Stable isotopes reveal an invasive plant contributes more than native sources to anuran larvae diets

Milica Radanovic, Joseph R. Milanovich, Kyle Barrett and John A. Crawford

ABSTRACT
Foraging theory suggests organisms increase their fitness through dietary and energetic choices. In nature, organisms choose to consume balanced optimal diets but as exotic plants invade ecosystems, new foraging options become available. Often these plants have different traits and can negatively affect fitness of foragers by lowering survival or growth due to toxicity. Wetland habitats are highly susceptible to plant invasions which can have negative effects on nutrient cycling. The purpose of this study was to examine whether invasion of purple loosestrife (Lythrum salicaria) alters tadpole foraging preference thus potentially impacting their survivorship and fitness. We designed a common garden experiment and utilized stable isotopic signatures to investigate the contribution of purple loosestrife and native hardwood detritus to larval wood frog (Rana sylvatica) and southern leopard frog (Rana sphenocephala) diets. Tadpoles derived a higher percentage of resources from purple loosestrife compared to native hardwood detritus when presented with both options, despite its high concentration of toxic secondary compounds. It is unknown if tadpoles can detect competing fitness signals that may be associated with higher nutrient and toxicity levels in purple loosestrife. Discovering the presence or absence of such signals will promote a greater understanding of selective foraging in changing environments.

KEYWORDS
Amphibians; mixSIAR; stable isotopes; optimal foraging; Rana sylvatica; Rana sphenocephala; Lythrum salicaria

Introduction
Foraging theory is used to explain the behaviors organisms exhibit when selecting food sources to increase fitness (Mangel & Clark 1986; Schoener 1987). Organisms are faced with choices of dietary composition, where to search for the optimal sources, how much time to spend foraging, and how far to move between nutrient sources when attempting to maximize their individual fitness (Pyke 1984; Searle et al. 2005; Bartumeus & Catalan 2009). Organisms selectively choose food sources to meet their preferred and balanced dietary needs (Bryant & Kuropat 1980; Sedinger & Raveling 1984; Felton et al. 2016), but selection can be influenced by other factors, such as predation risk and abundant food choices within their diverse habitat (Marco-Méndez et al. 2016), altering the effect the choices have on their fitness and survival. To achieve a balanced diet, organisms choose to consume sources that maximize energy (Felton et al. 2016), are nutrient rich (Felton et al. 2009; Rothman et al. 2011), and are void of toxins (Bryant & Kuropat 1980). Thus, organisms will discriminate against unpalatable and hard-to-digest foods, unless no other option is available (Felton et al. 2009,
2016) and to compensate for limited availability of highly nutritious food sources, organisms will consume excess amounts of the available low-quality resources (Cruz-Rivera & Hay 2000; Simpson et al. 2004; Robbins et al. 2007; Felton et al. 2016). Therefore, additional studies are required to explain complex foraging behavior in larger varieties of organisms and ecosystems.

Non-native invasive plants are an increasing feature of many ecosystems (Farnsworth & Ellis 2001; Wang et al. 2015). In cases where these novel resources have nutritive and toxic qualities that are unfamiliar to herbivores, it is unclear how (or if) foraging preferences will shift in invaded areas (Pyke 1984; Ehrenfeld et al. 2003; Marco-Méndez et al. 2016). The impact of invaders to wetlands is vigorously debated. Many studies argue that invasions have purely negative impacts on wetlands by reducing biodiversity, causing shifts in organismal consumption patterns, and altering the availability of nutrients (Ehrenfeld et al. 2003; Yakimowski et al. 2005; Roura-Pascual et al. 2011). Alternatively, a few studies suggest that invasions have a small or no impact on ecosystems and that it benefits from the added nutrients (Vilà et al. 2011). Purple loosestrife (*Lythrum salicaria*) is an invasive plant that has had negative impacts on different wetland systems such as decreases in biodiversity due to its high seed dispersion capabilities and fast growth rate (Farnsworth & Ellis 2001; Yakimowski et al. 2005). Additionally, purple loosestrife is composed of higher concentrations of secondary compounds (phenolic acid, tannins, etc.) compared to native species, which could negatively affect aquatic ecosystems (Rauha et al. 2001). Thus, ecologists need a better understanding of aquatic invaders’ influence on ecosystem processes—especially as it relates to the potential for invasive plants to alter organismal foraging decisions.

