

Intraspecific variation overrides origin effects in impacts of litter-derived secondary compounds on larval amphibians

Laura J. Martin · Bernd Blossey

Received: 15 February 2012 / Accepted: 8 February 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract Secondary compounds leached from plant litter can negatively affect aquatic amphibian larvae. Non-native plants and their potentially distinct secondary compounds may constitute cryptic threats to native amphibians. We used the availability of both native and introduced *Phragmites australis* (common reed) populations in North America to assess the importance of origin, intraspecific variation, and two purified classes of compounds (tannins and saponins; gradients 0–25 mg L⁻¹) on two common and widespread amphibians (*Ambystoma maculatum*, spotted salamander, and *Lithobates palustris*, pickerel frog). In experiments with purified compounds, high tannin concentrations reduced *A. maculatum* survival and developmental rate while high saponin concentrations reduced survival, developmental rate, and size of *L. palustris* and reduced *A. maculatum* developmental rate. In experiments using leaf litter extracts of 14 different *P. australis* populations, *A. maculatum* larval survival varied among populations but plant origin (native or introduced) did not explain this variation. In contrast to the lack of effects of purified saponins, increases in saponin concentrations in *P. australis* leachates significantly decreased *A. maculatum* survival. Our results suggest: (1) secondary compounds can impact larval amphibian survival and development in species-specific ways; (2) impacts of *P. australis* on *A. maculatum* vary among *P. australis* populations, reflecting intraspecific variation in secondary chemistry; and (3) origin (whether the plant is native or introduced) is

a poor predictor of *P. australis* effects on *A. maculatum*. Scientists and managers may need to move beyond considering origin as a predictive variable when managing plant communities to benefit amphibians.

Keywords Amphibian · Aquatic ecosystems · Chemical ecology · Invasive species · Plant–animal interaction

Introduction

Larval amphibians in littoral habitats of ponds and lakes develop in an environment that is determined to a significant degree by the make-up of adjacent aquatic and terrestrial plant communities. Amphibian larvae respond to “traits” of this environment, including hydroperiod, temperature, competitors, predators, diseases, food availability, and food quality, by changing foraging activity, developmental rates, and timing of metamorphosis (Babbitt and Jordan 1996; Skelly et al. 1999, 2002; Halverson et al. 2003; Rubbo and Kiesecker 2004; Kopp et al. 2006; Schiesari 2006; Williams et al. 2008; Maerz et al. 2010; Stoler and Relyea 2011; Cohen et al. 2012). Allochthonous litter provides the majority of energy and nutrients in near-shore habitats; changes in identity and quality of litter inputs (for example, in response to shifts in plant community composition following plant invasions) influence amphibian larval survival and two highly plastic responses often correlated with adult fitness: larval size at and timing of metamorphosis (Maerz et al. 2005, 2010; Brown et al. 2006; Williams et al. 2008; Watling et al. 2011a, c; Cohen et al. 2012; Earl et al. 2012; Earl and Semlitsch 2012).

Exposure of amphibians to novel plant species with potentially different chemical compounds may represent cryptic threats to amphibians (Maerz et al. 2005; Brown

Communicated by Anssi Laurila.

L. J. Martin (✉) · B. Blossey
Department of Natural Resources, Cornell University,
Ithaca, NY, USA
e-mail: ljm222@cornell.edu

et al. 2006; Watling et al. 2011a, c), but our understanding of the importance of these potential effects remains rudimentary (Kerby et al. 2010; Martin and Murray 2011). A recent surge in investigations assessing amphibian performance in environments with non-native plants has revealed strong species-specific effects ranging from negative, to neutral, to positive (Maerz et al. 2005, 2010; Brown et al. 2006; Williams et al. 2008; Rittenhouse 2011; Watling et al. 2011a, c; Adams and Saenz 2012; Cohen et al. 2012; Cotten et al. 2012; Davidson et al. 2012; Earl et al. 2012; Rogalski and Skelly 2012). Few of these studies explicitly test mechanisms other than plant origin (for example, differences in secondary chemistry or C:N:P ratios) that may help explain variation in amphibian performance (but see Maerz et al. 2005; Cohen et al. 2012). Furthermore, most studies use a single introduced species and compare it to a single or a few native species or groups of native species (Brown et al. 2006; Rittenhouse 2011; Watling et al. 2011a, b, c; Adams and Saenz 2012; Cotten et al. 2012). Although these experiments suggest that secondary compounds in invasive plants negatively impact larval amphibians, they do not test effects directly, nor do they compare closely related native and non-native plant species. Without phylogenetic controls, effects of plant origin can be conflated with variation of impacts due to phylogenetic distance (Agrawal and Kotanen 2003). In addition, functional traits such as litter chemistry are not always determined by taxonomic relationships or plant origin, and thus plant origin largely fails in predicting habitat quality (Meier and Bowman 2008; Leishman et al. 2010; Cohen et al. 2012). Finally, while intraspecific variation in plant chemistry is known to structure plant–insect interactions and their extended impacts on local food webs (LeRoy et al. 2006; Bailey et al. 2009; Smith et al. 2011), the importance of intraspecific trait variation has been largely ignored in community ecology (Violle et al. 2012). Interspecific differences in plant traits are assumed to be larger than intraspecific differences, but intraspecific variation can account for as much as 40 % of overall trait variation (Kattge et al. 2011), and both the extent and importance of this intraspecific variation are increasingly recognized (Violle et al. 2012).

We conducted two experiments to assess effects of: (1) plant origin (native or introduced); (2) plant intraspecific variation; and (3) purified plant derived tannins and saponins on two larval amphibians (*Ambystoma maculatum*, spotted salamander, and *Lithobates palustris*, pickerel frog). Both amphibian species are widespread and common in eastern North America (Hulse et al. 2001), and their distribution and habitat requirements overlap extensively with our focal plant species, *Phragmites australis* (common reed). We tested two amphibian species that belong to different functional groups (predatory salamander larvae

with external gills and an omnivorous tadpole) because differences in life history and physiology may lead to variation in species' tolerances of habitat conditions and secondary compounds (Ultsch et al. 1999; Brown et al. 2006). Both amphibians prefer forested landscapes and breed soon after ice out in the study area (April/May). The aquatic larvae of both species develop over an extended period from May to early fall before metamorphosing.

The breeding locations of both amphibians are being invaded by one of the most widespread angiosperms in the world, *P. australis*. This clonal grass has existed in North America for tens of thousands of years, but invasive European genotypes were introduced into North America in the nineteenth century (Saltonstall 2002). Over the past decades, introduced *P. australis* genotypes spread across much of northeastern North America and often replaced native genotypes that are recognized as a separate subspecies, *Phragmites australis americanus* (Saltonstall 2002; Saltonstall et al. 2004). The co-occurrence of native and introduced *P. australis* populations with great intraspecific variation in a number of important morphological traits and in insect herbivore preferences (Hansen et al. 2007; Park and Blossey 2008) provides a unique opportunity to assess origin and intraspecific variation for their impact on two native amphibians while controlling for phylogenetic effects.

