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Direct Effects of an Invasive European Buckthorn Metabolite on Embryo Survival and Development in *Xenopus laevis* and *Pseudacris triseriata*

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ABSTRACT.—**We demonstrate novel direct effects of an invasive plant metabolite on embryo development in the native Western Chorus Frog (*Pseudacris triseriata*) and a model organism, African Clawed Frog (*Xenopus laevis*). European buckthorn (*Rhamnus cathartica*) exhibits aggressive growth in amphibian breeding sites and releases the secondary metabolite, emodin, into soil and water. Emodin is known to have several deleterious, bioactive properties in mammals and birds, but its effects on amphibians have not been assessed. We used the FETAX (Frog Embryo Teratogenesis Assay–*Xenopus*) protocol to assess the effect of emodin on amphibian development in *X. laevis*, and modified the assay with *P. triseriata* to determine whether effects were consistent in a native species occurring within the range of the invasive *R. cathartica*. We detected and quantified emodin at amphibian breeding ponds that were infested heavily with buckthorn and have experienced recent declines in amphibian diversity and abundance. The *X. laevis* assay demonstrated significant embryo mortality and malformation in the presence of a gradient of concentrations of emodin including those detected in the environment. Teratogenicity indices (TI) >2 indicate the strong development-disrupting potential of emodin in amphibians. The *P. triseriata* assay produced similar patterns of embryo mortality and malformation as observed in the *X. laevis* assay. However, *P. triseriata* were more sensitive to emodin than *X. laevis* with TIs >40. Such effects may contribute to amphibian declines through depressed hatching success and poor larval survival and may represent an unrecognized impact of invasive plants more generally.**

Amphibian decline and poor recruitment have been attributed to environmental stressors including disease, contaminants, UV radiation, pH, and synergistic interactions of these stressors (Kiesecker et al., 2001; Blaustein and Kiesecker, 2002). Invasive exotic plants represent an additional factor that may contribute to amphibian decline (Martin and Murray, 2011). Despite the pervasiveness of invasive exotic plants and their encroachment into amphibian breeding habitats, few studies address the direct effects of invasive plants on amphibian survival, recruitment, and persistence (Martin and Murray, 2011). Recent studies examining direct effects of invasive plants on amphibians have focused on reduced growth, survival, foraging efficiency, and altered behavior in the larval stages of development (Maerz et al., 2005a, 2010; Watling et al., 2011a; Cotten et al., 2012). Studies of adult amphibian response to invasive species demonstrate indirect effects mediated through changes in microhabitat (Watling et al., 2011b). Effects of invasive plants on amphibian embryo survival and embryo development have not previously been studied. Here, we examine the direct effects of an invasive plant metabolite present in the breeding environment on amphibian embryo survival and development with a model species, the African Clawed Frog (*Xenopus laevis*), and the native Western Chorus Frog (*Pseudacris triseriata*) that occurs within the invaded range of European buckthorn (*Rhamnus cathartica*).

In this study, we focus on the potential for direct effects of a *R. cathartica* metabolite on embryo survival and embryo development in amphibians. *Rhamnus cathartica* produces the allelopathic anthraquinone emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) (Izhaki, 2002; Seltzner and Eddy, 2003). This secondary metabolite, which occurs in leaves, fruit, flowers, bark, and roots of *R. cathartica*, has known biological physiological effects in birds and mammals, including abortive and neurological effects (Litvinova and Fedorchenko, 1994; Lichtensteiger et al., 1997), purgation and feeding deterrence (Sherburne, 1972; Izhaki, 2002), damage to epithelial cells and inhibition of ion transport (Izhaki, 2002), and immunosuppressive and vasorelaxant effects (Huang et al., 1992). Emodin also inhibits growth and causes DNA damage in the bacterium *Helicobacter pylori* (Wang and Chung, 1997). Effects on amphibians are unknown, but release of emodin by *R. cathartica* and its release through decomposition of leaf litter may result in the leaching of emodin into soil and water in amphibian breeding pools and in the surrounding uplands. Given the aggressive growth of *R. cathartica* in moist soils and wetland edges (Knight et al., 2007), and encroachment of breeding pond basins, direct effects of emodin on amphibian embryo development may contribute to regional amphibian declines. Heavily infested sites in northern Illinois have undergone decreases in amphibian diversity and abundance over the past 30 years (Mierzwa and Nuzzo, 2000; Sacerdote, 2009; Sacerdote and King, 2009). In the Chicago region, common ephemeral pool-breeders such as *Ambystoma maculatum, Lithobates sylvaticus,* and *Pseudacris crucifer* have declined or been lost from the regional assemblage since the mid-1980s (Mierzwa and Nuzzo, 2000). Remaining pool-breeding amphibians including *Ambystoma laterale* and *P. triseriata* often have low hatching success in heavily invaded sites (Sacerdote and King, 2009).