Current rates of extinction resemble rates experienced during the previous five great extinction events leading to massive decreases in biodiversity and ecosystem services (Barnosky et al. 2011; Hocking & Babbitt 2014; Ceballos et al. 2015). Wetland taxa such as amphibians are especially vulnerable (Sodhi et al. 2008; Wake 2012; Hocking & Babbitt 2014). Of the more than 7000 species of amphibians, approximately 41% are facing extinction due to global change (Hocking & Babbitt 2014). Larval anurans (tadpoles) are uniquely positioned to be important in wetland ecosystems because they are typically found in large numbers, consume a variety of detrital resources, promote nutrient cycling and decomposition, and are consumed by many predators (Schiesari et al. 2009; Hocking & Babbitt 2014). Many amphibians have bi-phasic life cycles and are strongly connected to wetland environments due to their reliance upon aquatic systems for breeding sites, nutritional sources, and habitat increasing the impact plant invasions can have on their foraging patterns (Huckembeck et al. 2014; Burgett & Chase 2015).

Invasion of purple loosestrife (*L. salicaria*) introduces a nutrient-rich food source (Milanovich et al. 2016) with higher concentrations of secondary compounds (Rauha et al. 2001) to wetland ecosystems as a potential food source for tadpoles. Tadpoles need to consume a highly nutritious diet to prepare for and complete metamorphosis, which could be attracting tadpoles to choose purple loosestrife as part of their optimal diet (Kupferberg 1997). Foraging theory implies that tadpole fitness will increase when consuming optimal diets (Pyke 1984; Searle et al. 2005); however, the consumption and presence of purple loosestrife has been shown to decrease tadpole survivorship (Barrett et al. Forthcoming; Brown et al. 2006; Cohen et al. 2012; Milanovich et al. 2016). Tadpoles exposed to higher concentrations of secondary compounds associated with purple loosestrife experienced changes to metamorph size and duration of the development period (Cohen et al. 2012; Milanovich et al. 2016). Within wetlands invaded by purple loosestrife, tadpoles are presented with a choice between consuming an invasive plant that is more nutritious, but toxic, or native plants that do not have the same quality of nutrients. An examination into the contribution of invasive plants to tadpole diets can be used to further assess the impact of aquatic invaders on higher trophic levels.

The primary objective of our study was to quantify the contribution of purple loosestrife and native hardwood detritus to tadpole consumers at two locations in the United States. Specifically, we used stable isotopes to determine whether purple loosestrife invasion is influencing tadpole foraging strategies that are presumably evolved to optimize growth and survivorship. We hypothesized the
larvae of southern leopard frogs (*Rana sphenopehala*) and wood frogs (*Rana sylvatica*) would consume a higher percentage of native hardwood detritus compared to purple loosestrife to avoid negative impacts from secondary compounds despite the potential benefits of the higher nutrient concentrations in the invasive plant.

**Materials and methods**

**Focal species**

Purple loosestrife is an aquatic invasive plant introduced into North America in the early 1800s where cultivation for ornamental value has increased extensively since 1940 (Farnsworth & Ellis 2001). Currently, purple loosestrife has an extensive range across North American wetland habitats and is predicted to expand its known distribution (Lindgren & Walker 2012). Recent evidence suggests invasive purple loosestrife has higher phenolic (e.g. galic acid), nitrogen (N) and phosphorus (P) content when compared to several invasive and native aquatic plant species (Emery & Perry 1995; Templer et al. 1998; Stephens et al. 2013; Maurer 2014).

Wood frogs occur from the southern Appalachian Mountains of Georgia, north above the Arctic Circle, and west to Alaska. In Missouri (MO) and South Carolina (SC), breeding typically occurs between January and March in fish-free, ephemeral woodland pools or wetlands (Redmer & Trauth 2005). Wood frogs complete metamorphosis between 65 and 130 days; prior to metamorphosis larvae feed primarily on detritus, plant/algal material, and some zooplankton (Redmer & Trauth 2005; Schiesari et al. 2009; Schriefer & Williams 2013). Southern leopard frogs are most abundant in the southeastern portion of the United States with large densities in the Appalachian Highlands (Garrett & Barker 1987; Butterfield et al. 2005). Their southern range extends to Arkansas, Tennessee, Louisiana, Mississippi, Alabama, Georgia, South Carolina, and most of Missouri (Garrett & Barker 1987; Butterfield et al. 2005). In MO, adults breed in large numbers during autumn following rainfall (Doody & Young 1995; Johnson 2000; Butterfield et al. 2005). Southern leopard frogs complete metamorphosis 50–75 days following hatching and consume green algae, diatoms, and detritus as tadpoles (Wright 2002; Hillis 1982; Butterfield et al. 2005; Williams et al. 2008).