We selected tannins (polyphenols) and saponins (amphiphathic glycosides) as two classes of chemical compounds that are well studied for their effects on a variety of organisms but not necessarily for amphibian larvae (Kerby et al. 2010). Our target wetland plant species, *P. australis*, contains saponins that can act as feeding repellents for insects (Herlt et al. 2002) and shrimp (Chen et al. 1996). Saponins can swell gill lamella and interlamellar epithelia, lyse blood cells, and lower the surface tension between water and fish gills, leading to a slow death by preventing oxygen uptake (Sparg et al. 2004). High soybean-derived saponin concentrations kill *Bufo viridis* (European green toad) tadpoles within minutes (Ishaaya et al. 1969), and many have molluscicidal properties (Sparg et al. 2004). Similar to fish, many larval amphibians are obligate gill breathers (Burggren and Infantino 1994), or possess a limited ability to compensate for gill damage (Ultsch et al. 1999), and thus appear especially vulnerable to saponins.

Tannins are produced by many plant species that co-occur with *A. maculatum* and *L. palustris* (Maerz et al. 2005), and it is likely that no two plant species share the same tannin “portfolio” (Salminen and Karonen 2011). A recent review of toxicological studies (Kerby et al. 2010) revealed that larval amphibians appear particularly vulnerable to phenolics and at rather low concentrations ($<1 \text{ mg L}^{-1}$), yet the impacts of phenols on amphibians have been largely overlooked, despite their importance in

ecosystem processes such as decomposition (Kraus et al. 2003). The traditional view of tannin effects on consumers (derived from their protein precipitation capacity that rendered plant tissues non-nutritious) is being challenged and a more diversified and complicated view is emerging that includes tannin oxidation (Salminen and Karonen 2011). Oxidative stress in aquatic environments resulting from high tannin concentrations can also cause sub-lethal to lethal gill lesions in fish (Temminck et al. 1989), prevent sexual maturation in fish (Morrongiello et al. 2011), and has lethal consequences by damaging midgut epithelium cells in some dipteran larvae (Rey et al. 1999). The known effects of tannins and saponins on digestion, respiration, and overall physiology of multiple aquatic species suggest that larval amphibians may also be impacted by these plant-derived compounds.

We conducted two outdoor common garden experiments: in the first experiment, we reared individual larval *A. maculatum* and *L. palustris* in an aqueous gradient of purified tannins or saponins; in the second experiment, we reared *A. maculatum* larvae in leachate from seven native and seven introduced populations of *P. australis*. We were guided in our investigation by the following hypotheses: (1) amphibian survival and developmental rate will decrease as tannin and saponin concentrations increase; (2) negative effects of saponins will occur at lower concentrations for *A. maculatum* than for *L. palustris*; and (3) introduced *P. australis* populations will exhibit higher saponin concentrations, and have stronger negative effects on *A. maculatum* larval survival and development, than native *P. australis americanus* populations.

Materials and methods

We conducted two outdoor common garden experiments from May–August 2009. We collected multiple egg clutches of *A. maculatum* and *L. palustris* on 21 April and 6 May, respectively, from the Arnot Forest in Van Etten, NY (42.291977°N, 76.651890°W). We immediately transported egg clutches to the Cornell University Resource Ecology and Management facility, where we held clutches individually in 15-L plastic containers that floated in a large outdoor pool. We changed water every 2–4 days and fed hatching larvae with fish flakes ad libitum.

Saponin and tannin gradients

We reared *A. maculatum* and *L. palustris* larvae ($n = 20$ /treatment) in aged and filtered tap water (control) or added 1, 5, 10, 15, 20, or 25 mg L⁻¹ of commercially purified saponins (Sigma-Aldrich 84510, St. Louis, MO, USA) or tannic acid (Sigma-Aldrich 16201) for a total of 14

treatments. The range of our experimental concentrations exceeds reported early summer data (1–11 mg L⁻¹) for reactive phenolic compounds in ponds of the northeastern U.S. (Freda and Dunson 1986; Maerz et al. 2005; Earl et al. 2012) but remain well below typical discharge levels from tanneries or wood processing facilities (50 mg L⁻¹ and above) (Njau and Renalda 2010). Saponin concentrations in aquatic environments are largely unknown and concentrations of saponins produced by *Rumex fluviatilis* in the river Rhine fluctuated seasonally and differed among years but did not exceed 1.5 mg L⁻¹ (Wegner and Hamburger 2002). Our experimental concentrations purposefully spanned a larger range since we had no data on the potential vulnerability of our amphibian taxa.

Approximately 1 week after hatching, we randomly selected four larvae from each of five clutches for each treatment and placed them individually into 1-L plastic cups; *A. maculatum*: 15 May, Harrison stage 40 (Donavan 1980); *L. palustris*: 8 June, Gosner stage 26 (Gosner 1960). We floated cups in 1,100-L Rubbermaid stock tanks (52–65 cups/tank) as buffer against rapid temperature fluctuations and arranged them in a block design randomized by clutch and treatment across four tanks for *A. maculatum* and five tanks for *L. palustris*. We covered individual cups with a fine mesh and tanks with a clear plastic roof (leaving the sides open) to protect against dilution of treatments by rainfall. We added small pebbles to cups on 10 June to increase structural complexity.

We fed individual amphibians ad libitum [*A. maculatum*: *Daphnia pulex*, amphipods and chironomids; *L. palustris*: TopFin tropical flakes (Franklin, WI, USA) and Mazuri Rabbit Diet (Brentwood, MO, USA)]. Every 2 weeks, we recorded water temperature, dissolved oxygen, conductance, and pH of a random subset of cups (3/treatment) using a YSI 556 MPS (YSI Environmental, Yellow Springs, OH, USA). We recorded larval survival every 2–3 days and *A. maculatum* developmental stage at weeks 2, 5, and 10, after which we terminated the experiment. The literature on developmental stages of salamanders is far less extensive than for tadpole development; here, we use an expanded version of the Harrison series, the Donovan series, which describes developmental stages from uncleaved egg through metamorphosis (Donavan 1980). We recorded *L. palustris* developmental stage at week 10 (Gosner 1960) and final snout–vent length (SVL) of all surviving individuals for both species.

P. australis leachate and salamander larval performance

Between 25 November and 30 December 2008, we collected fully senescent leaves still attached to upright standing stems from seven native *P. australis americanus*

and seven introduced *P. australis* populations from across the United States (Table 1). We previously determined the native/introduced status of our collection populations using morphological characters developed by our group. Our regional samples included five native/introduced pairs collected within close proximity (1 km) of one another (Table 1). We stored all leaves dry in opaque paper bags at room temperature over the winter.

To assess effects of naturally occurring phytochemicals on larval amphibian performance, we leached randomly selected leaves from each population in aged and filtered tap water. We used 1 g dry litter L⁻¹, an approximation of wetland litter inputs in New York state (J. Dietrich, unpublished data), and similar to concentrations used in previous studies (Maerz et al. 2005; Brown et al. 2006). We gently rinsed leaves to dislodge foreign material before leaching and, after 48 h, filtered leachates through cheesecloth and transferred 800 mL to each experimental cup. We also prepared a no-litter control using aged tap water otherwise handled identically and arranged cups at random across nine cattle tanks (50 cups/tank).

On 15 May, we randomly selected three recently hatched *A. maculatum* larvae from each of ten clutches for each treatment and placed a single larva into each cup (15 treatments × 30 replicates/population for a total of 450 cups). We replaced leachates every 20 days to avoid waste accumulation and recorded *A. maculatum* survival, SVL, developmental stage, and abiotic variables (in five cups/treatment) as described above.

We estimated total saponin concentration for each *P. australis* population (Hostettmann and Marston 1995) by adding 0.058 g of phosphoric acid to 5 mL *P. australis* leachate immediately after 48 h leaching and shaking the

solution in a 10-mL graduated cylinder for 1 min. After an additional 1 min, we measured the volume of stable foam as an index of approximate saponin concentration. We compared these volumes to those of a standard curve generated with purified saponins.