If emodin produced by *R. cathartica* has teratogenic effects on amphibian embryos similar to those observed in mammals, survival rates for the aquatic life stage of amphibians may be decreased. Given the characteristically low survival rates of the aquatic life stage of pond-breeding amphibians, (Vonesh and De la Cruz, 2002), further decreases may limit recruitment in already declining populations and many breeding sites may become reproductive sinks. Such effects may be especially evident in species that did not co-evolve with *R. cathartica* as opposed to species that evolved in the native range of *R. cathartica*. For example, emodin in alder-leaved buckthorn (*Rhamnus alnifolia*) reduced feeding, prolonged development, and produced elevated mortality in nonnative Gypsy Moth larvae that do not share a native range with *R. alnifolia* (Trial and Dimond, 1979). Secondary compounds from invasive plants, as well as some native plants, are known to affect larval amphibians negatively (Cohen et al., 2012). Tannins from invasive purple loosestrife (*Lythrum salicaria*) are associated

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with poor larval performance in American Toad (*Anaxyrus americanus*) tadpoles (Maerz et al., 2005a) and may inactivate digestive enzymes and impair nutrient assimilation (Wynne-Edwards, 2001). Similarly, reduced larval survivorship and altered respiratory behavior occurs in native amphibians exposed to phenols from Amur honeysuckle, *Lonicera maacki* and litter from Chinese tallow (Watling et al., 2011a; Cotton et al., 2012).

We hypothesized that emodin from *R. cathartica* negatively impacts amphibian embryo development. Our objectives were to examine effects of emodin on the embryo development in the model species, *X. laevis*, through use of the FETAX (Frog Embryo Teratogenesis Assay–*Xenopus*) protocol (ASTM, 1998) and to modify the assay to examine effects of emodin on the native species, *P. triseriata*, which occurs within the invaded range of *R. cathartica*. Additional objectives included detection and quantification of emodin in the amphibian breeding environment such that the range of environmentally relevant concentrations could be determined.

Materials and Methods

Emodin Concentrations in the Environment: Pond Water.—We collected water samples from two ephemeral breeding pools in a flatwoods wetland site, MacArthur Woods, with historic buckthorn invasion, in which *P. triseriata* were breeding (Sacerdote and King, 2009). Pond 1 was surrounded by buckthorn sprouts encroaching into the pond basin, whereas sprouts around Pond 2 had been mechanically cleared in the winter of 2009. Ephemeral pools have been heavily encroached upon by *R. cathartica* in this region, and no sites without buckthorn were available for comparison. As a consequence, sites like Pond 2, from which *R. cathartica* has been cleared, represent the best available control. We recognize that additional investigation of replicate removal and invaded ponds may provide a clearer picture of variation in emodin concentration with restoration management. However, at the time of sampling, other ponds in the study area had undergone varying degrees of restoration management (e.g., herbicide, but not mechanical removal, some prescribed burning) and did not provide a clear comparison between invaded and cleared ponds.

Water samples were collected from Ponds 1 and 2 during early March 2011 while *P. triseriata* were breeding. Water samples were collected at random locations on the northern and southern ends of the pond within 2 m of the pond edge where amphibians typically deposit eggs. At each pond, four sets of water samples were collected in 50-ml glass sample bottles. Water samples were rotoevaporated to dryness. The resulting precipitate was resuspended in 95% methanol and filtered for HPLC analysis. Differences in mean emodin concentrations detected in Ponds 1 and 2 were examined using a *t*-test.

Emodin Concentrations in the Environment: Soil and Pond Sediment.—We spiked samples of clean blasting sand with emodin in 95% methanol (4°C) solution to test the effectiveness of benzene as a solvent for recovering emodin from soil. Benzene (18°C) was mixed with the spiked sand, agitated in a water bath for 1 h, centrifuged, and rotoevaporated to dryness. Each precipitate was resuspended in 95% methanol, and HPLC was applied. Benzene has been used as a solvent for isolation and extraction of emodin and similar anthraquinones in several studies (Sherburne, 1972; Abou-Chaar and Shamlian, 1980; Manojlovic et al., 2006). In our study, benzene extraction recovered only 20% of the sample from soil; however, this was more effective than other solvents tested (methanol, ethanol, pentane, and hexane).

We collected five sets of field samples of hydric clay soils immediately adjacent to small (<1 m in height) buckthorn sprouts in amphibian breeding sites. Three replicates of 5-cm^3 soil samples were collected from buckthorn-infested areas surrounding each of five ephemeral ponds, two of which provided the water samples. However, soil-sample collection occurred prior to any *R. cathartica* removal. Samples were taken at the soil surface, placed in Whirl-Pak bags (Whirl-Pak®, Nasco) on ice, and stored frozen until 24 h before extraction. Emodin was extracted with benzene, and the precipitate was resuspended in 95% methanol and filtered for HPLC analysis. We also sampled four 10-m transects radiating from mature *R. cathartica* stems on the edge of four additional ephemeral ponds, extending into the pond basins. We collected three replicate soil samples at the stem and every 2 m from the stem. Soil and pond sediment samples were collected as described above with sample collection beginning within 1 m of breeding pond edges and ending in the pond sediment. Emodin concentrations in soil and pond sediments are presented without correction for recovery success.

