time in the middle or top of the container during the first half of the experiment. However, in the latter half of the experiment, tadpoles were almost exclusively found on the bottom of their containers. Further, warmer temperatures often cause an increase in activity level, potentially increasing the risk of predation, while also increasing metabolic rate and reducing energy reserves. Zhao et al. (2014) found that under warmer temperatures, tadpole size increased, but predation risk did not decrease. Therefore, the behavioral experiments conducted here may not have been rigorous or complete enough to fully assess the impact of *R. cathartica*, triclopyr, and warmer temperature on tadpole behavior (Duelmann and Trueb 1994).

The presence of *R. cathartica* appears to be an important factor impacting tadpole size, development, and morphology. The beneficial effects observed here and previously (Chapter 1) may be a byproduct of the using tea bags to soaking fresh buckthorn and buckthorn litter weekly, which inadvertently allowed for plant tissue nutrients to permeate through the pores in the tea bag and provide excess food. Tadpoles consuming this plant leachate along with *ad lib* commercial food likely converted the excess food into increased size. Additionally, buckthorn leaf litter was much more brittle and smaller than the fresh buckthorn and it is possible that more particles could have easily penetrated the tea bag producing more nutrients available for tadpoles. However, the excess plant material had the trade-off of lower dissolved oxygen. The decreased dissolved oxygen likely contributed to the immediate mortality of tadpoles placed in the buckthorn litter treatments in experiment I (1 g/L concentration) and may partially account for the significantly lower survival rates of experiment II. Dissolved oxygen measurements of similar conditions (initial 1 g/L buckthorn litter) revealed nearly anoxic levels (range for 20°C 0.13-0.36 mg/L and at 25°C was 0.10-0.17 mg/L), which alone or perhaps coupled with

inadvertent bacterial contamination on the leaf litter likely caused the death of the young and fragile tadpoles. Decreased dissolved oxygen associated with the breakdown of invasive plant material has been reported by others, with lower oxygen levels with larger quantities of invasive plant material (Cohen et al. 2012; Earl et al. 2012; Saenz et al. 2013). The genus *Lithobates* have lower oxygen consumption rates and therefore can tolerate lower oxygen levels, but other genera may not be able to withstand the lower oxygen levels associated with *R. cathartica* (Bennett 1978). Therefore, the effects of *R. cathartica* on amphibians may elicit genera-specific or species-specific responses in amphibians, which have been observed in previous studies using other invasive plants (Maerz et al. 2005; Cohen et al. 2012).

Under the ambient temperature tadpoles in buckthorn litter and buckthorn litter + herbicide had significantly longer intestines. Longer intestines have been associated with increased digestion efficiency to deal with lower quality or quantity of food resources (Relyea and Auld 2004; Venesky et al. 2014). However, since tadpoles in these treatments had access to extra food that permeated the tea bags, decreased quantity of food cannot explain the increased intestine length. It is unclear whether *R. cathartica* represents a high quality food item, but it is believed that the secondary chemical emodin present in *R. cathartica* deters herbivory and acts as a laxative in mammals and birds (Izaki 2002). Stoler and Relyea (2013) found that higher nitrogen content of plant material induced shorter intestines in wood frog tadpoles (*Lithobates sylvaticus*). However, since *R. cathartica* has high nitrogen percentage, results from this study appear to contradict that previous finding (Heneghan et al. 2004; Stoler and Relyea 2013). Intestine length was greatly reduced in the elevated temperature, which may partially explain why tadpoles in this environment exhibited reduced final size.

The buckthorn + herbicide treatments displayed similar growth trends as the invasive plant treatments (fresh buckthorn or buckthorn litter) and were generally larger than control or herbicide treatments. Increased food available in those groups probably explains why tadpole weight and length of buckthorn + herbicide treatments correspond closely with the invasive plant treatments. However, distinctions between the individual buckthorn treatments and the buckthorn + herbicide treatment are evident in differences in tadpole morphology, especially within the elevated temperature. For example, in the elevated temperature tadpoles in the fresh buckthorn + herbicide treatment had smaller bodies and were closer in resemblance to the control or herbicide treatment. Likewise in the ambient temperature, tadpoles raised in the buckthorn litter treatment had longer and deeper tails than those grown in the buckthorn litter + herbicide treatment. Therefore, the presence of the herbicide may have slight, but not overwhelming constraints on tadpole size.

It was hypothesized that triclopyr would be toxic to tadpoles and thus, we would see significantly reduced growth and high mortality. However, under both the ambient and elevated temperature tadpole survival when exposed to herbicide was 100%. Reductions in growth and morphology were observed between tadpoles exposed to herbicide grown under the elevated temperature compared to control tadpoles in the ambient temperature. Under the ambient temperature, morphology of tadpoles exposed to herbicide was similar to tadpoles in the control treatment. In addition, within the elevated temperature the herbicide tadpoles often displayed similar growth and morphology as tadpoles in the control treatment. In chapter I, a reduction of final weight, final length, and tail muscle depth was found only at the higher concentration (0.9 mg ai/L of herbicide, but not with the lower concentration (0.2 mg ai/L). It appears that the elevated temperature has a much greater effect on tadpole growth and morphology than direct

exposure to triclopyr herbicide alone, which again is likely due to increased metabolism experienced in the warmer temperature.

The specific uptake and mechanism that would cause reduction in size of tadpoles exposed to triclopyr is largely unknown. It is believed that triclopyr has a low accumulation potential due to rapid breakdown and it mostly eliminated from the body in urine (Timchalk et al. 1990). However, the TBEE formulation of triclopyr was found to accumulate in the muscle tissue of fish and reduce maternal fitness (Johansen and Geen 1990; Getsinger et al. 2000; Petty et al. 2003; Carney et al. 2007). Hayes et al. (2002) found that exposure to the herbicide atrazine, induced gonadal changes and increased rates of hermaphrodites in tadpoles. The effects of triclopyr on reproduction or endocrine disruption were outside the scope of this research; but future experiments are needed in order to fully grasp the potentially effects that triclopyr could have on amphibians populations.

If the experiments performed here were terminated at four weeks, as are commonly performed in ecotoxicology experiments, the results would have been skewed and represented only part of what actually occurred. Consequently, it is difficult to determine if the trends observed at the conclusion of the experiment would have persisted until metamorphosis or into adulthood. Exactly how the temperature-associated reduced size or the potentially increased number of abnormal tadpoles found here will impact amphibian populations is not certain. Therefore, if the full picture of how herbicides, invasive plants and/or climate change truly an impact an organism is to be understood, longer experiments covering multiple life-stages are imperative.

Caruso et al. (2014) found that six species of *Plethodon* salamanders have been decreasing in size in areas that have experienced the greatest warming and drying. Reduced size

can increase predation, desiccation, disease, lower survival, and reduce fitness (Berven and Gill 1983; Werner 1986; Semlitsch et al. 1988; Morey and Reznick 2001; Downie et al. 2004; Cabrera-Guzmán et al. 2013). While it is possible for smaller metamorphs to increase terrestrial growth, it often has the trade-off of higher predation risk due to increased activity (Morey and Reznick 2001). Additionally, smaller adults typically produce smaller clutch sizes thereby directly impact population sizes, ultimately leading to population declines (Woolbright 1983; Berven and Gill 1983; Reading 2007). It is evident that much more research needs to be conducted before the impacts of climate change are understood. More experimental studies examining interactions of climate change and other stressors are needed, especially in mesocosms that better assess more natural conditions that organisms experience. This study is the first to simultaneously address the potential effects of invasive plants, herbicides and chemical management for invasive plants under projected temperature increases. Since invasive the range of many invasive plants are likely to expand and cause the use of herbicides to control them to rise, more studies focusing on these effects should help land managers make better management decision (Hellman et al. 2008).

LITERATURE CITED

Adams, M.J., D.A.W. Miller, E. Muths, P.S. Corn, E.H. C. Grant, L.L Bailey,G.M. Fellers, R.N. Fisher, W.J. Sadinski, H. Waddle, and S.C. Walls. 2013. Trends in amphibian occupancy in the United States. PLoS ONE 8: e64347.

Alford, R.A., and S.J. Richards. 1999. Global amphibians declines: a problem in applied ecology. Annual Review of Ecology and Systematics 30: 133-165.

Arendt, J. 2010. Morphological correlates of sprint swimming speeds in five species of spadefoot toad tadpoles: comparison of morphometric methods. Journal of Morphology 271: 1044-1052.