Data analysis

We constructed all models of larval performance in JMP 9.0 and SAS 9.2 (SAS Institute, Cary, NC, USA). To test for abiotic differences between treatments in experiments 1 and 2, we used one-way ANOVA with independent contrasts. To calculate the probability of survival from time-to-death data, we constructed a Cox proportional hazards regression model testing for effects of treatment, clutch, and tank. This method is semi-parametric requiring no choice of probability distribution for time-to-death and estimates effects of covariates by maximum partial likelihood (Cox 1972; Allison 1995). In these analyses, a larva killed during the experiment represented one complete observation, whereas survivors were right-censored at the time the experiment was terminated. Differences between risk ratios were tested with effect likelihood ratios.

We constructed ordinal logistic regressions of final Gosner stage (*L. palustris*, week 10) or Donovan stage (*A. maculatum*, weeks 2, 5, 10) using the GENMOD platform in SAS 9.2, conducting independent contrasts by comparing least square means, to analyse whether treatment, clutch, or tank affected development of surviving individuals. We modeled *A. maculatum* developmental stages separately for weeks 2, 5, and 10 because mortality was not random; therefore, missing values among sampling weeks would cause unnecessary loss of power when

Table 1 Population, abbreviation, origin, and geographical coordinates of 14 *Phragmites australis* populations used to assess *Ambystoma maculatum* performance

Pair	Population	Abbreviation	Origin	Lat (°N)	Long (°W)
1	Agawam Lake, MA	MA-N	Native	42.2664	73.3266
1	Agawam Lake, MA	MA-I	Introduced	41.5857	70.6371
	Blackstone River, RI	RI-I	Introduced	41.9763	71.4838
	Syracuse, NY	NYS-I	Introduced	43.0777	76.0493
	Waubay NWR, SD	SD-N	Native	45.4114	97.3614
2	Nonesuch River, ME	ME-I	Introduced	43.5561	70.3320
2	Nonesuch River, ME	ME-N	Native	43.5610	70.3297
	Aransas NWR, TX	TX-N	Native	28.3024	96.8061
3	Eastern VA Rivers NWR	VA-I	Introduced	37.9173	76.8591
3	Eastern VA Rivers NWR	VA-N	Native	38.0710	76.9401
4	Douglas County, WI	WI-N	Native	46.4180	92.0847
4	Douglas County, WI	WI-I	Introduced	46.4899	92.1833
5	Caldwell Pond, NY	NY-N	Native	43.6997	76.1893
5	Caldwell Pond, NY	NY-I	Introduced	43.6988	76.1906

Populations within close proximity (1 km) of one another designated as pairs

analyzing all weeks at once. We constructed a mixed model with treatment and clutch as fixed effects and tank as a random effect to test whether final SVL of surviving individuals differed among treatments. We tested for significant differences between treatments with Tukey's HSD.

To compare differences in *A. maculatum* performance among *P. australis* populations we analysed the full dataset, along with two subsets: (1) all *P. australis* populations without the water control [testing effects of *P. australis* origin (native vs. introduced) and population nested within origin], and (2) those ten *P. australis* populations for which native and introduced samples were geographically paired (testing effects of *P. australis* origin, collection location, and their interaction). Models of larval performance were otherwise identical to those described for the gradient experiment. We used linear regression to assess whether survival, developmental stage, and final SVL were functions of estimated population saponin concentration.

Results

We observed high survival (~70 %) in control treatments, indicating that experimental conditions provided suitable larval environments. Abiotic variables did not differ significantly among treatments in the gradient or the litter leachate experiments (Table 2). We therefore exclude them as explanatory factors in models of larval performance.

Saponin and tannin gradients

For *A. maculatum*, probability of survival was significantly reduced in tannin treatments ≥ 20 mg L⁻¹ ($\chi^2 = 46.43$, $P < 0.0001$; Fig. 1) and developmental rates were decreased in tannin concentrations ≥ 5 mg L⁻¹ by week 2 ($\chi^2 = 23.72$, $P = 0.0006$) and ≥ 1 mg L⁻¹ by week 5 ($\chi^2 = 46.43$, $P < 0.0001$; Fig. 1). This pattern continued until the termination of the experiment at week 10 ($\chi^2 = 27.82$, $P < 0.0001$) but tannin concentrations did not affect SVL of surviving *A. maculatum* (Fig. 1).

Probability of *A. maculatum* survival was not significantly affected by saponin concentration ($\chi^2 = 7.33$, $P = 0.292$; Fig. 1) but developmental stage was reduced in saponin treatments ≥ 5 mg L⁻¹ in weeks 2 and 5 ($\chi^2 = 13.90$, $P = 0.0307$ and $\chi^2 = 45.50$, $P < 0.0001$, respectively). By week 10, only the 25 mg L⁻¹ treatment differed from the control ($\chi^2 = 24.72$, $P = 0.0004$). Final *A. maculatum* SVL was not affected by saponin concentration (Fig. 1).

Purified tannins did not affect *L. palustris* probability of survival ($\chi^2 = 5.92$, $P = 0.432$), final development stage ($\chi^2 = 6.74$, $P = 0.344$), or final SVL ($F_{6,92} = 1.20$, $P = 0.313$). In contrast, probability of *L. palustris* survival was significantly reduced in saponin treatments ≥ 15 mg L⁻¹ ($\chi^2 = 13.74$, $P = 0.0327$). However, probabilities of survival among the 15, 20, and 25 mg L⁻¹ treatments were not significantly different from each other (risk ratios = 5.17–6.18). Development was also slightly retarded in the 20 and 25 mg L⁻¹ saponin treatments compared to the control ($\chi^2 = 27.47$, $P = 0.0012$; Fig. 1), and final *L. palustris* SVL was negatively correlated with saponin concentration ($R_{\text{adj}}^2 = 0.40$, $F_{1,80} = 54.33$, $P < 0.0001$; Fig. 1).

P. australis leachate

Probability of *A. maculatum* survival ranged from 0.2–0.67, and was affected by both *P. australis* population ($\chi^2 = 29.21$, $P = 0.0098$) and clutch ($\chi^2 = 72.10$, $P < 0.0001$), but was significantly lower than the water control in all treatments but WI-N and MA-N (Fig. 2). Larvae died through the duration of our experiment, but the greatest mortality occurred between days 21 and 36 (Fig. 3). Population and clutch also affected developmental stage in week 2 (clutch: $\chi^2 = 42.65$, $P < 0.0001$; population: $\chi^2 = 37.21$, $P = 0.0007$) and week 5 (clutch: $\chi^2 = 32.36$, $P = 0.0002$; population: $\chi^2 = 51.85$, $P < 0.0001$), whereas population but not clutch was significant in week 10 ($\chi^2 = 33.32$, $P = 0.0026$). Intraspecific variation among *P. australis* populations had a strong influence on larval development, which was delayed in four populations by week 2 and eight

Table 2 Temperature (°C), conductance ($\mu\text{S l}^{-1}$), pH, and dissolved oxygen (mg l^{-1}) in two experiments to assess effects of saponin and tannin gradients, or origin (native or introduced) on *A. maculatum* and *L. palustris* performance