Emodin Concentrations in the Environment: Leachate.—We examined the leaching of emodin from *R. cathartica* leaves into water by placing 25 g (wet weight) of *R. cathartica* leaves into two 1-l glass beakers of distilled water. Although this ratio of leaf litter to water is likely greater than that in nature, we wanted to ensure detection of the emodin compound to examine changes in the concentrations of leachate through time. Beakers were left uncovered at room temperature. Two replicate 1.5-ml water samples were collected from each beaker at 24, 48, 72, 96, and 168 h (1 week). Water samples were rotoevaporated to dryness. The resulting precipitate was resuspended in 95% methanol and filtered for HPLC analysis.

Xenopus laevis Assay.—We assessed the teratogenicity of emodin on embryos of *X. laevis* using the Frog Embryo Teratogenesis Assay–*Xenopus* (FETAX) protocol (ASTM 1998) employing a gradient of emodin that included concentrations detected in the environment. Ovulation and mating were induced by priming two breeding pairs of *X. laevis* with human chorionic gonadotropin injections (Koss and Wakeford, 2000). We sorted resulting embryos into replicates containing 20 embryos per 100 × 20 mm petri dish with 16 ml of treatment solution. Replicates consisted of embryos from two individual breeding pairs such that any clutch-related mortality or malformation could be identified if necessary. The control treatments included 90% FETAX solution as a negative control, 5.5-ppm 6-aminonicotinamide as a low positive control and 2,500-ppm 6-aminonicotinamide as a high positive control. Experimental treatments included 0.1-ppm, 0.5-ppm, 1.0-ppm, 10.0-ppm, 50.0-ppm, and 100.0-ppm emodin. We used the standard HPLC grade emodin (Sigma) in the experimental treatments to eliminate any confounding effects from other metabolites. Solutions were replaced, and dead embryos were removed every 24 h. Embryos were fixed in 3% formalin and scored for malformations following Bantle et al. (1990).

Pseudacris triseriata Assay.—*Pseudacris triseriata* was selected as a native comparative species because it breeds in the ephemeral pool habitats subject to aggressive *R. cathartica* encroachment. The breeding phenology of *P. triseriata* coincides with peak emodin production by *R. cathartica* (early March through April, Sherburne, 1972; Izahki, 2002). Other amphibian species in the region that breed at this time are either ranked as Illinois Species

in Greatest Need of Conservation (*Acris crepitans*, *Ambystoma laterale*) or have declined or been extirpated locally.

To obtain *P. triseriata* embryos, breeding sites were visited in early March when calling commenced. Four amplexed pairs of *P. triseriata* were placed in 5-gallon buckets of distilled water in the field during mating and returned to the breeding pond following egg deposition, thus preventing egg exposure to emodin in the pond environment. A small portion (<10%) of each clutch was transported to the laboratory in coolers and immediately sorted by clutch into experimental treatments. Care was taken such that the jelly surrounding each embryo was left intact. Remaining embryos were returned to the breeding pond. We replicated all treatments and scoring methods used in the FETAX experiment. The number of replicate embryos in each breeding pair block varied because of differences in clutch size with Blocks 1–4 containing 21, 14, 9, and 16 embryos per treatment, respectively.

Statistical Analysis of Xenopus laevis and Pseudacris triseriata Assays.—To calculate probability functions for mortality and malformation with increasing emodin concentrations, we performed logistic regression with a binomial distribution and a logit link function in SPSS 18.0 (SPSS, Inc.). The number of deaths and the number of malformations in each block, relative to the total number of trials, were treated as dependent response variables. For the *X. laevis* assay, we used mating pair (block) as a factor and emodin concentration as a covariate, testing for block-by-concentration interaction. For malformations, there was no significant block-by-concentration interaction; thus, we repeated the analysis solely with main effects. For the *P. triseriata* assay, there was no block-by-concentration interaction effect for either mortality or malformation, so we repeated both logistic regressions testing only main effects of block and emodin concentration. Resulting logistic regression equations for *X. laevis* and *P. triseriata* assays were used to estimate the median lethal concentration at which 50% mortality is observed (LC-50) and median effective concentration at which 50% malformation is observed (EC-50). The ratio of LC-50 to EC-50 was used to estimate the Teratogenicity Index (TI), a measure of developmental hazard with values >1.5 signifying a greater potential for embryos to be malformed in the absence of significant mortality (DuMont et al., 1983).

Results

Emodin Concentration in the Environment.—Water samples from Ponds 1 and 2 did not significantly differ in emodin concentrations detected ($t = 1.39$, df = 6, $P = 0.21$) with mean (SD) concentrations of 0.017 ppm (± 0.015) and 0.004 ppm (± 0.008), respectively. We detected a mean (SD) concentration of 2.007 ppm (± 0.227) emodin from the five hydric soil sample locations adjacent to buckthorn sprouts. Emodin was detected in soil samples radiating from mature stems into seasonal pool basins at mean (SD) concentrations of 0.206 ppm (± 0.018) at 0 m and 0.303 ppm (± 0.014) at 2 m from the stem across four pond sediment samples but was undetectable at 4, 6, 8, and 10 m. Buckthorn leachate samples had detectable emodin concentrations of 0.602 ppm after 24 h. Emodin in leachate samples decreased over time to 0.204-ppm emodin at 48 and 72 h and <0.100-ppm emodin at 96 h. Emodin was not detectable at 168 h.