**Experimental design**

Six mesocosm tanks (133 L) at Lindenwood University’s Daniel Boone Field Station in St. Charles County, MO, and 12 mesocosm tanks at Clemson University’s Environmental Toxicity facility in Anderson County, SC, were set up to simulate natural aquatic environments. Native hardwood detritus was collected following senescence during November 2013 from wetland sites where egg masses were collected for this study. SC native hardwood detritus consisted of scarlet oak (*Quercus occinea*), white oak (*Quercus alba*), and American sweetgum (*Liquidambar styraciiflua*), while MO native hardwood detritus consisted of black oak (*Quercus velutina*), post oak (*Quercus stellata*), and white ash (*Fraxinus americana*). Whole purple loosestrife plants were collected following senescence in November 2013 from Porter County, Indiana. In December 2013, all 18 mesocosms were filled with 120 L of tap water and a 50/50 mixture of 75 g of purple loosestrife, and 75 g of native hardwood detritus similar to other experiments and left to overwinter (Stoler & Relyea 2011, 2013a). Mesocosms were covered with 60% mesh shade cloth and inoculated 24 hours later with 1 L of natural wetland water from respective sites where egg masses were collected.

In spring 2014, mesocosms were again inoculated with 1 L of natural wetland water and 5 g of Purina® Rabbit Chow approximately 10 days prior to the addition of tadpoles (Stoler & Relyea 2013b). Wood frog and southern leopard frog egg masses were collected from wetlands near the SC study site and only wood frog egg masses were collected in MO (February–March for SC and March–April for MO). The egg masses were stored in holding tanks, at both locations, until frog tadpoles hatched and reached Gosner stage 21, the desired developmental stage for tank release.
Twenty tadpoles were randomly distributed to the each of the 18 experimental tanks at this stage and were allowed to complete metamorphosis. A 0.5 m × 0.1 m piece of floating foam was placed in each tank during metamorphosis to provide habitat for the metamorphic individuals. Tanks were checked daily to collect metamorphic individuals and the experiment ended when two weeks passed without a metamorphic individual found in each tank. The first two and last two individual wood frogs and southern leopard frogs from each tank were euthanized in a benzocaine solution (Oragel®, 1 g/L) and were placed in a 60 °C oven until dried (approximately seven days). Gut content analysis was not performed on metamorphic individuals; however, stable isotope analysis provides biomass accumulations from long-term dietary patterns (Rudnick & Resh 2005; Araújo et al. 2007). Prior to addition to the mesocosm tanks, five native hardwood leaves and five samples of whole purple loosestrife plants were randomly selected and dried at 60 °C. Dried tadpoles and dried plant samples were homogenized with a ball mill and sent to the University of Georgia Analytical Chemistry Laboratory (Athens, Georgia) for carbon (13C) and nitrogen (15N) stable isotope analyses following the methods of Milanovich et al. (2012). Lipid proportions of tadpoles are known to be less than 5%; therefore, we did not conduct lipid processing or correct for lipid content (Post et al. 2007; Jefferson et al. 2014). Stable isotopic values are expressed in parts per thousand (%o).

**Stable isotope mixing models**

Proportional isotopic contributions of native hardwood versus purple loosestrife detritus to the isotopic composition of the tadpoles in each treatment were estimated using δ13C and δ15N. Specifically, a Bayesian mixing model software package, MixSIAR (version 2.1.3), was used for stable isotopic analysis to represent the contribution of different sources of plant material to the isotopic composition of wood frog and southern leopard frog tadpoles up to metamorphosis (Stock & Semmens 2013). The Markov Chain Monte Carlo parameters were set at the very long test length (chain length = 1,000,000; burn = 700,000; thin = 300; chains = 3). Unlike other organisms, tadpoles’ gut lengths differ based on the nutritional quality that they are exposed to during their larval period (Latney & Clayton 2014). Tadpoles presumably forage more on nutrient-rich resources during their larval periods in order to maximize energy for growth and development (Schiesari et al. 2009). To account for the differences in the quality and utilization of purple loosestrife and native hardwood detritus, we corrected for trophic enrichment between plant material resources and tadpoles by using a range of calculated discrimination factors: (a) 0.0‰ and 0.0‰; (b) 1.2‰ and 2.6‰; (c) 1.69‰ and 1.98‰; and (d) 2.0‰ and 2.4‰ for δ13C and δ15N, respectively (Schiesari et al. 2009; Jefferson et al. 2014; J. C. Maerz, University of Georgia, unpublished data). Fractionation factors show the degree to which lighter isotopic values are being used by biological functions and assimilated in the consumer’s biomass. Changing those values results in different ratios and concentrations of heavy to light isotopes in the consumer’s isotopic signature. If tadpoles are primarily consuming purple loosestrife, then the changing fractionation factors should not drastically alter their isotopic signatures (O’Leary 1981). If the mixing models show tadpoles are still deriving the majority of their nutrients from purple loosestrife after correcting the fractionation values, then tadpoles are most likely consuming purple loosestrife and rapidly utilizing its higher nutritional values for biological development leading to higher concentrations of lighter isotopes in its biomass (Schiesari et al. 2009).