Abiotic variable	Gradient experiment			<i>Phragmites</i> experiment		
	Control	Saponin	Tannin	Control	Native popns	Intro popns
Temperature (°C)	20.9 ± 0.50	21.2 ± 0.56	21.2 ± 0.56	20.9 ± 1.00	20.7 ± 0.89	20.8 ± 0.97
Conductance ($\mu\text{S l}^{-1}$)	0.5 ± 0.27	0.6 ± 0.27	0.6 ± 0.28	0.5 ± 0.08	0.5 ± 0.09	0.6 ± 0.09
pH	7.6 ± 0.40	8.4 ± 0.39	7.9 ± 0.21	8.6 ± 0.80	8.7 ± 0.85	8.7 ± 0.88
Dissolved oxygen (mg l^{-1})	10.2 ± 0.33	10.8 ± 0.37	10.4 ± 0.71	10.2 ± 0.66	10.1 ± 0.89	10.3 ± 1.29

Data are means ± 1 SE of subsamples across treatments (Experiment 1: 3 reps/treatment, 13 treatments, 5 sampling dates, $n = 195$; Experiment 2: 5 reps/treatment, 15 treatments, 5 sampling dates; $n = 375$)

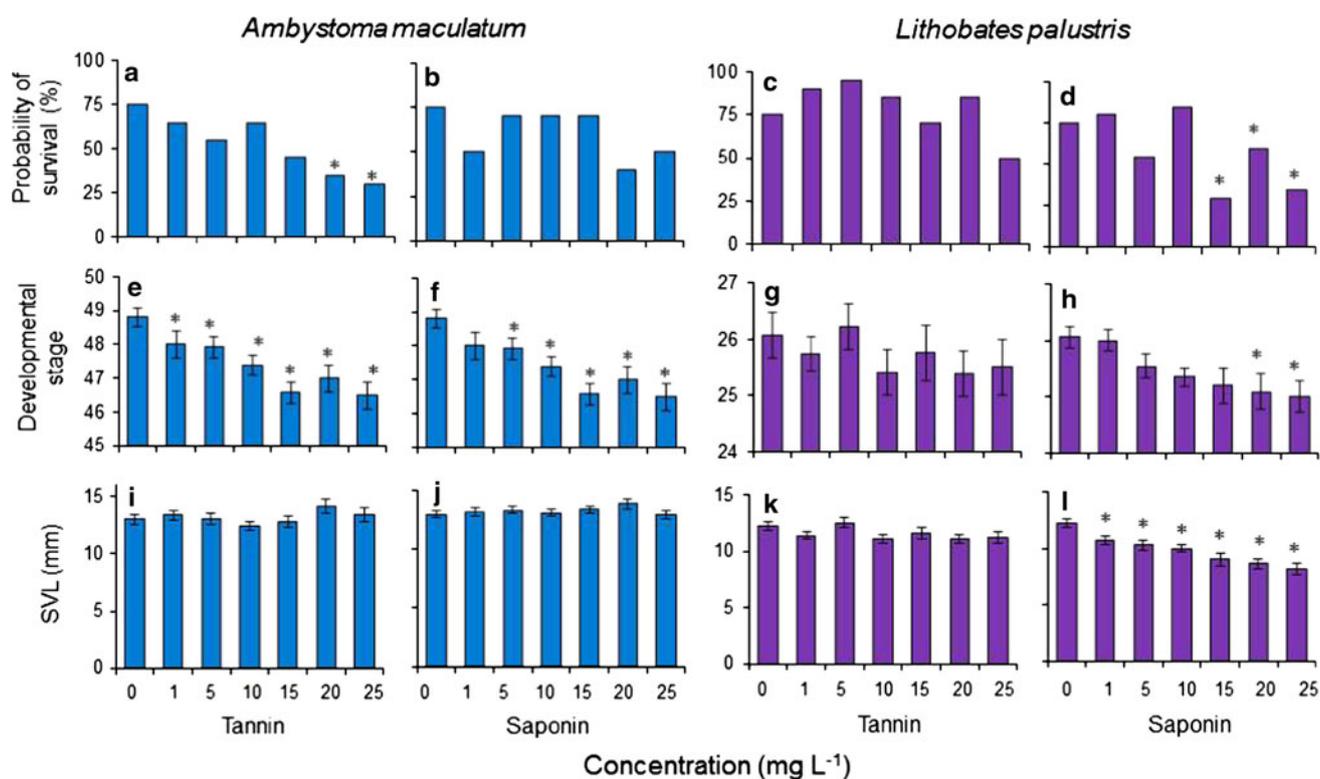


Fig. 1 Effect of tannin and saponin concentrations (mg L^{-1}) on **a–d** probability of survival **e–h** developmental stage (week 5 Donovan stage for *A. maculatum*; week 10 Gosner stage for *L. palustris*), and **i–l** snout–vent length (mm) of *Ambystoma maculatum*

populations by week 5 (Fig. 2); by week 10, differential mortality had obscured these effects (Fig. 2). Final SVL also varied by population ($F_{14,187} = 3.04$, $P = 0.0003$), and was significantly larger in SD-N and WI-I than in the control (Fig. 2).

When analyzing the dataset with the control treatment removed, we found significant effects of clutch on probability of survival ($\chi^2 = 44.44$, $P = 0.0001$), but neither *P. australis* origin ($\chi^2 = 2.46$, $P = 0.117$) nor population within origin ($\chi^2 = 12.55$, $P = 0.483$) were significant. Similarly, although clutch had a significant effect on weeks 2 and 5 developmental stage (week 2: $df = 9$, $N = 438$, $\chi^2 = 38.19$, $P < 0.0001$; week 5: $df = 9$, $N = 173$, $\chi^2 = 25.74$, $P = 0.002$), origin was not significant. By week 10, many individuals had died, and neither clutch nor origin influenced the final developmental stage of surviving individuals. Final SVL was the only response variable significantly affected by origin ($F_{1,154} = 4.44$, $P = 0.0368$) and population within origin ($F_{12,154} = 1.92$, $P = 0.0359$): Larvae reared in leachate of native populations were slightly smaller (mean \pm SE = 14.16 ± 0.17 mm) than those reared in leachate of introduced populations (14.65 ± 0.16 mm). We found significant differences in SVL among native populations ($F_{6,154} = 2.62$, $P = 0.0192$) but not among introduced populations

and *Lithobates palustris*. Data shown as mean \pm 1SE of 5–15 individually reared larvae per treatment (initial $n = 20$ per treatment). Asterisk treatments significantly different from control, $P \leq 0.05$

($F_{6,154} = 1.22$, $P = 0.298$). When we modeled only those ten *P. australis* treatments paired by collection location, we found no effect of collection location, origin, or their interaction on probability of survival, developmental stage, or final SVL.

Foam height was a reliable predictor of saponin concentration ($R^2 = 0.98$, $P < 0.0001$; Fig. 4). Saponin concentration in *P. australis* leachates ranged from 0.45 to 5.87 mg L^{-1} . *Ambystoma maculatum* survival was strongly negatively correlated with estimated saponin concentration ($R^2_{\text{adj}} = 0.69$, $F = 31.89$, $P < 0.0001$) and developmental stage was weakly negatively correlated with estimated saponin concentration in week 5 ($R^2_{\text{adj}} = 0.29$, $F = 6.75$, $P = 0.022$) but not in weeks 2 ($R^2_{\text{adj}} = 0.15$, $F = 3.43$, $P = 0.087$) or 10 ($R^2_{\text{adj}} = 0.14$, $F = 3.32$, $P = 0.091$; Fig. 4). Final SVL was positively but weakly correlated with saponin concentration ($R^2_{\text{adj}} = 0.27$, $F = 6.06$, $P = 0.029$).