Xenopus laevis Mortality and Malformation.—Negative controls in the *X. laevis* assay exhibited a mean (SD) mortality of 3.2% (± 2.7) and a mean rate of stunted growth of 11.2% (± 2.2) as the only observed developmental abnormality. Low positive controls exhibited a mean mortality of 12.6% (±14.0) and a mean malformation rate of 93.9% (±10.5) with severe axial and notochord malformations. High positive controls exhibited a mean mortality of 100% within 24 h and, thus, had a mean malformation rate of 0% as the embryos ceased development.

In all emodin treatments, mortality and malformation occurred prior to 96 h, with mortality typically occurring by 48 h and malformations visible between 48 and 72 h. There were significant positive emodin concentration (Wald $\chi^2 = 11.588$, df = 1, $P = 0.001$) and block-by-emodin concentration interaction effects (Wald $\chi^2 = 5.078$, df = 1, $P = 0.024$) but no significant block effect (Wald $\chi^2 = 0.408$, df = 1, $P = 0.523$) on mortality (Fig. 1A). There was a significant positive effect of emodin concentration (Wald $\chi^2 = 12.375$, df = 1, $P < 0.001$) but no significant block effect (Wald $\chi^2 = 0.028$, df = 1, $P = 0.867$) on malformation (Fig. 1B). Logistic regression equations were generated relating emodin concentration to mortality and malformation for Blocks 1 and 2 and used to calculate LC-50 and EC-50 (Tables 1, 2). LC-50s ranged from 5.96–7.12 ppm; EC-50s ranged from 1.21–2.90 ppm; and Teratogenicity Indices ranged from 2.45–4.92 (Table 2).

Pseudacris triseriata Mortality and Malformation.—Negative controls exhibited a mean (SD) mortality of 8.0% (± 6.0) and a mean malformation rate of 1.7% (± 3.5) limited to stunted growth. Low positive controls exhibited a mean mortality of 1.8% (± 3.5) and a mean malformation rate of 94.7% (± 6.7) with severe notochord and axial malformations. High positive controls exhibited mean mortality of 100% within 24 h and, thus, a mean malformation rate of 0%.

As with the FETAX, in all emodin treatments, mortality and malformation occurred prior to 96 h, with mortality typically occurring by 48 h and malformations visible between 48 and 72 h. There were significant positive emodin concentration (Wald $\chi^2 = 11.897$, df = 1, $P = 0.001$), and block (Wald $\chi^2 = 9.914$, df = 3, $P = 0.019$) effects on mortality (Fig. 1C). There was a significant positive emodin concentration effect (Wald $\chi^2 = 25.752$, df = 1, $P < 0.001$) but no significant block effect (Wald $\chi^2 = 1.902$, df = 3, $P = 0.593$) on malformation (Fig. 1D). Logistic regression equations were generated, relating emodin concentration, mortality, and malformation for Blocks 1–4 and used to calculate LC-50, EC-50, and TI for each block (Tables 1, 2). LC-50s ranged from 2.09–4.26 ppm; EC-50s ranged from 0. 05–0.07 ppm; and Teratogenicity Indices ranged from 41.80–60.85 (Table 2).

Severity of Malformations.—For both *X. laevis* and *P. triseriata*, severity of malformations increased with rising emodin concentrations (Figs. 2, 3). The most severe types of malformations and the greatest number of malformations per embryo were observed in the 10.0-ppm emodin treatment for *X. laevis* (Fig. 2) and the 1.0-ppm treatment for *P. triseriata* (Fig. 3; Bantle et al., 1990). Mortality occurred within 24 h, and embryos failed to develop in concentrations >10 ppm for *X. laevis* and concentrations >1 ppm for *P. triseriata*. In both species, malformed embryos in the 0-ppm treatment (negative control) displayed only stunted growth or minor axial asymmetries. Malformations in emodin treatments included stunted growth, axial asymmetries, axial tail malformations, gut malformations, axial notochord malformations, and in the most severe cases, abdominal edema, optic malformations, and severe facial asymmetry (Figs. 2, 3).

Discussion

Direct Effects of Invasive Plant Metabolites on Amphibians.—This study demonstrates a novel aspect of the invasion of *R. cathartica*