Battaglin, W.A., K.C. Rice, M.J. Focazio, S. Salmons, R.X. Barry. 2009. The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005-2006. Environmental Monitoring and Assessment 155: 281-307.

Beebee, T.J.C., and R.A. Griffiths. 2005. The amphibian decline crises: a watershed for conservation biology? Biological Conservation 125: 271-285.

Bennett, A.F. 1978. Activity metabolism of the lower vertebrates. Annual Reviews of Physiology 400: 447-469.

Berven, K.A., and D.E. Gill. 1983. Interpreting geographic variation in life-history traits. Integrative and Comparative Biology 23: 85-97.

Blaustein, A.R., and J.M. Kiesecker. 2002. Complexity in conservation: lessons from the global decline of amphibian populations. Ecology Letters 5: 597-608.

Blaustein, A.R., J.M. Romansic, J.M. Kiesecker, and A.C. Hatch. 2003. Ultraviolet radiation, toxic chemicals and amphibian population declines. Diversity and Distributions 9: 123-140.

Blaustein, A.R., L.K. Belden, D.H. Olson, D.M. Green, T.L. Root, and J.M. Kiesecker. 2001. Amphibian breeding and climate change. Conservation Biology 15: 1804-1809.

Blaustein, A.R., S.C. Walls, B.A. Bancroft, J.A. Lawler, C.L. Searle, and S.S. Gervasi. 2010. Direct and indirect effects of climate change on amphibian populations. Diversity 2: 281-313.

Bloomfield, J.P., R.J. Williams, D.C. Gooddy, J.N. Cape, and P. Guha. 2006. Impacts of climate change on the fate and behavior of pesticides in surface and groundwater-a UK perspective. Science of the Total Environment 369: 163-177.

Blossey, B. 1999. Before, during and after: the need for long-term monitoring in invasive plant species management. Biological Invasions 1: 301-311.

Boone, M.D., and C.M. Bridges. 1999. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). Environmental Toxicology and Chemistry 18: 1482-1484.

Boone, M.D., and S.M. James. 2003. Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. Ecological Applications 13: 829-841.

Bradley, B.A., D.M. Blumenthal, D.S. Wilcove, and L.H. Ziska. 2010. Predicting plant invasions in an era of global change. Trends in Ecology and Evolution 25: 310-318.

Bridges, C.M. 2002. Tadpoles balance foraging and predator avoidance: effects of predation, pond drying, and hunger. Journal of Herpetology 36: 627-634.

Bridges, C.M., and R.D. Semlitsch. 2000. Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. Conservation Biology 14: 1490-1499.

Brown, C.J., B. Blossey, J.C. Maerz, and S.J. Joule. 2006. Invasive plant and experimental venue affect tadpole performance. Biological Invasions 8: 327-338.

Brunelli, E., I. Bernabò, C. Berg, K. Lundstedt-Enkel, A. Bonacci, and S. Tripepi. 2009. Environmentally relevant concentrations of endosulfan impair development, metamorphosis and behavior in *Bufo bufo* tadpoles. Aquatic Ecology 91: 135-142.

Brunner, E., S., Domhof, and F. Langer. 2002. Nonparametric analysis of longitudinal data in factorial experiments. New York: John Wiley.

Cabrera-Guzmán, E., M.R. Crossland, G.P. Brown, and R. Shine. 2013. Larger body size at metamorphosis enhances survival, growth and performance of young cane toads (*Rhinella marina*). PLoS ONE 8: e70121.

Carey, C., and M.A. Alexander. 2003. Climate change and amphibian declines: is there a link? Diversity and Distributions 9: 111-121.

Carney, E.W., R. Billington, and S.M. Barlow. 2007. Developmental toxicity evaluation of triclopyr butoxyethyl ester and triclopyr triethylamine salt in the CD rat. Reproductive Toxicology 23: 165-174.

Caruso, N.M., M.W. Sears, D.C. Adams, and K.R. Lips. 2014. Widespread rapid reductions in body size of adult salamanders in response to climate change. Global Change Biology 20: 1751-1759.

Chen, C.Y., K.M. Hathaway, D.G. Thompson, and C.L. Folt. 2008. Multiple stressor effects of herbicide, pH, and food on wetland zooplankton and a larval amphibian. Ecotoxicology and Environmental Safety 71: 209-218.

Chen, C-C., and B.A. McCarl. 2001. An investigation of the relationship between pesticide usage and climate change. Climatic Change 50: 475-487.

Cohen, J.S., J.C. Maerz, and B. Blossey. 2012. Traits, not origin explain impacts of plants on larval amphibians. Ecological Applications 22: 218-228.

Cohen, J.S., J.C. Maerz, and B. Blossey. 2012. Traits, not origin, explain impacts of plants on larval amphibians. Ecological Applications 22: 218-228.

Corn, P.S. 2005. Climate change and amphibians. Animal Biodiversity and Conservation 28: 59-67.

Cotten, T.B., M.A. Kwiatkowski, D. Saenz, and M. Collyer. 2012. Effects of an invasive plant, Chinese tallow (*Triadica sebifera*), on development and survival of Anuran larvae. Journal of Herpetology 46: 186-193.

Crump, M.L. 1989. Effect of habitat drying on developmental time and size at metamorphosis in *Hyla pseudopuma*. Copeia 1989: 794-797.

Delanoy, L., and O.W. Archibold. 2007. Efficacy of control measures for European buckthorn (*Rhamnus cathartica* L.) in Saskatchewan. Environmental Management 40: 709-718.

Dillon, M.E., G. Wang, and R.B. Huey. 2010. Global metabolic impacts of recent climate warming. Nature 467: 704-707.

Donnelly, M.A., and M.L. Crump. 1998. Potential effects of climate change on two Neotropical amphibian assemblages. Climatic Change 39: 541-561.

Downie, J.R., R. Bryce, and J. Smith. 2004. Metamorphic duration: an under-studied variable in frog life histories. Biological Journal of the Linnean Society 83: 261-272.

Duelmann, W.E., and L. Trueb. 1994. Biology of amphibians. Baltimore: The Johns Hopkins University Press.

Dukes, J.S., and H.A. Mooney. 1999. Does global change increase the success of biological invaders? Trends in Ecology and Evolution 14: 135-139.

Earl, J.E., P.O. Castello, K.E. Cohagen, and R.D. Semlitsch. 2014. Effects of subsidy quality on reciprocal subsidies: how leaf litter species changes frog biomass export. Oecologia 175: 209-218.

Egea-Serrano, A., R.A. Relyea, M. Tejedo, and M. Torralva. 2012. Understanding of the impact of chemicals on amphibians: a meta-analytic review. Ecology and Evolution 2: 1382-1397.

Gale, S.W. 2000. Control of the invasive exotic *Rhamnus cathartica* in temperate North American forests. Restoration and Reclamation Review 6: 1-13.

Getsinger, K.D., D.G. Petty, J.D. Madsen, J.G. Skogerboe, B.A. Houtman, W.T. Haller, and A.M. Fox. 2000. Aquatic dissipation of the herbicide triclopyr in Lake Minnetonka, Minnesota. Pest Management Science 56: 388-400.

Gillooly, J.F., J.H. Brown, G.B. West, V.M. Savage, and E.L. Charnov. 2001. Effects of size and temperature on metabolic rate. Science 293: 2248-2251.

Gitay, H., A.Suárez, R. Watson, O. Anisimov, F.S. Chapin, R.V. Cruz, M. Finlayson, W. Hohenstein, G. Insarov, Z. Kundzewicz, R. Leemans, C. Magadza, L. Nurse, I. Noble, J. Price, N.H. Ravindranath, T. Root, B. Scholes, A. Villamizar, X. Rumei, O. Canziani, O. Davidson, D. Griggs, J. McCarthy, and M. Prather. 2002. Climate change and biodiversity. IPCC Technical Paper V. Intergovernmental Panel on Climate Change. Geneva.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183-190.

Greenburg, D.A., and D.M. Green. Effects of an invasive plant on population dynamics in toads. Conservation Biology 27: 1049-1057.

Harris, M.L, C.A. Bishop, J. Struger, B. Ripley, and J.P. Bogart. 1998. The functional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in orchard wetlands. II. Effects of pesticides and eutrophic conditions on early life stage development.
Environmental Toxicology and Chemistry 17: 1351-1363.

Hayes, T.B., P. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. P. Mai, Y. Marjuoa, J. Parker, and M. Tsui. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environmental Health Perspectives 114: 40-50.