Discussion

We used two unique approaches in our assessment of impacts of plant-derived compounds and plant invasions on

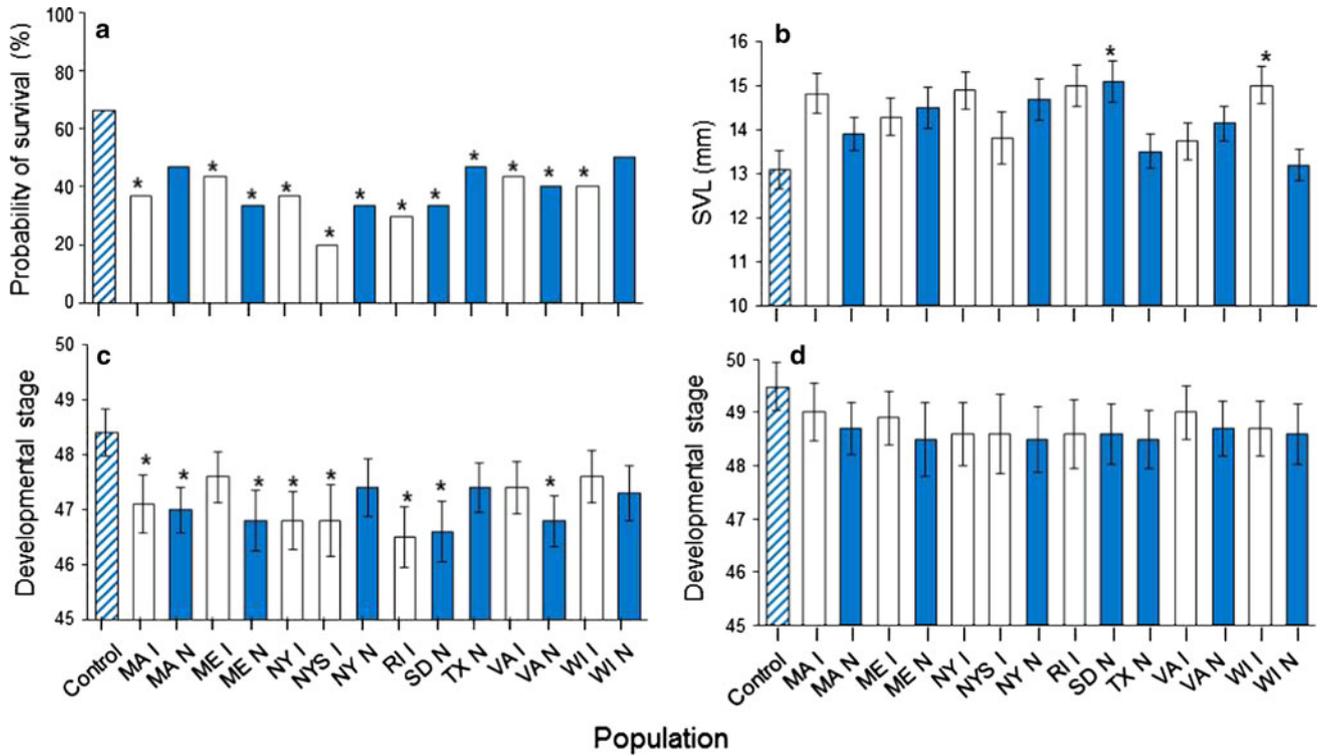
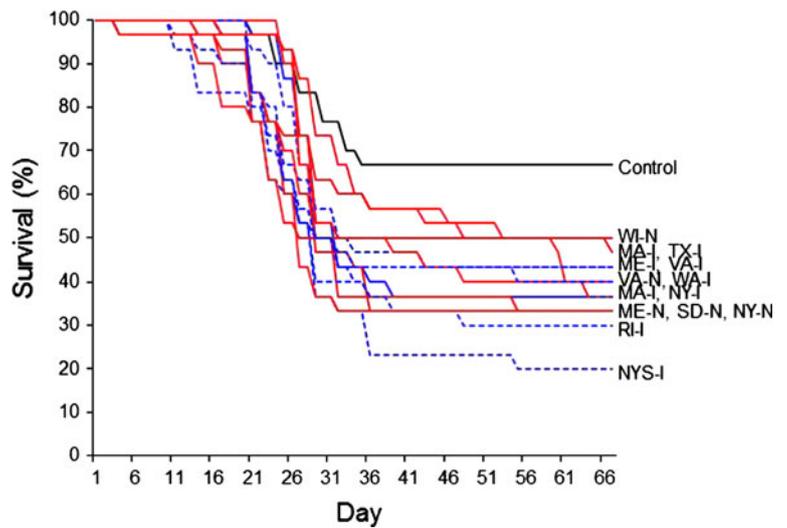


Fig. 2 Larval *A. maculatum* **a** probability of survival, **b** SVL (mm), and Donovan developmental stage **c** at week 5 and **d** at week 10 when reared in *Phragmites australis* leachate from 14 different native (*N*, solid bars) and introduced (*I*, white bars) populations and a water

control (hatched bars). Data shown as mean \pm 1SE of 6–20 individually reared larvae per treatment (initial $n = 30$ per treatment). Asterisk treatments significantly different from control, $P \leq 0.05$. See Table 1 for population abbreviations

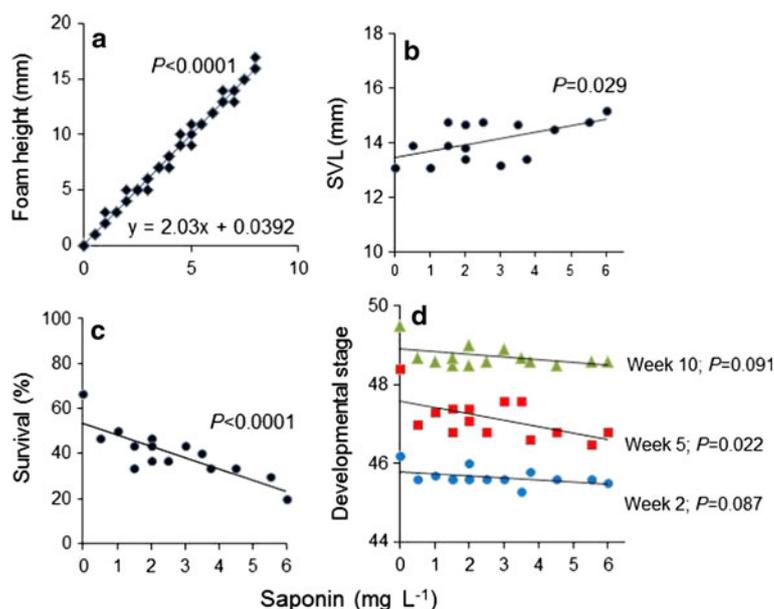
Fig. 3 Larval *A. maculatum* survival (%) in a water control and leachates of 14 different native (*N*, solid lines) and introduced (*I*, dotted lines) *P. australis* populations over a 2-month experimental period. Data are totals of 30 individually reared larvae per population. See Table 1 for population abbreviations



native larval amphibians: (1) gradients of purified tannins and saponins; and (2) phylogenetic control with multiple genotypes of a plant species. Both approaches yielded important new insights into how secondary compounds, plant origin, and plant phytochemical phenotype affect amphibian larvae. We demonstrated that both saponins and tannins can negatively impact larval amphibians. The magnitude of this effect varied across amphibian species

and was a function of concentration. In the tannin experiment, we found delayed development of *A. maculatum* at concentrations as low as 1 mg L^{-1} , at the low end of recorded tannin concentrations in U.S. wetlands (Freda and Dunson 1986; Maerz et al. 2005; Earl et al. 2012), suggesting a potentially important cryptic influence of phenolic compounds that may have been previously overlooked. We have little information about saponin

Fig. 4 **a** Standard curve of foam height as a function of saponin concentration. *Ambystoma maculatum* **b** SVL, **c** survival (%), and **d** Donovan development stage at week 2 (blue points), 5 (red points), and 10 (green points) as a function of estimated saponin concentration (mg L^{-1}) for 15 *P. australis* populations. Data are means of 6–20 individually reared larvae per population with initial $n = 30$ per population (color figure online)



concentrations in field settings, but in our experiments the highest saponin concentrations reduced survival, developmental rate, and size of *L. palustris* and reduced *A. maculatum* developmental rate. Most importantly, we demonstrated high intra-specific variation in saponin concentrations for *P. australis*. Reduced *A. maculatum* survival was a function of increasing saponin concentrations (Fig. 4) but not origin of the leaf material (native or introduced).