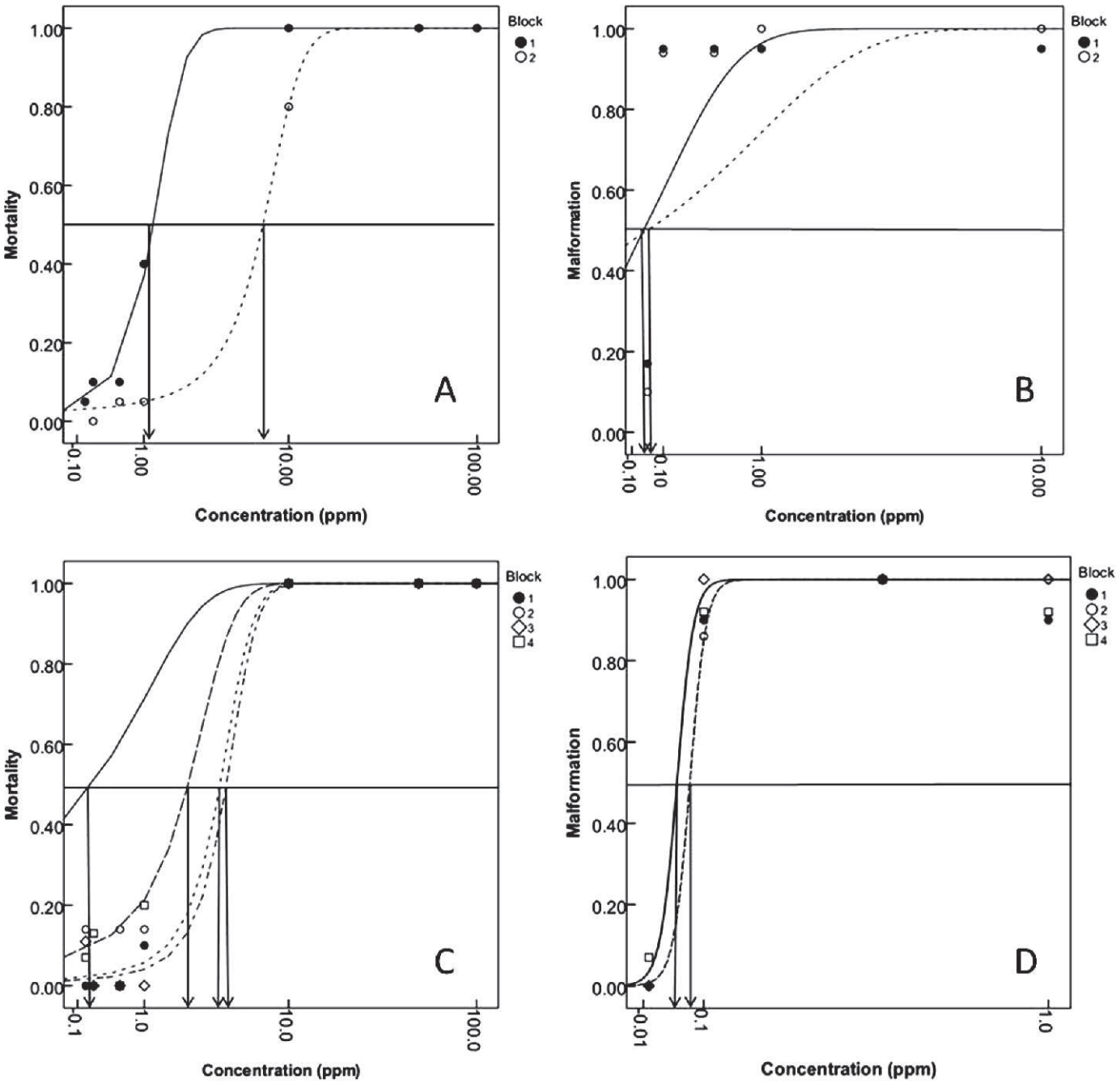

FIG. 1. Probability of *Xenopus laevis* embryo mortality (A) and malformation (B) and *Pseudacris triseriata* embryo mortality (C) and malformation (D) with increasing emodin concentrations. In A and B, filled circles and open circles indicate the raw proportions of mortality and malformation for Blocks 1 and 2, respectively. The solid and broken curves represent the logistic regression equations for Blocks 1 and 2, respectively. For C and D, filled circles, open circles, open diamonds, and open squares indicate the raw proportions of mortality and malformations for Blocks 1, 2, 3, and 4, respectively. The curves represent the logistic regression equations for Blocks 1–4, from left to right. Arrows and reference lines indicate the LC-50 for each block in A and C and the EC-50 for each block in B and D. In D, arrows overlap for blocks 1 and 2 and blocks 3 and 4.

through the ability of the secondary metabolite emodin to induce malformation and mortality during amphibian embryo development in both a model amphibian, *X. laevis*, and in a native species, *P. triseriata*. Previous documentation of effects of invasive plant metabolites from purple loosestrife (*Lythrum salicaria*), cattails (*Typha* spp.), Amur honeysuckle (*Lonicera maacki*), and Chinese tallow (*Triadica sebifera*) on amphibians focused on the larval stage and involved reduced growth, reduced survivorship, and altered behavior (Maerz et al., 2005a, 2010; Watling et al., 2011a; Cotten et al., 2012). We believe that the observed effects of emodin from *R. cathartica* represent an additional environmental stressor that may contribute to regional amphibian declines through reduced hatching success in heavily invaded areas.

More generally, our results demonstrate the ability of invasive plants to introduce chemicals such as emodin into the environment with which native species have not co-evolved (Wynne-Edwards, 2001; Maerz et al., 2005a). Plant traits (increased C : N ratio, phenolics in leaf litter) can result in reduced larval amphibian survivorship whether those plants are native or invasive (Cohen et al., 2012). Although *R. cathartica*

TABLE 1. Coefficients of logistic regression equations $y = 1/1 + e^{-(a+bx)}$ relating mortality and malformation (y) to emodin concentration (x) for embryos of *Xenopus laevis* and *Pseudacris triseriata*. Each block represents a clutch from a different mating pair.