Hayes, T.B., P. Falso, S. Gallipeau, and M. Stice. 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. Journal of Experimental Biology 213: 921-933.

Hecnar, S.J., and R.T. M'Closkey. 1996. Amphibians species richness and distribution in relation to pond water chemistry in south-western Ontario, Canada. Freshwater Biology 36: 7-15.

Heimpel, G.E., L.E. Frelich, D.A. Landis, K.R. Hopper, K.A., Hoelmer, Z. Sezen, M.K. Asplen, and K.Wu. 2010. European buckthorn and Asian soybean aphid as components of an extensive invasional meltdown in North America. Biological Invasions 12: 2913-2931.

Hellmann, J.J., J.E. Byers, B.G. Bierwagen, and J.S. Dukes. 2008. Five potential consequences of climate change for invasive species. Conservation Biology 22: 534-543.

Heneghan, L., C. Rauschenberg, F. Fatemi, and M. Workman. 2004. European buckthorn (*Rhamnus cathartica*) and its effects on some ecosystem properties in an urban woodland. Ecological Restoration 22: 275-280.

Hickman, C.R., and J.I. Watling. 2014. Leachates from an invasive shrub causes risk-prone behavior in a larval amphibian. Behavioral Ecology 25: 300-305.

Hof, C., M.B. Araújo, W. Jetz, and C. Rahbek. 2011. Additive threats from pathogens, climate and land-use change for global amphibian diversity. Nature 480: 516-521.

Howe, C.M., M. Berrill, B.D. Pauli, C.C. Helbing, K. Werry, and N. Veldhoen. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry 23: 1928-1938.

Izhaki, I. 2002. Emodin-a secondary metabolite with multiple ecological functions in higher plants. New Phytologist 155: 205-217.

Johnson, P.T.J., V. J. McKenzie, A.C. Peterson, J.L. Kerby, J. Brown, A.R. Blaustein, and T. Jackson. 2011. Regional decline of an iconic amphibian associated with elevation, land-use change, and invasive species. Conservation Biology 25: 556-566.

Johnson, W.G., T.L. Lavy, and E.E. Gbur. 1995. Sorption, mobility and degradation of triclopyr and 2,4-D on four soils. Weed Science 43: 678-684.

Jones, D.K., J.I. Hammond, R.A. Relyea. 2009. Very highly toxic effects of endosulfan across nine species of tadpoles: lag effects and family-level sensitivity. Environmental Toxicology and Chemistry 28: 1939-1945.

Kattwinkel, M., J-V Kühn, K. Foit, and M. Liess. 2011. Climate change, agricultural insecticide exposure, and risk for freshwater communities. Ecological Applications 21: 2068-2081.

King, K.C., J.D. McLaughlin, A.D. Gendron, B.D. Pauli, I. Giroux, B. Rondeau, M. Boily, P. Juneau, and D.J. Marcogliese. 2007. Impacts of agriculture on the parasite communities of northern leopard frogs (*Rana pipiens*) in southern Quebec, Canada. Parasitology 134: 2063-2080.

Klionsky, S.M., K.L. Amatangelo, and D.M. Waller. 2011. Above-and belowground impacts of European buckthorn (*Rhamnus cathartica*) on four native forbs. Restoration Ecology 19: 728-737.

Knight, K.S., J.S. Kurylo, A.G. Endress, J.R. Stewart, and P.B. Reich. 2007. Ecology and ecosystem impacts of common buckthorn (*Rhamnus cathartica*): a review. Biological Invasions, 9: 925-937.

Kreutzweiser, D. P., S. B. Holmes, and D. C. Eichenberg. 1994. Influence of exposure duration on the toxicity of triclopyr ester to fish and aquatic insects. Archives of Environmental Contamination and Toxicology 26: 124-129.

Lawler, S.P. 1989. Behavioural responses to predators and predation risk in four species of larval anurans. Animal Behaviour 38: 1039-1047.

Li, Y., J.M. Cohen, and J.R. Rohr. 2013. Review and synthesis of the effects of climate change on amphibians. Integrative Zoology 8: 145-161.

Lips, K.R., J. Diffendorfer, J. R. Mendelson III, and M.W. Sears. 2008. Riding the wave: reconciling the roles of disease and climate change in amphibian declines. PLoS Biology 6: e72 0441-0454.

Mack, R.N., D. Simberloff, W.M. Lonsdale, H. Evans, M. Clout, and F.A Bazzaz. 2000. Biotic invasions: causes, epidemiology, global consequences, and control. Ecological Applications 10: 689-710.

Maerz, J.C., C.J. Brown, C.T. Chapin, and B. Blossey. 2005. Can secondary compounds of an invasive plant affect larval amphibians? Functional Ecology 19: 970-975.

McMenamin, S.K., E.A. Hadly, and C.K. Wright. 2008. Climatic change and wetland desiccation cause amphibian decline in Yellowstone National Park. Proceedings of the national Academy of Sciences USA 105: 16988-16993.

Morey, S., and D. Reznick. 2001. Effects of larval density on postmetamorphic spadefoot toads (*Spea hammondii*). Ecology 82: 510-522.

Netherland, M.D., and K.D. Getsinger. 1992. Efficacy of triclopyr on Eurasian watermilfoil: concentration and exposure time effects. Journal of Aquatic Plant Management 30: 1-5.

Newman, R.A. 1992. Adaptive plasticity in amphibian metamorphosis. BioScience 42: 671-678.

Noland, R., and G.R. Ultsch. 1981. The roles of temperature and dissolved oxygen in microhabitat selection by the tadpoles of a frog (*Rana pipiens*) and a toad (*Bufo terrestris*). Copeia 1981: 645-652.

Norris, L.A., M.L. Montgomery, and L.E. Warren. 1987. Triclopyr persistence in western Oregon hill pastures. Bulletin of Environmental Contamination and Toxicology 39: 134-141.

Noyes, P.D., M.K. McElwee, H.D. Miller, B.W. Clark, L.A. Van Tiem, K.C. Walcott, K. N. Erwin, and E.D. Levin. The toxicology of climate change: environmental contaminants in a warming world. Environment International 35: 971-986.

NRCS. Pest management-invasive plant control buckthorns-*Rhamnus cathartica* and *Frangula alnus*. Conservation Practice Job Sheet NH-595.

O'Regan, S.M., W.J. Palen, and S.C. Anderson. 2014. Climate warming mediates negative impacts of rapid pond drying for three amphibian species. Ecology 95: 845-855.

Orizaola, G., E. Dahl, and A. Laurila. 2014. Compensatory growth strategies are affected by the strength of environmental time constraints in anuran larvae. Oecologia 174: 131-137.

Ouellet, M., J. Bonin, J. Rodrigue, J-L. DesGranges, and S. Lair. 1997. Hindlimb deformities (Ectromelia, Ectrodactyly) in free-living anurans from agricultural habitats. Journal of Wildlife Diseases 33: 95-104.

Parmesan, C. 2006. Ecological and evolutionary responses to recent climate change. Annual Review of Ecology, Evolution, and Systematics 37: 637-669.

Pergams, O.R.W., and J.E. Norton. 2006. Treating a single stem can kill the whole shrub: a scientific assessment of buckthorn control methods. Natural Area Journal 26: 300-309.

Perkins, P.J., H.J. Boermans, and G.R. Stephenson. 2000. Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis assay-*Xenopus*. Environmental Toxicology and Chemistry 19: 940-945.

Petty, D.G., K.D. Getsinger, and K.B. Woodburn. 2003. A review of the aquatic environmental fate of triclopyr and its major metabolites. Journal of Aquatic Plant Management 41: 69-75.

Potvin, C., and D. Roff. 1993. Distribution-free methods: viable alternatives to parametric statistics. Ecology 74: 17-28.

Pounds, J.A., M.R. Bustamante, L.A. Coloma, J.A. Consuegra, M.P.L. Fogden, P.N. Foster, E. La Marca, K.L. Masters, A. Merino-Viteri, R. Puschendorf, S.R. Ron, G.A. Sánchez-Azofeifa, C.J. Still, and B.E. Young. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439: 161-167.

Reading, C.J. 2007. Linking global warming to amphibian declines through its effects on female body condition and survivorship. Oecologia 151: 125-131.

Relyea, R.A. 2005. The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecological Applications 15: 1118-1124.

Relyea, R.A. 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159: 363-376.

Relyea, R.A. 2012. New effects of Roundup on amphibians: predators reduce herbicide mortality; herbicides induce antipredator morphology. Ecological Applications 22: 634-647.