Concentrations of secondary compounds, including tannins and saponins, vary among plant species and genotypes and even vary temporally within a plant species (Salminen et al. 2004; Yarnes et al. 2006). Differences in these phytochemical phenotypes are a function of genetic as well as environmental (climate, soils, past and present herbivory, water, nutrient availability, light, etc.) factors (Haviola et al. 2012). This mosaic of different genotypes and their interaction with abiotic and biotic factors forms the basis of rapid evolutionary interactions (Agrawal et al. 2012) and affects decomposition, invertebrate consumers, and higher trophic levels (Müller et al. 2006; Bailey et al. 2009). Despite the increasingly recognized importance of intra-specific variation in plant traits (Kattge et al. 2011; Violle et al. 2012), our study, to the best of our knowledge, is the first to demonstrate the biological significance of this variation in litter traits to developing amphibians.

The results for *P. australis* and *A. maculatum* strongly indicate that intra-specific variation in plant traits is more important than evolutionary origin, at least in the context of this experiment. Similar results were reached in a mesocosm experiment involving three native and three introduced plant species and five native larval amphibians (Maerz et al. 2010). Survival, developmental rate and identity of species able to complete metamorphosis was a

function of plant litter C:N ratio but not origin (Maerz et al. 2010), and the same results were obtained in a large follow-up field study (Cohen et al. 2012). These results question the validity of using origin as an explanatory factor when assessing impacts of plants without attention to functional plant traits. Reports of negative impacts when comparing effects of a single introduced plant such as *L. salicaria*, *Lonicera maackii*, or *Triadica sebifera* to a single native species or a mixture of native species may be the result of selecting particular species (or phytochemical phenotypes) from the regional species pool for comparisons and not representative of generalizable origin effects (Brown et al. 2006; Rittenhouse 2011; Watling et al. 2011a; Adams and Saenz 2012; Cotten et al. 2012).

The accumulating evidence that native species are not by default of better quality for larval amphibian development requires a fundamental rethinking of our assessment of invasive species impacts and approaches to amphibian conservation or habitat management. At a minimum, future work should assess core functional traits (C:N, for example) and not assume that all individuals of a species, or species within a genus, are of similar quality or chemical composition. While this will increase the size of experimental investigations, it is more likely to uncover fundamental mechanisms that are responsible for differences in amphibian performance in different habitats. We do not question the importance of many introduced species as potential drivers of habitat deterioration, but we need a more refined impact assessment before making management choices.

Biological invasions will continue to reshape plant community composition with important but currently widely unknown consequences for water chemistry. If a wetland plant community was to be simplified through

replacement of mixed vegetation with monoculture, as often happens with *P. australis* invasion, the particular chemistry of that wetland would likely change dramatically with diverse consequences for different biota. Our observed species-specific effects are consistent with previous studies in which larval amphibians have demonstrated interspecific variation in their responses to plant treatments (Skelly et al. 2002; Maerz et al. 2005, 2010; Watling et al. 2011b; Cohen et al. 2012; Cotten et al. 2012; Earl et al. 2012; Earl and Semlitsch 2012). At present, we can only speculate about the potential mechanisms that may explain these differences, among them different tolerances to plant secondary compounds and their toxicity (Kerby et al. 2010), as well as behavioral plasticity (such as surface air breathing) that may ameliorate effects that are not immediately lethal (Ultsch et al. 1999; Brown et al. 2006; Maerz et al. 2005). Although our experiment was not designed to test for physiological mechanisms of phytochemical impact, the fact that death occurred gradually throughout the duration of the experiment is reminiscent of the “slow death by oxygen deprivation” known to occur when saponins lower the surface tension between water and fish gills (Sparg et al. 2004).

An important question is whether effects we demonstrated in our experiment have field relevance. Unlike field settings, small-scale investigations allow for manipulation of single variables (Chalcraft et al. 2005; Drake and Kramer 2012); however, small containers do not capture the complexity of natural systems and their results sometimes but not always hold true under field conditions (Skelly 2002; Melvin and Houlahan 2012). Furthermore, commercially available compounds are not always good surrogates for the diversity of secondary compounds such as tannins that are found in plants and litter (Rautio et al. 2007; Salminen and Karonen 2011). Our results comparing *P. australis* leachate with commercially available saponin clearly illustrate this issue. *Ambystoma maculatum* was sensitive to much lower saponin concentrations in *P. australis* leachate than in the gradient experiment, suggesting that (1) saponins derived from certain *P. australis* populations may have different biological activities than those derived from *Quillaja saponaria* (the source of commercial saponins), or (2) that leachates contained additional compounds that contributed to negative effects on *A. maculatum* larvae.

We are confident that our approach can be extrapolated to field conditions. Previous work used similar experimental designs to assess effects of litter quality on tadpole performance (Brown et al. 2006; Maerz et al. 2005, 2010). The importance of plant traits detected in mesocosms was also a significant factor for predicting tadpole performance under field conditions where tadpoles experienced challenging hydroperiods and abiotic conditions (Cohen et al.

2012). This evidence and results of a recent meta-analysis (Melvin and Houlahan 2012) suggest that we may expect even stronger effects in the field where smaller and stressed individuals usually fail to metamorphose.

Today, 32 % of amphibian species are globally threatened, as compared with 12 % of birds and 23 % of mammals, due to a combination of stressors (Stuart et al. 2004). Our experiments suggest that the phytochemical genotypes of individual plants may have tangible effects on survival and fitness of amphibians. Since our results show that plant chemistry is not necessarily related to a plant's native/non-native origin, nativity may not be a sufficient (or even relevant) criterion when managing amphibian habitat.

Acknowledgments We thank A. Laurila, C. Hickmann and two anonymous reviewers for their suggestions and V. Nuzzo, C. Kraft, A. Davalos, J. Maerz, J. Cohen, and E. Feldman for critical feedback. We thank F. Vermeulen for statistical advice, many land managers for collecting litter, and B. Whitmore, S. Biddlecomb, S. Rainford, C. Thurston, I. Conti-Jerpe, W. Simmons, W. Dietrich and J. Dietrich for technical assistance. L.J.M. is supported by the NSF GRFP; additional funding was provided by the NY Department of Transportation. This project was approved by Cornell University's Institutional Animal Care and Use Committee (Protocol 00-26-03).