		Mortality		Malformation	
Species	Block	*a*	*b*	*a*	*b*
Xenopus laevis	1	3.448	0.484	1.257	0.433
	2	2.885	0.484	0.525	0.433
Pseudacris triseriata	1	4.144	0.972	4.846	66.289
	2	3.783	0.972	4.912	66.289
	3	2.283	0.972	3.254	66.289
	4	2.034	0.972	3.353	66.289

TABLE 2. Median lethal concentrations (LC-50, ppm), median effective concentrations (EC-50, ppm), and resulting Teratogenicity Indices (TI) for emodin in the *Xenopus laevis* and *Pseudacris triseriata* assays. Each block represents a clutch from a different mating pair. LC-50 and EC-50 were obtained by setting $y = 0.50$ in logistic equations in Table 1.

Experiment	Block	LC-50	EC-50	TI
Xenopus laevis	1	7.12	2.90	2.45
Xenopus laevis	2	5.96	1.21	4.92
Pseudacris triseriata	1	4.26	0.07	60.85
Pseudacris triseriata	2	3.89	0.07	55.57
Pseudacris triseriata	3	2.34	0.05	46.80
Pseudacris triseriata	4	2.09	0.05	41.80

shares the trait of increased C : N ratio, the release of emodin in the breeding pond environment is a trait that is not shared with native plants. Emodin production may characterize other successful invasive plants that negatively impact amphibians. For example, invasive giant knotweed (*Polygonum sachalineae*) reduces amphibian foraging success (Maerz et al., 2005b) and also releases emodin (Izhaki, 2002). Reduced herbivory by phytophagous insects on introduced *R. cathartica* in Canada compared to Europe (Trial and Dimond, 1979) has been interpreted as evidence of a long period of co-evolution between phytophagous insects and *R. cathartica* in Europe, allowing for insect adaptations to emodin and other feeding deterrents (Izhaki, 2002). Thus, although some plant traits may negatively impact amphibians regardless of plant origin, other traits, including the production of novel compounds, may be unique to invasives.

The *X. laevis* assay demonstrated significant levels of embryo mortality and malformation at concentrations of emodin detected in the amphibian breeding environment. The *P. triseriata* assay demonstrated similar deleterious effects of emodin as were observed in *X. laevis*. Moreover, Teratogenicity Indices >1.5 for *X. laevis* and >40 for *P. triseriata* demonstrate that emodin poses a developmental hazard for both species. *Pseudacris triseriata embryos* were more sensitive to emodin than *X. laevis* with mortality and malformation occurring at lower emodin concentrations. For *P. triseriata*, the EC-50 for emodin was below the minimum concentration of emodin examined in our gradient (0.1 ppm). Consequently, even the lower concentrations of emodin detected in pond water were within the range expected to induce malformations. These experiments demonstrate that emodin may cause significant amphibian embryo mortality and malformation at concentrations detected in pond sediments, in the soil surrounding buckthorn plants, and in pond water.