Relyea, R.A. 2000. Trait-mediated indirect effects in larval anurans: reversing competition with the threat of predation. Ecology 81: 2278-2289.

Relyea, R.A., and J.R. Auld. 2004. Having the guts to compete: how intestinal plasticity explains costs of inducible defences. Ecology Letters 7: 869-875.

Relyea, R.A., and K. Edwards. 2010. What doesn't kill you makes you sluggish: how sublethal pesticides alter predator-prey interactions. Copeia 4: 558-567.

Relyea, R.A., and N. Diecks. 2008. An unforeseen chain of events: lethal effects of pesticides on frogs at sublethal concentrations. Ecological Applications 18: 1728-1742.

Rogalski, M.A., and D.K. Skelly. 2012. Posititve effects of nonnative invasive *Phragmites australis* on larval bullfrogs. PLoS ONE 7: E44420.

Rohr, J.R., A.A. Elskus, B.S. Shepherd, P.H. Crowley, T.M. McCarthy, J.H. Niedzwiecki, T. Sager, A. Sih, and B.D. Palmer. 2003. Lethal and sublethal effects of atrazine, carbaryl, endosulfan, and octylphenol on the streamside salamander (*Ambystoma barbouri*). Environmental Toxicology and Chemistry 22: 2385-2392.

Rohr, J.R., and B.D. Palmer. 2013. Climate change, multiple stressors, and the decline of ectotherms. Conservation Biology 27: 741-751.

Rohr, J.R., T.M. Sesterhenn, and C. Stieha. 2011. Will climate change reduce the effects of a pesticide on amphibians?: partitioning effects on exposure and susceptibility to contaminants. Global Change Biology 17: 657-666.

Sacerdote, A.B., and R.B. King. 2014. Direct effects of an invasive European buckthorn metabolite on embryo survival and development in *Xenopus laevis* and *Pseudacris triseriata*. Journal of Herpetology 48: 51-58.

Saenz, D., E.M. Fucik, and M.A. Kwiatkowski, M.A. 2013. Synergistic effects of the invasive Chinese tallow (*Triadica sebifera*) and climate change on aquatic amphibian survival. Ecology and Evolution 3: 4828-4840.

Schlicter, L.C. 1981. Low pH affects the fertilization and development of *Rana pipiens* eggs. Canadian Journal of Zoology 59: 1693-1699.

Scott, D.E., E.D. Casey, M.F. Donovan, and T.K. Lynch. 2007. Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. Oecologia 153: 521-532.

Semlitsch, R.D., D.E. Scott, and J.H.K. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology 69: 184-192.

Smith, B.E., and D.A. Keinath. 2007. Northern Leopard Frog (*Rana pipiens*): a technical conservation assessment. [Online]. USDA Forest Service, Rocky Mountain Region. Available: http://www.fs.fed.us/r2/projects/scp/assessments/northernleopardfrog.pdf.

Sparling, D.W., G.M. Fellers, and L.L. McConnell. 2001. Pesticides and amphibian population declines in California, USA. Environmental Toxicology and Chemistry 20: 1591-1595.

Stephenson, G.R., K.R. Solomon, C.S. Bowhey, and K. Liber. 1990. Persistence, leachability, and lateral movement of triclopyr (Garlon) in selected Canadian forestry soils. Journal of Agricultural and Food Chemistry 38: 584-588.

Stoler, A.B., and R.A. Relyea. 2013. Leaf litter quality induces morphological and developmental changes in larval amphibians. Ecology 94: 1594-1603.

Stuart, S.N., J.S. Chanson, N.A. Cox, B.E. Young, A.S.L. Rodrigues, D.L. Fischman, and R.W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306: 1783-1786.

Teplitsky, C., S. Plenet, J-P Léna, N. Mermet, E. Malet, and P. Joly. 2005. Escape behaviour and ultimate causes of specific induced defences in an anuran tadpole. Journal of Evolutionary Biology 18: 180–190.

Timchalk, C., M.D. Dryzga, and P.E. Kastl. 1990. Pharmacokinetics and metabolism of triclopyr (3,5,6-trichloro-2-pyridinyloxyacetic acid) in Fischer 344 rats. Toxicology 62: 71-87.

Touchon, J.C., R.R. Jiménez, S.H. Abinette, J.R. Vonesh, and K.M. Warkentin. 2013. Behavioral plasticity mitigates risk across environments and predators during anuran metamorphosis. Oecologia 173: 801-811.

Venesky, M.D., S.M. Hanlon, K. Lynch, M.J. Parris, and J.R. Rohr. 2013. Optimal digestion theory does not predict the effect of pathogens on intestinal plasticity. Biology Letters 9: 20130038.

Wake, D.B. and V.T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Science USA 105: 11466-11472.

Walther, G-R., E. Post, P. Convey, A. Menzel, C. Parmesan, T.J.C. Beebee, J-M. Fromentin, O. Hoegh-Guldberg, and F. Bairlein. 2002. Ecological responses to recent climate change. Nature 416: 389-395.

Walther, G-R., Roques, A., Hulme, P.E., Sykes, M.T., Pyšek, P., Kuhn, I., Zobel, M., Bacher, S., Botta-Dukát, Z., Bugmann, H., Czúcz, B., Dauber, J., Hickler, T., Jarošík, V., Kenis, M., Klotz, S., Minchin, D., Moora, M., Nentwig, W., Ott, J., Panov, V.E., Reineking, B., Robinet, C., Semenchenko, V., Solarz, W., Thuiller, W., Vilà, M., Vohland, K. & Settele, J. (2009). Alien species in a warmer world: risks and opportunities. Trends in Ecology and Evolution 24: 686-693.

Wan, M.T., D.J. Moul, and R.G. Watts. 1987. Acute toxicity to juvenile Pacific salmonids of Garlon 3A™, Garlon 4™, triclopyr, triclopyr ester and their transformation products: 3,5,6-trichloro-2-pyridinol and 2-methoxy-3,5,6-trichloropyridine. Bulletin of Environmental Contamination and Toxicology 39: 721-728.

Watling, J.I., C.R. Hickman, E. Lee, K. Wang, and J.L. Orrock, J.L. 2011. Extracts of the invasive shrub *Lonicera maackii* increase mortality and alter behavior of amphibian larvae. Oecologia 165: 153-159.

Werner, E.E. 1986. Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. The American Naturalist 128: 319-341.

Wilbur, H.M., and J.P. Collins. 1973. Ecological aspects of amphibian metamorphosis Science 182: 1305-1314.

Wojtaszek, B.F., T.M. Buscarini, D.T. Chartrand, G.R. Stephenson, and D.G. Thompson. 2005. Effect of Release® herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. Environmental Toxicology and Chemistry 24: 2533-2544.

Woolbright, L.L. 1983. Sexual selection and size dimorphism in anuran amphibia. The American Naturalist 121: 110-119.

Yahnke, A.E., C.E. Grue, M.P. Hayes, and A.T. Troiano. 2013. Effects of the herbicide imazapyr on juvenile Oregon spotted frogs. Environmental Toxicology and Chemistry 32: 228-235.

Zhao, J., Y. Yang, X. Xi, C. Zhang, and S. Sun. 2014. Artifical warming facilitates growth but not survival of Plateau frog (*Rana kukunoris*) tadpoles in presence of gape-limited predatory beetles. PLoS ONE 9: e98252.