MixSIAR and other mixing models use Bayesian inference to help solve linear mixing models, which can determine diet composition of consumers based on the isotopic signatures of the consumers and their sources (Parnell et al. 2010). In short, these models are fit using MCMC sampling which generates proportions of the consumer’s diet based on the isotopic signatures of the consumers and their sources (McClellan et al. 2010). Mixing models use isotopic values and discrimination factors to account for the organism’s assimilated diet and thus estimate what portions of the diet compose its separate or combined tissues (Parnell et al. 2010; Phillips 2012). Using fractionation values improves accuracy, but does add additional variability (Parnell et al. 2013). The MixSIAR
program is equipped to handle concentration dependence, uncertainties regarding consumer–source discrimination, uncertainties due to isotopic variability, multiple sources of nutrients, and model error options (process error or process and residual error; Semmens & Moore 2008). Although Bayesian mixing model software has improved, issues can still be associated with stable isotope analysis. Such analyses require extensive prior information for the models to give a correct output (Phillips et al. 2014). For example, all the sources available to the consumer should be present and the fractionation values unique to the study organism must be known (Semmens & Moore 2008; Caut et al. 2009). Ecological systems are complex and it is difficult to account for all errors and uncertainties without the proper prior information (Semmens & Moore 2008).

Results

In the SC treatments, the δ15N of purple loosestrife detritus was significantly enriched (121%; \( t_{(5.353)} = -3.504, p = 0.015 \)) compared to native hardwood detritus; whereas the δ13C signature of purple loosestrife detritus is only 1.74% higher than that of native hardwood detritus and not significantly different (\( t_{(7.98)} = -1.694, p = 0.129 \)). Similar differences were documented in the MO treatments, with a marginally significant difference of 90.17% in δ15N between purple loosestrife and native hardwood detritus (\( t_{(5.789)} = -2.137, p = 0.078 \)), and a non-significant difference of −0.06% in δ13C (\( t_{(7.409)} = 0.05, p = 0.961 \)). Isotopic signature plots of 13C and 15N at the SC and MO treatments were similar (Figure 1(A−L)), but show tadpoles and both detritus sources are misaligned—suggesting a source outside of the two primary detritus sources was contributing to tadpole biomass. Tadpole stable isotope concentrations were more enriched in 15N for both frog species compared to available detritus sources at Clemson and Lindenwood (Figure 1(A−L)).

The stable isotope mixing models for the treatments at both sites estimate contributions from native hardwood detritus to the composition of wood frog, or southern leopard frog tadpole tissue was measurably lower compared to the biomass derived from purple loosestrife—despite both detritus types comprising 50% of available resources and after accounting for variation in discrimination factors (Table 1).

Discussion

Our results suggest that when given a choice, two frog species actively select resources derived from purple loosestrife over native hardwood detritus. Such a choice may result from the tadpole’s ability to recognize detritus that has higher nutritive value (Pfennig 2000). Nevertheless, purple loosestrife is known to decrease survival of larval anurans due to a high concentration of secondary compounds (Maerz et al. 2005; Brown et al. 2006). Foraging theory states that organisms will make dietary choices that will increase their fitness, but frog species in this study chose to consume plant matter that is decreasing their survival rates suggesting other factors could be influencing their choices (Barrett et al. Forthcoming; Pyke 1984; Milanovich et al. 2016).