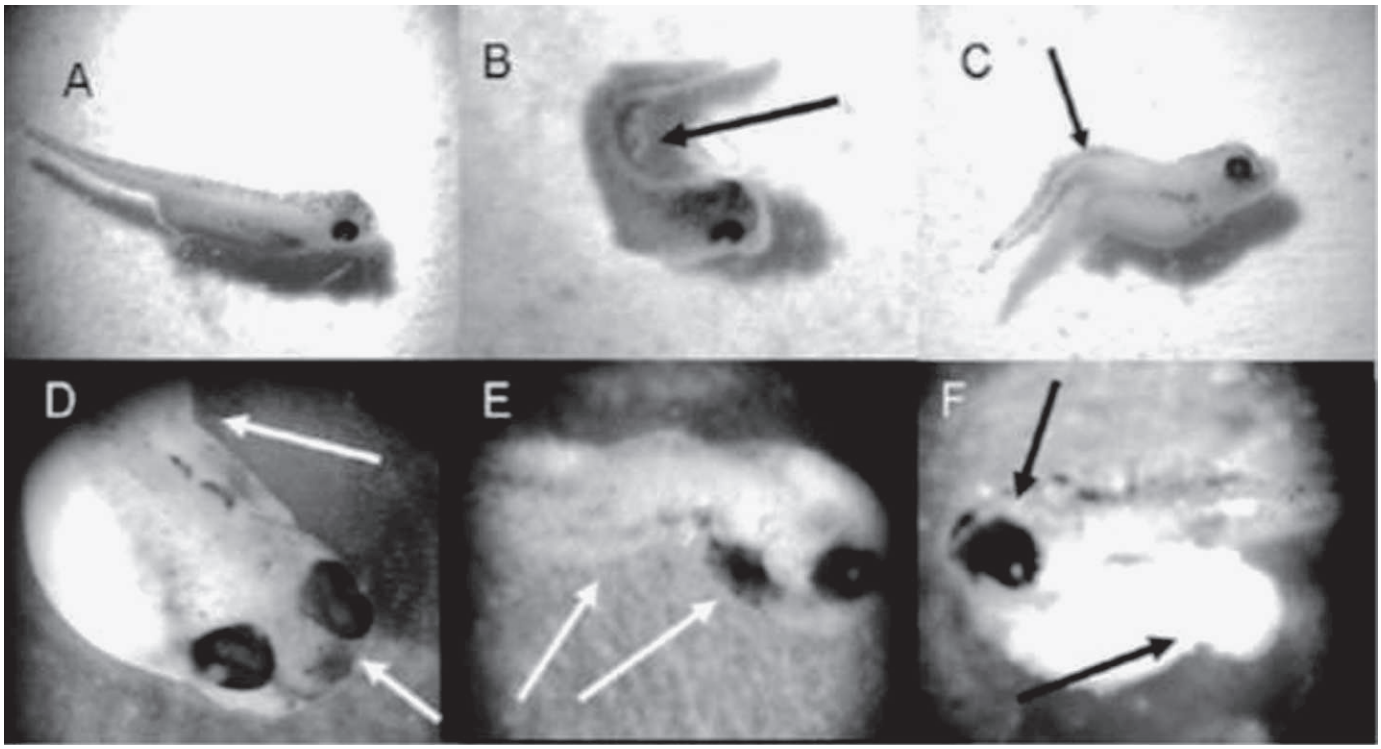

FIG. 2. Examples of typical emodin-induced malformations in *Xenopus laevis*. (A) Normally developing embryo. (B) Axial tail and notochord malformations in 0.1-ppm treatment. (C) Severe "wavy tail" notochord malformation in 0.5-ppm treatment. (D) Axial notochord malformation and facial asymmetry in 1.0-ppm treatment. (E) Severe optic and gut malformation in 10.0-ppm treatment. (F) Severe facial asymmetry, optic malformation, and notochord malformation in 10.0-ppm treatment.

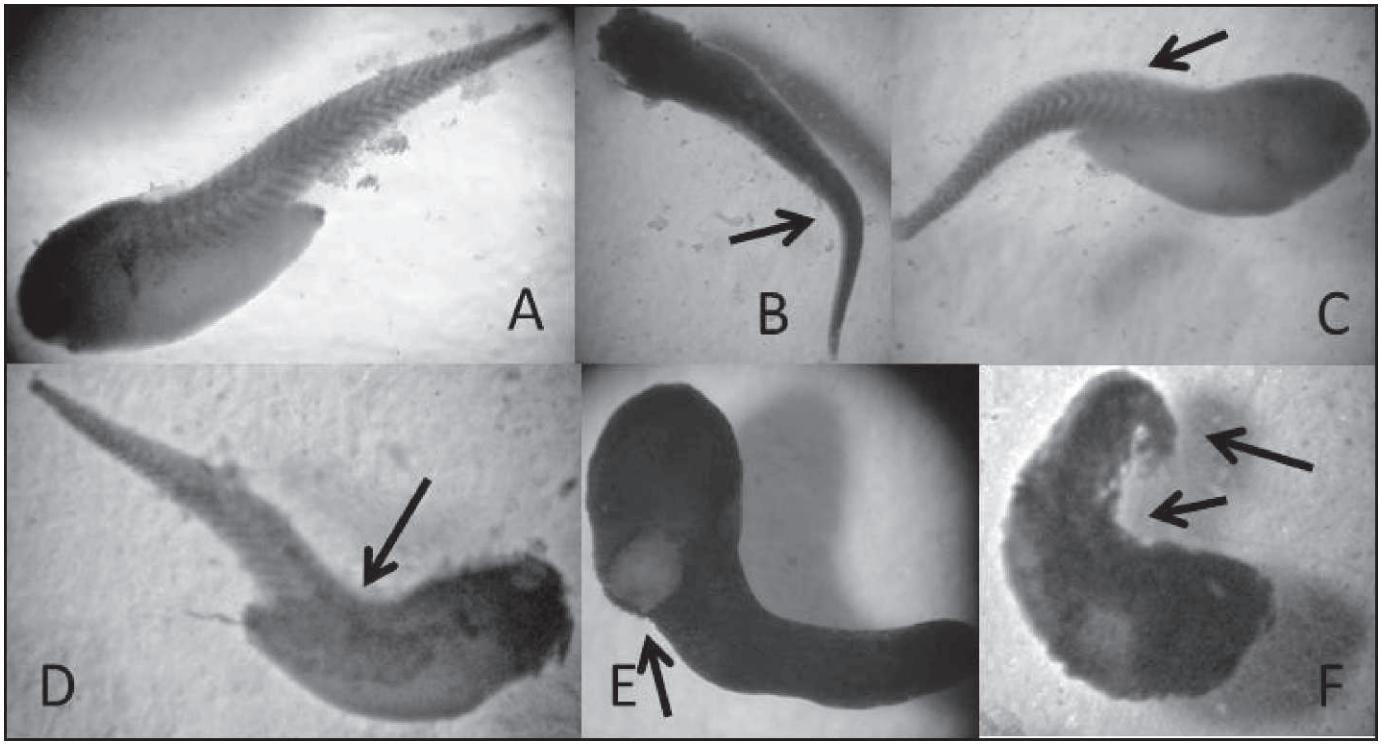

FIG. 3. Examples of typical emodin-induced malformations in *Pseudacris triseriata*. (A) Normally developing embryo. (B) Axial tail and notochord malformations in 0.1-ppm treatment. (C) Severe "wavy tail" notochord malformation in 0.5-ppm treatment. (D) Axial notochord malformation and facial asymmetry in 1.0-ppm treatment. (E) Severe gut malformation in 1.0-ppm treatment. (F) Severe tail malformation, notochord malformation, and facial asymmetry in 1.0-ppm treatment.