References

- Adams CK, Saenz D (2012) Leaf litter of invasive Chinese tallow (*Triadica sebifera*) negatively affects hatching success of an aquatic breeding anuran, the southern leopard frog (*Lithobates sphenoccephalus*). *Can J Zool* 90:991–998. doi:10.1139/z2012-067
- Agrawal AA, Kotanen PM (2003) Herbivores and the success of exotic plants: a phylogenetically controlled experiment. *Ecol Lett* 6:712–715
- Agrawal AA, Hastings AP, Johnson MTJ, Maron JL, Salminen JP (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science* 338:113–116. doi:10.1126/science.1225977
- Allison P (1995) Survival analysis using the SAS system. SAS Institute, Cary
- Babbitt K, Jordan F (1996) Predation on *Bufo terrestris* tadpoles: effects of cover and predator identity. *Copeia* 2:485–488
- Bailey JK et al (2009) From genes to ecosystems: a synthesis of the effects of plant genetic factors across levels of organization. *Philos Trans R Soc Lond B* 364:1607–1616. doi:10.1098/rstb.2008.0336
- Brown CJ, Blossey B, Maerz JC, Joule SJ (2006) Invasive plant and experimental venue affect tadpole performance. *Biol Inv* 8:327–338
- Burggren WW, Infantino RL (1994) The respiratory transition from water to air-breathing during amphibian metamorphosis. *Am Zool* 34:238–246
- Chalcraft DR, Binckley CA, Reseraris WJ Jr (2005) Experimental venue and estimation of interaction strength: comment. *Ecology* 86:1061–1067
- Chen JC, Chen KW, Chen JM (1996) Effects of saponin on survival, growth, molting and feeding of *Penaeus japonicus* juveniles. *Aquaculture* 144:165–175. doi:10.1016/s0044-8486(96)01301-4
- Cohen JS, Maerz JC, Blossey B (2012) Traits, not origin, explain impacts of plants on larval amphibians. *Ecol Appl* 22:218–228

- Cotten TB, Kwiatkowski MA, Saenz D, Collyer M (2012) Effects of an invasive plant, Chinese Tallow (*Triadica sebifera*), on development and survival of anuran larvae. *J Herpetol* 46:186–193. doi:[10.1670/10-311](https://doi.org/10.1670/10-311)
- Cox DR (1972) Regression models and life tables. *J R Stat Soc* 34:187–220
- Davidson EW, Larsen A, Palmer CM (2012) Potential influence of plant chemicals on infectivity of *Batrachochytrium dendrobatidis*. *Dis Aquat Org* 101:87–93. doi:[10.3354/dao02505](https://doi.org/10.3354/dao02505)
- Donavan L (1980) Morphological features of the stages in the development of *Ambystoma talpoideum* (Holbrook) from the fertilized egg to the adult. PhD thesis, University of Southern Mississippi
- Drake JM, Kramer AM (2012) Mechanistic analogy: how microcosms explain nature. *Theor Ecol* 5:433–444. doi:[10.1007/s12080-011-0134-0](https://doi.org/10.1007/s12080-011-0134-0)
- Earl JE, Semlitsch RD (2012) Reciprocal subsidies in ponds: does leaf input increase frog biomass export? *Oecologia* 170:1077–1087. doi:[10.1007/s00442-012-2361-5](https://doi.org/10.1007/s00442-012-2361-5)
- Earl JE, Cohagen KE, Semlitsch RD (2012) Effects of leachate from tree leaves and grass litter on tadpoles. *Environ Tox Chem* 31:1511–1517. doi:[10.1002/etc.1829](https://doi.org/10.1002/etc.1829)
- Freda J, Dunson WA (1986) Effects of low pH and other chemical variables on the local distribution of amphibians. *Copeia*. 454–466
- Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190
- Halverson MA, Skelly DK, Kiesecker JM, Freidenburg LK (2003) Forest mediated light regime linked to amphibian distribution and performance. *Oecologia* 134:360–364
- Hansen DL, Lambertini C, Jampeetong A, Brix H (2007) Clone-specific differences in *Phragmites australis*: effects of ploidy level and geographic origin. *Aquat Bot* 86:269–279
- Haviola S et al (2012) Genetic and environmental factors behind foliar chemistry of the mature mountain birch. *J Chem Ecol* 38:902–913. doi:[10.1007/s10886-012-0148-0](https://doi.org/10.1007/s10886-012-0148-0)
- Herlt AJ, Mander LN, Pongoh E, Rumampuk RJ, Tarigan P (2002) Two major saponins from seeds of *Barringtonia asiatica*: putative antifeedants toward *Epilachna* sp larvae. *J Natural Products* 65:115–120. doi:[10.1021/np000600b](https://doi.org/10.1021/np000600b)
- Hostettmann K, Marston A (1995) Saponins. University of Cambridge Press, Cambridge
- Hulse A, McCoy C, Censky E (2001) Amphibians and reptiles of Pennsylvania and the Northeast. Cornell University Press, Ithaca
- Ishaaya I, Birk Y, Bondi A, Tencer Y (1969) Soyabean saponins. 9. Studies of their effects on birds, mammals and cold blooded organisms. *J Sci Food Agric* 20:433–436. doi:[10.1002/jfsa.2740200716](https://doi.org/10.1002/jfsa.2740200716)
- Kattge J et al (2011) TRY—a global database of plant traits. *Glob Change Biol* 17:2905–2935. doi:[10.1111/j.1365-2486.2011.02451.x](https://doi.org/10.1111/j.1365-2486.2011.02451.x)
- Kerby JL, Richards-Hrdlicka KL, Storfer A, Skelly DK (2010) An examination of amphibian sensitivity to environmental contaminants: are amphibians poor canaries? *Ecol Lett* 13:60–67. doi:[10.1111/j.1461-0248.2009.01399.x](https://doi.org/10.1111/j.1461-0248.2009.01399.x)
- Kopp K, Wachlevski M, Eterovick PC (2006) Environmental complexity reduces tadpole predation by water bugs. *Can J Zool* 84:136–140. doi:[10.1139/z05-186](https://doi.org/10.1139/z05-186)
- Kraus TEC, Dahlgren RA, Zasoski RJ (2003) Tannins in nutrient dynamics of forest ecosystem—a review. *Plant Soil* 256:41–66. doi:[10.1023/a:1026206511084](https://doi.org/10.1023/a:1026206511084)
- Leishman MR, Thomson VP, Cooke J (2010) Native and exotic invasive plants have fundamentally similar carbon capture strategies. *J Ecol* 98:28–42. doi:[10.1111/j.1365-2745.2009.01608.x](https://doi.org/10.1111/j.1365-2745.2009.01608.x)
- LeRoy CJ, Whitham TG, Keim P, Marks JC (2006) Plant genes link forests and streams. *Ecology* 87:255–261. doi:[10.1890/05-0159](https://doi.org/10.1890/05-0159)
- Maerz JC, Brown CJ, Chapin CT, Blossey B (2005) Can secondary compounds of an invasive plant affect larval amphibians? *Funct Ecol* 19:970–975
- Maerz JC, Cohen JS, Blossey B (2010) Does detritus quality predict the effect of native and non-native plants on the performance of larval amphibians? *Freshw Biol* 55:1694–1704. doi:[10.1111/j.1365-2427.2010.02404.x](https://doi.org/10.1111/j.1365-2427.2010.02404.x)
- Martin LJ, Murray BR (2011) A predictive framework and review of the ecological impacts of exotic plant invasions on reptiles and amphibians. *Biol Rev* 86:407–419. doi:[10.1111/j.1469-185X.2010.00152.x](https://doi.org/10.1111/j.1469-185X.2010.00152.x)
- Meier CL, Bowman WD (2008) Links between plant litter chemistry, species diversity, and below-ground ecosystem function. *Proc Nat AcadSci USA* 105:19780–19785. doi:[10.1073/pnas.0805600105](https://doi.org/10.1073/pnas.0805600105)
- Melvin SD, Houlihan JE (2012) Tadpole mortality varies across experimental venues: do laboratory populations predict responses in nature? *Oecologia* 169:861–868. doi:[10.1007/s00442-012-2260-9](https://doi.org/10.1007/s00442-012-2260-9)
- Morrongiello JR, Bond NR, Crook DA, Wong BBM (2011) Eucalyptus leachate inhibits reproduction in a freshwater fish. *Freshw Biol* 56:1736–1745. doi:[10.1111/j.1365-2427.2011.02605.x](https://doi.org/10.1111/j.1365-2427.2011.02605.x)
- Müller MS, McWilliams SR, Podlesak D, Donaldson JR, Bothwell HM, Lindroth RL (2006) Tri-trophic effects of plant defenses: chickadees consume caterpillars based on host leaf chemistry. *Oikos* 114:507–517
- Njau KN, Renalda M (2010) Performance of horizontal subsurface flow constructed wetland in the removal of tannins. *Can J Civil Eng* 37:496–501. doi:[10.1139/09-161](https://doi.org/10.1139/09-161)
- Park MG, Blossey B (2008) Importance of plant traits and herbivory for invasiveness of *Phragmites australis* (Poaceae). *Am J Bot* 95:1557–1568
- Rautio P, Bergvall UA, Karonen M, Salminen JP (2007) Bitter problems in ecological feeding experiments: Commercial tannin preparations and common methods for tannin quantifications. *Biochem Syst Ecol* 35:257–262. doi:[10.1016/j.bse.2006.10.016](https://doi.org/10.1016/j.bse.2006.10.016)
- Rey D, Pautou MP, Meyran JC (1999) Histopathological effects of tannic acid on the midgut epithelium of some aquatic Diptera larvae. *J Invertebr Pathol* 73:173–181. doi:[10.1006/jipa.1998.4810](https://doi.org/10.1006/jipa.1998.4810)
- Rittenhouse TAG (2011) Anuran larval habitat quality when reed canary grass is present in wetlands. *J Herpetol* 45:491–496
- Rogalski MA, Skelly DK (2012) Positive effects of non-native invasive *Phragmites australis* on larval bullfrogs. *PLoS One* 7:e44420. doi:[10.1371/journal.pone.0044420](https://doi.org/10.1371/journal.pone.0044420)
- Rubbo MJ, Kiesecker JM (2004) Leaf litter composition and community structure: translating regional species changes into local dynamics. *Ecology* 85:2519–2525. doi:[10.1890/03-0653](https://doi.org/10.1890/03-0653)
- Salminen JP, Karonen M (2011) Chemical ecology of tannins and other phenolics: we need a change in approach. *Funct Ecol* 25:325–338. doi:[10.1111/j.1365-2435.2010.01826.x](https://doi.org/10.1111/j.1365-2435.2010.01826.x)
- Salminen JP, Roslin T, Karonen M, Sinkkonen J, Pihlaja K, Pulkkinen P (2004) Seasonal variation in the content of hydrolyzable tannins, flavonoid glycosides, and proanthocyanidins in oak leaves. *J Chem Ecol* 30:1693–1711. doi:[10.1023/B:JOEC.0000042396.40756.b7](https://doi.org/10.1023/B:JOEC.0000042396.40756.b7)
- Saltonstall K (2002) Cryptic invasion by non-native genotypes of the common reed, *Phragmites australis*, into North America. *Proc Nat Acad Sci USA* 99:2445–2449
- Saltonstall K, Peterson PM, Sorong RJ (2004) Recognition of *Phragmites australis* subsp. *americanus* (Poaceae: Arundinoideae) in North America: evidence from morphological and genetic analyses. *SIDA* 21:683–692
- Schiesari L (2006) Pond canopy cover: a resource gradient for anuran larvae. *Freshw Biol* 51:412–423. doi:[10.1111/j.1365-2427.2005.01497.x](https://doi.org/10.1111/j.1365-2427.2005.01497.x)