Purple loosestrife is morphologically similar to native cattails that tadpoles consume in undisturbed wetlands which can lead to the two plant species having similar environmental cues attracting tadpoles (Blossey et al. 2004). If tadpoles are choosing to consume purple loosestrife because of false environmental cues and their survival and fitness are decreasing due to that choice, then purple loosestrife invasions create putative ecological traps (Barrett et al. Forthcoming; Battin 2004; Maerz et al. 2005; Brown et al. 2006; Milanovich et al. 2016). An ecological trap can arise when an organism chooses a maladaptive habitat due to false environmental cues that leads to a decline in reproductive fitness and/or survival (Battin 2004; Robertson & Hutto 2006; Gilroy & Sutherland 2007). Ecological traps have been observed in terrestrial and aquatic ecosystems affecting a wide range of species (Dwernychuk & Boag 1972; Nordby et al. 2009; Severns 2011; Robertson 2012). Decreased habitat quality is the primary driver of ecological traps, as many habitats have been altered to the point where previously useful cues no longer offer an honest signal of environmental quality (Schlaepfer
et al. 2002; Battin 2004; Severns 2011; Fletcher et al. 2012; Robertson 2012; Robertson et al. 2013). Further support of this hypothesis would require evidence that the tadpoles truly can detect higher nutritive value in purple loosestrife and choose to consume it during foraging for an optimal and balanced diet (Pyke 1984; Searle et al. 2005).

Figure 1. Dual isotope plot of $\delta^{13}C$ and $\delta^{15}N$ signatures (mean ± SE) of native and invasive detritus and tadpoles across each treatment in South Carolina (A, D, G, J for leopard frogs and B, E, H, K for wood frogs) and Missouri (C, F, I, L). (A–C) No discrimination factors for $\delta^{13}C$ and $\delta^{15}N$ are used; (D–F) discrimination factors of 1.2%o and 2.6%o for $\delta^{13}C$ and $\delta^{15}N$, respectively, are used; (G–I) discrimination factors of 1.69%o and 1.98%o for $\delta^{13}C$ and $\delta^{15}N$, respectively, are used; and (J–L) discrimination factors of 2.0%o and 2.4%o for $\delta^{13}C$ and $\delta^{15}N$, respectively, are used.
Tadpoles are known to be opportunistic grazers and will consume a variety of resources including algae, detritus, and zooplankton (Seale 1980; Huckembeck et al. 2014). Although we limited possible resources with respect to detritus, the mesocosms were inoculated with pond water and likely contained a variety of algae and plankton. This could explain the variation of $^{13}$C values across basal resources and the disjunct nature of the $^{13}$C and $^{15}$N plots (Figure 1). Following correction using several reported discrimination factors, our results suggest the contribution from purple loosestrife is not solely due to consumption of plants, but potentially other sources that are deriving energy from purple loosestrife such as algae or zooplankton (Table 1; Figure 1). A consumer’s $^{13}$C value should align with their nutritional sources (Phillips 2012), but when the signatures do not align it can be due to carbon sources of low abundance being overlooked (Wilkinson et al. 2013). Another explanation for the $^{13}$C differences could be due to how tadpoles utilize their gained nutrients and the changes in tadpole gut length due to the nutritional value of those resources (Latney & Clayton 2014). Unlike other organisms, tadpoles have limited time to reach metamorphosis and need to efficiently use their nutrients for rapid growth (Schiesari et al. 2009). The nutrients within tadpoles are reused and recycled for the tadpoles to be as efficient as possible and these processes could lead to the $^{13}$C signatures not aligning completely (J. C. Maerz, University of Georgia, unpublished data). Discrimination of $^{13}$C by larval anurans varies due to nutritional quality of diet (Schiesari et al. 2009; Jefferson et al. 2014). After adjusting for various correction factors, our results still suggest that purple loosestrife is the main food source despite the change in gut structure and the quality of the nutrient sources.

Despite the fact that secondary compounds found in purple loosestrife lead to a lower survival rate relative to native detritus (Barrett et al. Forthcoming; Maerz et al. 2005; Brown et al. 2006; Milanovich et al. 2016), anuran larvae are choosing to consume the invasive plant. The preference for purple loosestrife we describe here may result from the higher nutrient levels found in the plant (Milanovich et al. 2016), which promote faster times to metamorphosis and larger overall tadpoles (Milanovich et al. 2016, Barrett et al. Forthcoming). The fitness implications of the competing outcomes for growth and survivorship probabilities are realized at the individual level. Whether or not such tradeoffs can be detected by the tadpoles is an open question, and research into it will help resolve the extent to which our results are consistent with current understanding of selective foraging (Pyke 1984; Fryxell 1991).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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