Potential for Exposure to Emodin.—Emodin is found throughout *R. cathartica* tissues, including leaves, roots, bark, and fruit (Izhaki, 2002; Tsahar, 2002) and is detectable in the amphibian breeding environment. Our quantification of emodin through HPLC analysis from pond sediment and soil surrounding breeding pools produced mean values ranging from 0.206–2.007 ppm (uncorrected for recovery success and soil type). Although we continue to refine techniques to extract emodin from soils and improve recovery, this is not the first study to document the presence of this compound in the environment. Emodin concentrations of 55 mg/kg dry mass have been reported from soils in which invasive giant knotweed (*Polygonum sachalineae*) was growing (Izhaki, 2002). Furthermore, emodin persisted in dried knotweed litter four months after defoliation at a concentration of 213 mg/kg dry mass (Inoue et al., 1992). Improved recovery and detection will provide better estimates of environmental concentrations of emodin such that we may better understand its movement and persistence in nature.

The concentration of emodin in pond water and sediments is expected to vary depending on the timing of emodin inputs, its precipitation from solution and accumulation in sediments, and its eventual breakdown. Seasonal variation in emodin concentrations in vegetative tissue of *R. cathartica* and other emodin-producing plants have been documented, with emodin representing 50% of the total anthraquinones in leaves in April and May and then decreasing in concentration through the summer (Sherburne, 1972; Trial and Dimond, 1979; Izhaki, 2002; Tsahar et al., 2002). Soil minerals, light, and water availability affect seasonality of emodin production in *R. cathartica* (Izhaki, 2002). As a result, the concentrations we detected in our study site may vary from other regions and vary throughout the amphibian breeding season. Species whose breeding coincides with pulses of emodin production in early spring may have greater risk of teratogenic effects. Similarly, differences in egg mass structure (single vs. clumped eggs) and oviposition site (on leaf litter or pond substrates vs. attached to emergent or floating vegetation) may cause exposure to vary across native species.

Potential for Indirect Effects of Rhamnus cathartica on Amphibians.—*Rhamnus cathartica* invasion has several known indirect effects on wildlife. *Rhamnus cathartica* alters soil properties such that leaf litter and soil moisture are greatly reduced and soil is increasingly acidified (Heneghan et al. 2002; Kurylo et al., 2007; Klionsky, 2010). These changes in soil properties result in increased prevalence of soil arthropods and nonnative earthworms, which accelerate decomposition of leaf litter (Klionsky et al., 2010) and result in boom–bust cycles of soil arthropod populations (Heneghan et al., 2007). Changes in earthworm and soil arthropod densities may impact the prey availability to amphibians (Migge-Kleian et al., 2006). Furthermore, Kurylo et al. (2007) suggest that there is a midwestern ecotype of *R. cathartica* that is predisposed to establishment of dense monocultures in wetlands. Thus, amphibian communities may be indirectly impacted by the changes in hydroperiod, soil moisture, leaf litter cover, and subsequent changes in humidity and temperature. Similar indirect effects resulting from invasion of Amur honeysuckle affect microhabitat suitability and influence the presence and abundance of Green Frogs (*Lithobates clamitans*) (Watling et al., 2011b). Over time, these habitat changes may restrict interwetland movements of amphibians (Gibbons, 2003; Porej et al., 2004). Alteration of habitat structure through changes in native vegetative encroachment has reduced the distribution and abundance of the Natterjack Toad (*Bufo calamita*), and such effects may be expected as invasive plants form dense monocultures (Beebee, 1977; Martin and Murray, 2011). Loss of

amphibian diversity and abundance no doubt has many causes. However, control of the spread of *R. cathartica* helps maintain native plant diversity, habitat structure, and soil moisture regimes that benefit amphibians (Klionsky et al., 2010).

The results of our *X. laevis* and *P. triseriata* assays document direct effects of an invasive plant metabolite, emodin, on amphibian embryo survival and development. This and other studies of direct and indirect effects of secondary plant compounds on amphibians demonstrate the need for more research to assess persistence of invasive plant metabolites in the environment and the subsequent ecosystem changes that may result as invasives are cleared. Additional studies of the impacts of invasive plants on amphibian survival, physiology, and behavior at multiple life stages are necessary. Future research should focus on identifying amphibian species within the invaded range of *R. cathartica* and other nonnative plants and monitoring population responses as habitat structure and composition changes. Because emodin exposure results in amphibian embryo mortality and malformation, it represents an additional threat to population persistence. Further management activities to control the spread of this invasive in North American natural areas are warranted.

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