Table 2. Repeated-measures MANOVA examining the effects of treatment and temperature on tadpole weight and length and repeated-measures ANOVA on the effects of treatment, temperature, time, treatment × time, temperature × time, treatment ×temperature, and treatment × temperature × time on tadpole weight and length in Experiment I

Source of variation	**Weight**		**Length**	
	DF	F	DF	F
Repeated measures MANOVA				
Treatment	27, 427	4.56**	27, 424	4.45**
Temperature	9, 148	54.96**	9, 147	50.96**
Repeated measures ANOVA				
Treatment	3, 1158	62.50**	3, 1158	21.17**
Temperature	1, 1158	334**	1, 1158	0.21
Time	8, 1158	1779**	8, 1158	2557**
Treatment x time	24, 1158	3.3**	24, 1158	1.68*
Temperature x time	8, 1158	68.48**	8, 1158	23.59**
Treatment x temperature	3, 1158	30.22**	3, 1158	30.64**
Treatment x temperature x time	24, 1158	1.04	24, 1158	1.76*

* P<0.05; ** P<0.0001

Table 3. Results from repeated-measures MANOVA examining the effects of group, treatment and temperature on weight and length and repeated-measures ANOVA on the effects of group, treatment, temperature, time, treatment x time, temperature x time, treatment x temperature, and treatment x temperature x time on tadpole weight and length in Experiment II

Source of variation	**Weight**		**Length**	
	DF	F	DF	F
Repeated measures MANOVA				
Treatment	16, 120	5.32**	16, 120	6.19**
Temperature	8, 61	15.41**	8, 61	20.10**
Repeated measures ANOVA				
Treatment	2, 532	48**	2, 532	32.41**
Temperature	1, 532	137.26**	1, 532	18.92**
Time	7, 532	686.34**	7, 532	1192.45**
Treatment x time	14, 532	6.25**	14, 532	4.37**
Temperature x time	7, 532	20.80**	7, 532	9.56**
Treatment x temperature	2, 532	0.98	2, 532	2.35
Treatment x temperature x time	14, 532	0.65	14, 532	0.82

* P<0.05; ** P<0.0001

Table 4. ANOVA results for the effects of treatment, temperature, and treatment × temperature on tadpole morphology in Experiment I

Morphological Measurement	Treatment	Temperature	Treatment × Temperature
Tail depth	<0.0001	<0.0001	0.1952
Tail length	0.0204	0.7000	0.0122
Body length	<0.0001	<0.0001	0.7866
Body depth	<0.0001	<0.0001	0.5383
Body width	<0.0001	<0.0001	0.7226
Muscle width	0.0008	<0.0001	0.0001
Muscle depth	<0.0001	<0.0001	0.0575
Denticle width	0.1231	<0.0001	0.3128
Denticle length	0.0297	0.6894	0.7019
Intestine length	0.0061	<0.0001	0.0163

Table 5. ANOVA results for the effects of treatment, temperature, and treatment × temperature on tadpole morphology in Experiment II

Morphological Measurement	Treatment	Temperature	Treatment × Temperature
Tail depth	<0.0001	<0.0001	0.2559
Tail length	<0.0001	0.0407	0.2808
Body length	0.0002	<0.0001	0.5702
Body depth	0.0012	<0.0001	0.7264
Body width	0.0004	<0.0001	0.7308
Muscle width	0.0007	<0.0001	0.5676
Muscle depth	<0.0001	<0.0001	0.1914
Denticle width	0.2263	0.2053	0.3844
Denticle length	0.3671	0.0426	0.0323
Intestine length	<0.0001	<0.0001	0.0338

Table 6. Ranges of pH, dissolved oxygen (mg/L), and conductivity measured (µS/cm) in Experiment I

Treatment	Temperature	pH	Dissolved oxygen (mg/L)	Conductivity (µs/cm)
Control	Ambient	7.00 - 7.46	3.52 - 4.94	487 - 580
Fresh buckthorn	Ambient	7.12 - 7.88	2.96 - 3.84	538 - 606
Herbicide	Ambient	6.78 - 7.80	3.65 - 5.09	487 - 595
Fresh buckthorn + herbicide	Ambient	7.28 - 7.93	3.40 - 3.74	539 - 607
Control	Elevated	7.39 - 7.68	3.22 - 5.01	476 - 592
Fresh buckthorn	Elevated	7.52 - 8.07	3.55 - 4.15	520-617
Herbicide	Elevated	7.30 - 7.94	3.75 - 4.30	479 - 597
Fresh buckthorn + herbicide	Elevated	7.56 - 8.08	3.55 - 4.06	524 - 618

Table 7. Ranges of pH, dissolved oxygen (mg/L), and conductivity (µS/cm) measured in Experiment II

Treatment	Temperature	pH	Dissolved oxygen (mg/L)	Conductivity (µs/cm)
Control	Ambient	6.97 - 7.59	3.54 - 4.70	503 - 559
Buckthorn litter	Ambient	7.34 - 7.93	3.38 - 3.85	569 - 620
Buckthorn litter + herbicide	Ambient	7.48 - 8.00	3.33 - 4.11	569 - 625
Control	Elevated	7.52 - 7.73	3.39 - 4.02	494 - 571
Buckthorn litter	Elevated	7.37 - 8.06	3.03 - 3.69	569 - 628
Buckthorn litter + herbicide	Elevated	7.49 - 8.10	3.10 - 3.69	573 - 632

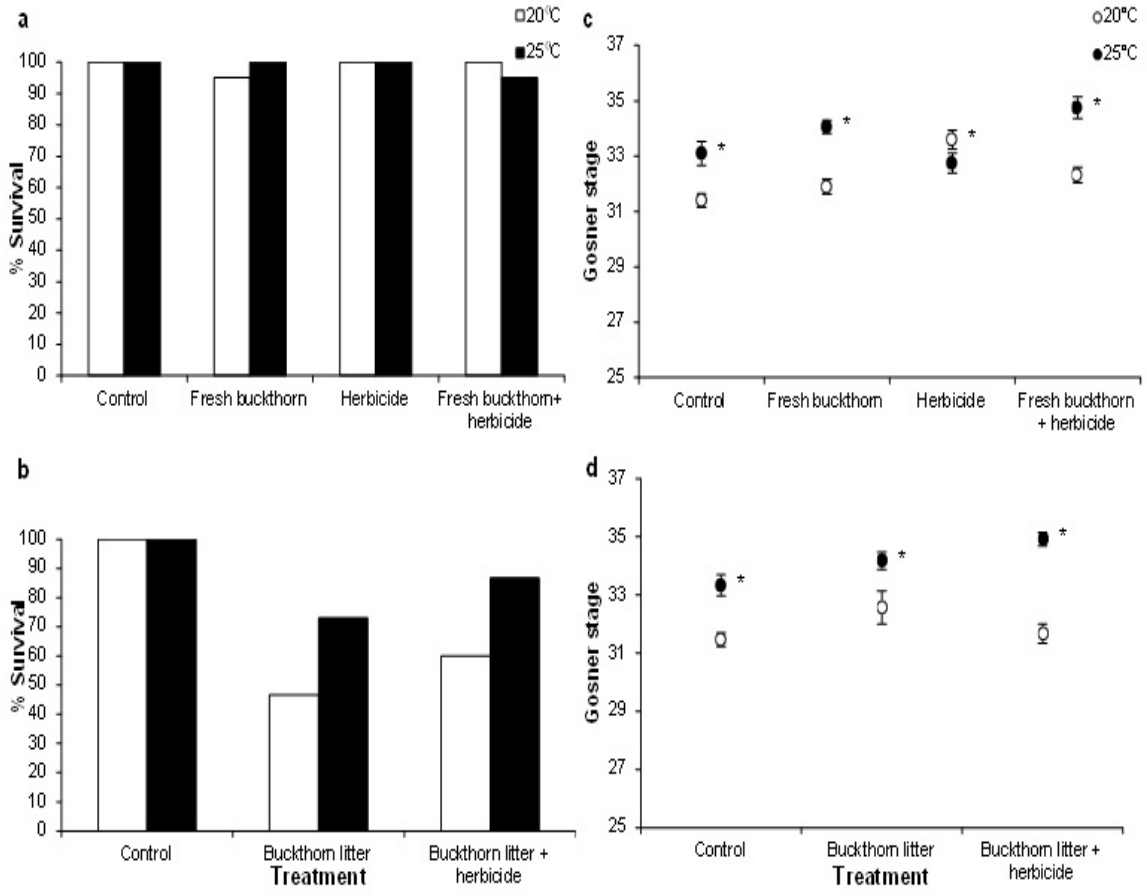

Figure 4. Results from Fisher's exact test on tadpole survival for Experiment I (a) and Experiment II (c), along with final Gosner developmental stage for Experiment I (b) and Experiment II (d). Error bars represent standard error and asterisks indicate a significant difference from the control ambient temperature (20°C)