- Skelly DK (2002) Experimental venue and estimation of interaction strength. *Ecology* 83:2097–2101
- Skelly DK, Werner EE, Cortwright SA (1999) Long-term distributional dynamics of a Michigan amphibian assemblage. *Ecology* 80:2326–2337. doi:10.1890/0012-9658(1999)[2326:ltddoa]2.0.co;2
- Skelly DK, Freidenburg LK, Kiesecker JM (2002) Forest canopy and the performance of larval amphibians. *Ecology* 83:983–992
- Smith DS, Bailey JK, Shuster SM, Whitham TG (2011) A geographic mosaic of trophic interactions and selection: trees, aphids and birds. *J Evol Biol* 24:422–429. doi:10.1111/j.1420-9101.2010.02178.x
- Sparg SG, Light ME, van Staden J (2004) Biological activities and distribution of plant saponins. *J Ethnopharmacol* 94:219–243. doi:10.1016/j.jep.2004.05.016
- Stoler AB, Relyea RA (2011) Living in the litter: the influence of tree leaf litter on wetland communities. *Oikos* 120:862–872. doi:10.1111/j.1600-0706.2010.18625.x
- Stuart SN et al (2004) Status and trends of amphibian declines and extinctions worldwide. *Science* 306:1783–1786
- Temminck JHM, Field JA, Vanhaastrecht JC, Merkelbach RCM (1989) Acute and sub-acute toxicity of bark tannins in carp (*Cyprinus carpio* L). *Water Res* 23:341–344. doi:10.1016/0043-1354(89)90100-0
- Ultsch GR, Bradford DF, Freda J (1999) Physiology: coping with the environment. In: McDiamid R, Altig R (eds) *Tadpoles: the biology of anuran larvae*. University of Chicago Press, Chicago, pp 189–214
- Violle C et al (2012) The return of the variance: intraspecific variability in community ecology. *Trends Ecol Evol* 27:244–252. doi:10.1016/j.tree.2011.11.014
- Watling JI, Hickman CR, Lee E, Wang K, Orrock JL (2011a) Extracts of the invasive shrub *Lonicera maackii* increase mortality and alter behavior of amphibian larvae. *Oecologia* 165:153–159. doi:10.1007/s00442-010-1777-z
- Watling JI, Hickman CR, Orrock JL (2011b) Invasive shrub alters native forest amphibian communities. *Biol Conserv* 144:2597–2601. doi:10.1016/j.biocon.2011.07.005
- Watling JI, Hickman CR, Orrock JL (2011c) Predators and invasive plants affect performance of amphibian larvae. *Oikos* 120:735–739. doi:10.1111/j.1600-0706.2010.19255.x
- Wegner C, Hamburger M (2002) Occurrence of stable foam in the upper Rhine River caused by plant-derived surfactants. *Environ Sci Technol* 36:3250–3256. doi:10.1021/es025532p
- Williams BK, Rittenhouse TAG, Semlitsch RD (2008) Leaf litter input mediates tadpole performance across forest canopy treatments. *Oecologia* 155:377–384. doi:10.1007/s00442-007-0920-y
- Yarnes CT, Boecklen WJ, Tuominen K, Salminen JP (2006) Defining phytochemical phenotypes: size and shape analysis of phenolic compounds in oaks (Fagaceae, *Quercus*) of the Chihuahuan Desert. *Can J Bot* 84:1233–1248. doi:10.1139/b06-076