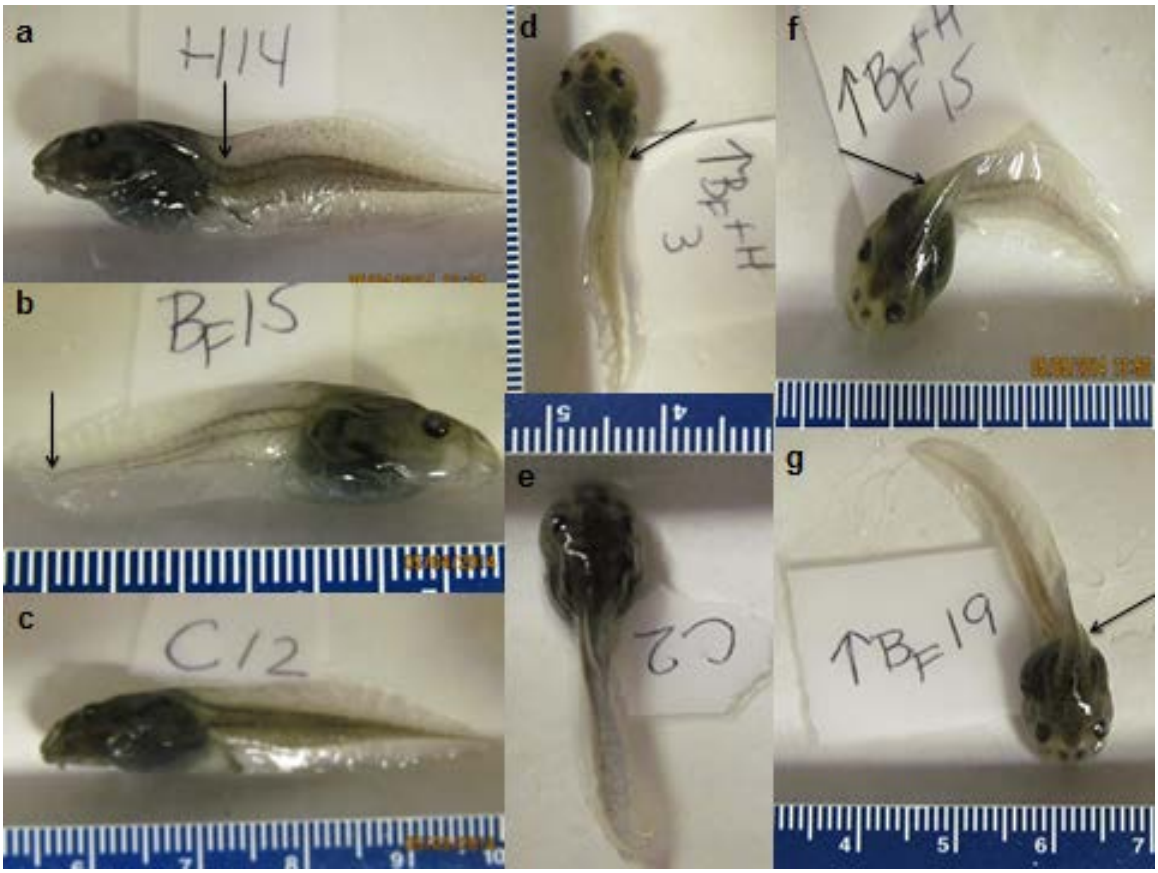

Figure 5. A sample of post-mortem abnormalities observed in experiment I: a) lateral view of herbicide a tadpole exposed to herbicide under ambient temperature exhibiting tail abnormality, b) lateral view of a tadpole exposed to fresh buckthorn under ambient temperature with an abnormality at the tip of the tail, c) lateral view of a control treatment tadpole exposed to ambient temperature, d) dorsal view of a tadpoles exposed to fresh buckthorn + herbicide and elevated temperature exhibiting a crooked tail, e) dorsal view of a control tadpole under ambient temperature, f) dorsal view of a tadpole exposed to fresh buckthorn + herbicide in the elevated temperature with a bent tail, and g) a dorsal view of a tadpole exposed to elevated fresh buckthorn and elevated temperature with a bent tail

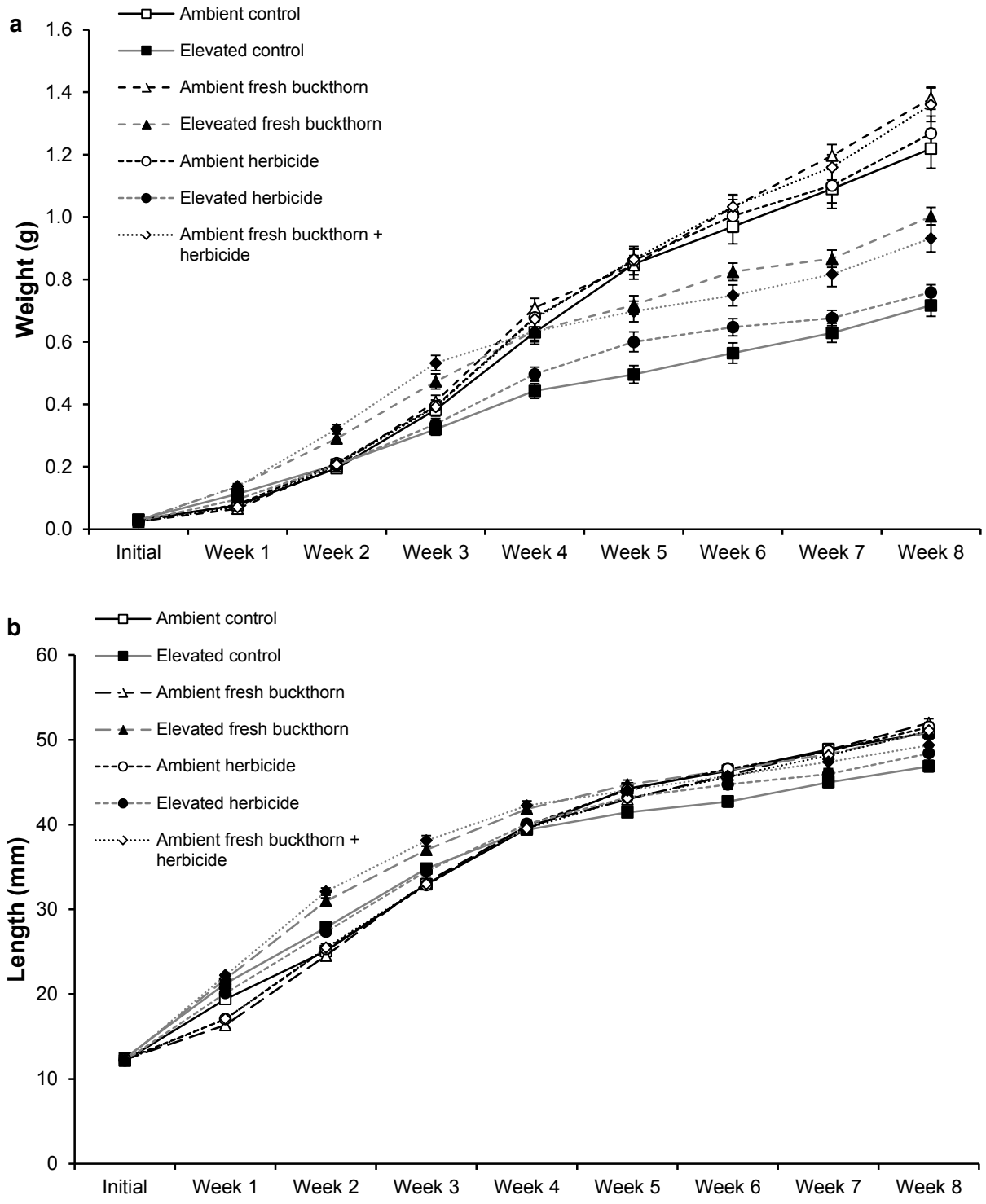

Figure 6. All tadpole weights (a) and lengths (b) shown from the initial weight until the experiment was terminated on week 8

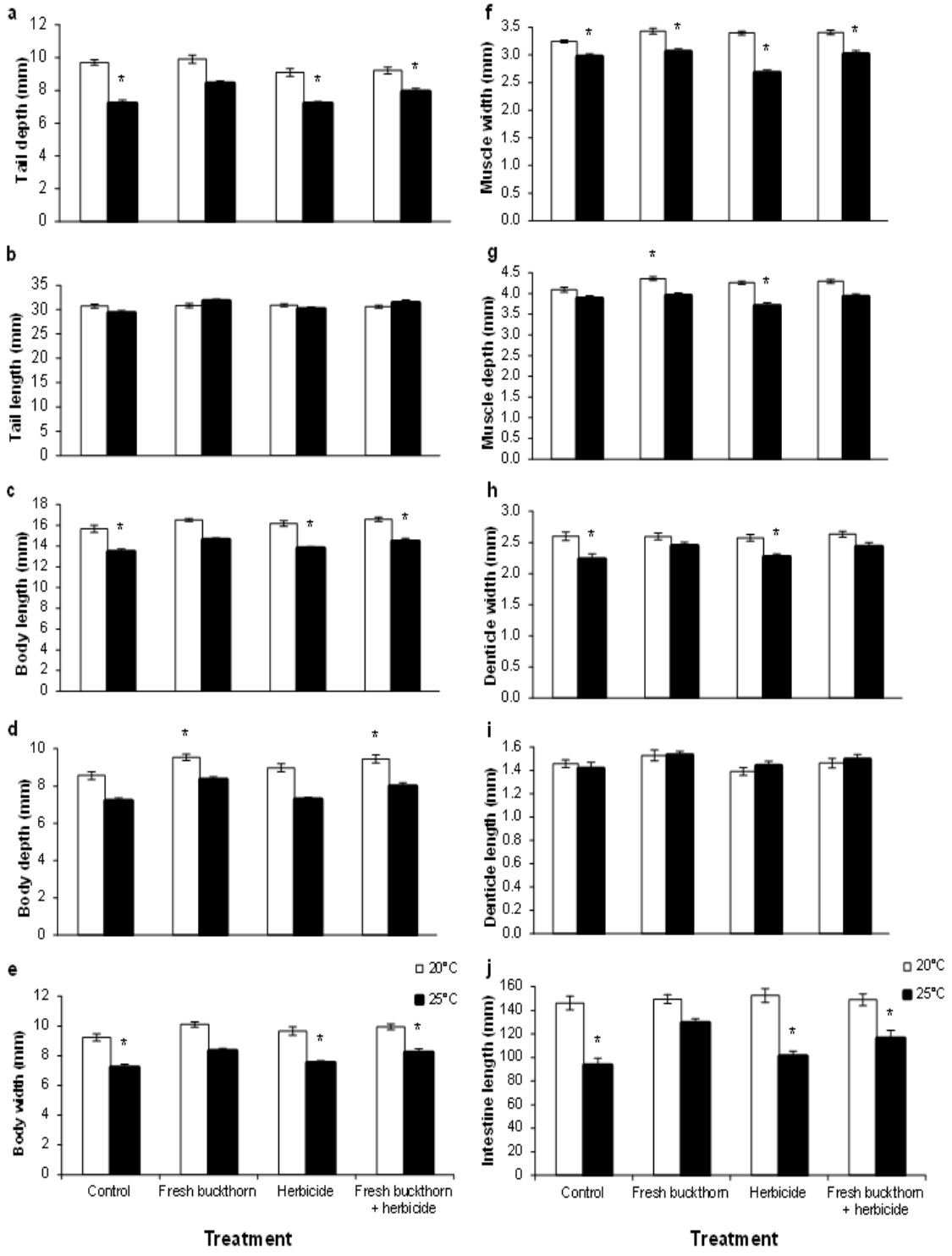

Figure 7. Post-mortem morphological measurements of experiment I: a) mean tail depth (mm), b) mean tail length (mm), c) mean body length (mm), d) mean body depth (mm), e) mean body width (mm), f) mean muscle width (mm), g) mean muscle depth (mm), h) mean denticle width (mm), i) mean denticle length, and j) mean intestine length. Standard error bars and asterisks denote significant difference from the 20°C control

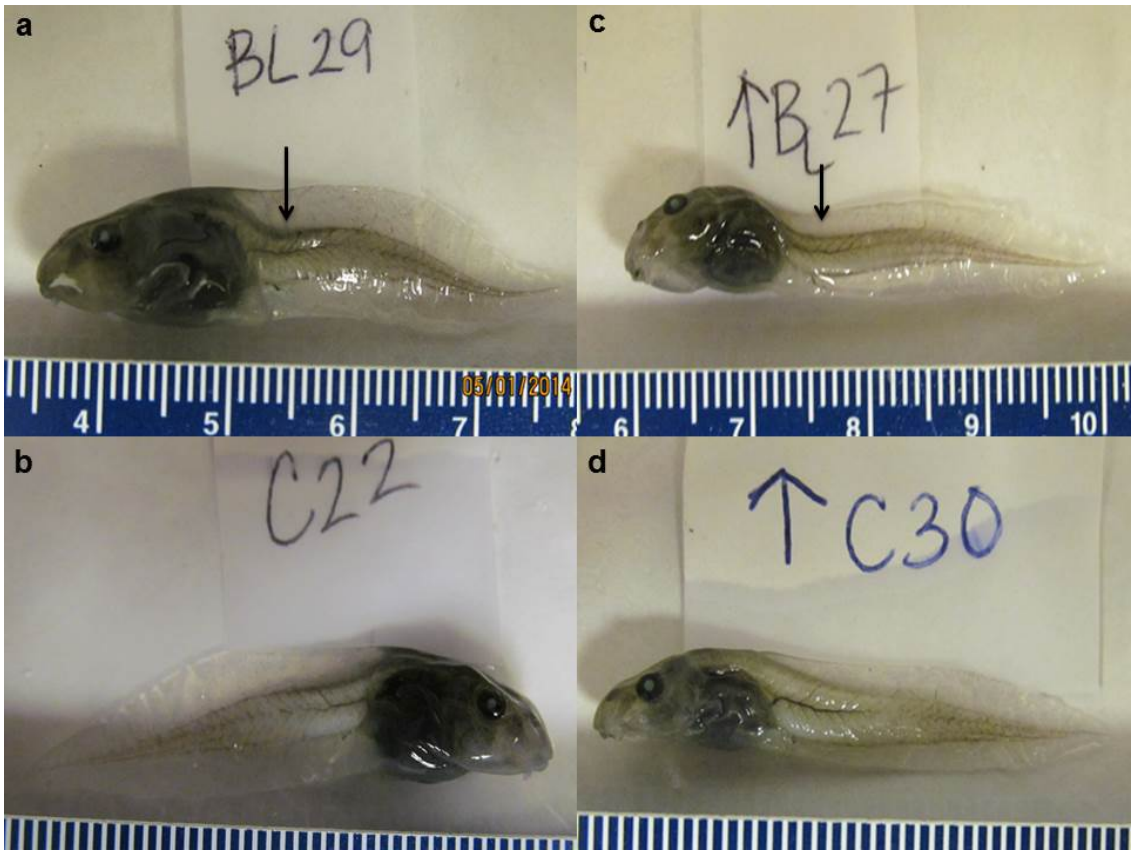

Figure 8. Abnormalities observed in experiment II, a) lateral view of an ambient buckthorn litter tadpole exhibiting a tail abnormality, b) lateral view of an ambient control tadpole for comparison, c) lateral view of an elevated buckthorn litter tadpole with an abnormal tail, and d) the lateral view of an elevated control tadpole

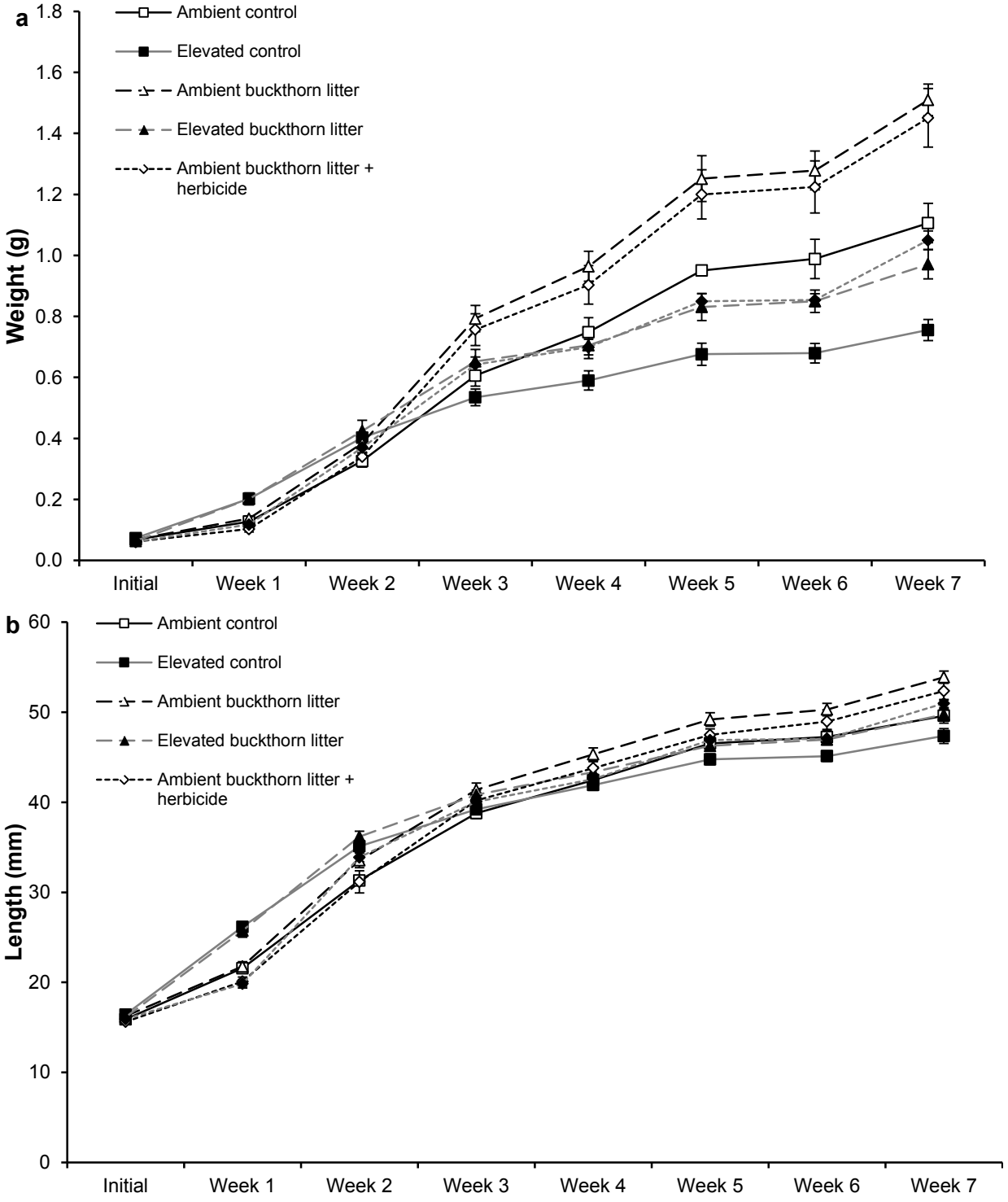

Figure 9. All tadpole weights (a) and lengths (b) shown from the initial weight) until the Experiment II was terminated on week 7

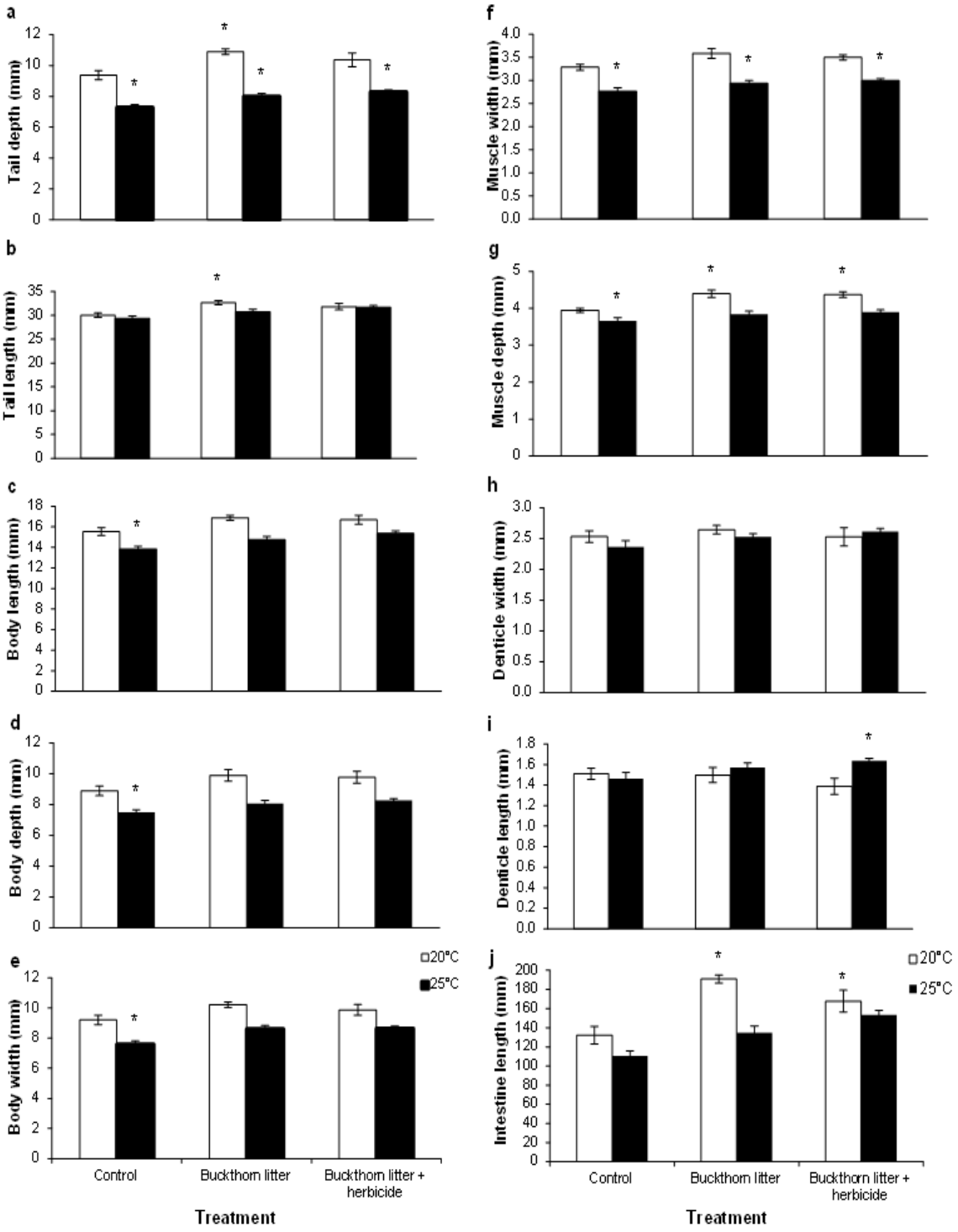

Figure 10. Experiment II post-mortem morphological measurements: a) mean tail depth (mm), b) mean tail length (mm), c) mean body length (mm), d) mean body depth (mm), e) mean body width (mm), f) mean muscle width (mm), g) mean muscle depth (mm), h) mean denticle width (mm), i) mean denticle length, and j) mean intestine length. Standard error bars and asterisks denote significant difference from the 20°C control

APPENDIX A:

2013 INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

BGSU
Bowling Green State University

Office of Research Compliance
309A University Hall
Bowling Green, OH 43403
Phone: (419) 372-7716
Fax: (419) 372-6916
E-mail: hsrb@bgsu.edu

January 15, 2013

Dr. Maria Gabriela Bidart-Bouzat
Biological Sciences
Bowling Green State University

Re: IACUC Protocol 12-010

Title:
Determining the effects of herbicides and invasive plants on tadpole development and adaptation

Dear Dr. Bidart-Bouzat:

On **January 10, 2013** the above referenced protocol received **final approval** after review of the requested modifications by the IACUC. The modifications have been incorporated into the official copy of your protocol (see attached).

This approval expires on January 9, 2014, by which time renewal must be requested if you wish to continue work on the protocol. The Office of Research Compliance will send notification reminding you of the need for renewal in advance of that date.

Please have all members of your research team read the approved version of the protocol. Please also remember to keep a copy of the approved protocol in the animal facility room(s) in which your animals are housed and in any associated procedure rooms (contact the UAF staff for assistance in this regard).

Please consult with the staff of the Animal Facility about your requirements to get started on this project. Good luck with your project.

Sincerely,

Hillary Harms, Ph.D.
IACUC Administrator

APPENDIX B:
2014 INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

BGSU
Bowling Green State University

Office of Research Compliance
309A University Hall
Bowling Green, OH 43403-0183
Phone: (419) 372-7716
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January 22, 2014

Dr. Maria Gabriela Bidart-Bouzat
Biological Sciences
Bowling Green State University

Re: Annual Renewal of IACUC Protocol 12-010

Title:
Determining the effects of herbicides and invasive plants on tadpole development and adaptation

Dear Dr. Bidart-Bouzat:

On January 22, 2014 the annual renewal for the above referenced protocol was **approved** after review by the IACUC. This renewal is in effect for one calendar year and expires on January 21, 2015. Please consult with the staff of the Animal Facility about any special needs you might have to continue with this project.

Comment(s):
Note that in item 5 of the annual renewal form, the first paragraph is provided twice. Please edit this in future submissions.

Sincerely,

Hillary Snyder, Ph.D.
IACUC Administrator