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Phenotypic variation of toxin levels in a chemically defended amphibian: The ecological and evolutionary significance of tetrodotoxin

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Phenotypic variation of toxin levels in a chemically defended amphibian: The ecological and evolutionary significance of tetrodotoxin

A dissertation submitted in partial satisfaction of the requirements for
the degree of Doctor of Philosophy in Biology

by

Gary Bucciarelli

2015

ABSTRACT OF THE DISSERTATION

Phenotypic variation in space and time of toxin levels in an amphibian:
The ecological and evolutionary significance of tetrodotoxin

by

Gary Bucciarelli
Doctor of Philosophy in Biology
University of California, Los Angeles, 2015
Professor Howard Bradley Shaffer, Chair

The global decline of amphibian populations is well documented, yet researchers have only begun to understand how these declines may affect biological, environmental, ecological, and evolutionary processes. In the first chapter, recently published primary literature is reviewed to describe the complexities of amphibian population declines, with a focus on the interactions of nonnative species, anthropogenic disturbances, and climate change. To begin to understand the potential consequences of the decline of endemic California herpetofauna, the ecological role of a Species of Special Concern, the California newt (*Taricha torosa*) was evaluated in streams of the Santa Monica Mountains. The California newt possesses a potent neurotoxin, tetrodotoxin (TTX), which is known to elicit antipredator behavior in conspecific larvae. Given these results, potential variation of individual and breeding population TTX levels may affect ecological and evolutionary processes. In chapter 2, TTX levels of adult *T. torosa* from three breeding localities in the

Santa Monica Mountains were quantified to test for variation in amounts of TTX between sites using a novel non-destructive sampling method, a newly developed technique to extract TTX, and an adapted high performance liquid chromatography system with a detection limit of 48 picograms TTX (0.15 picomoles). The amounts of TTX from newts in these three streams suggest fine geographic variation in toxin levels. In chapter 3, the potential ecological effects of newts and TTX were tested using field and laboratory bioassays to determine if newt chemical cues and TTX affect the foraging behavior of macroinvertebrates. Field bioassays indicate that macroinvertebrates move away from point sources of newt chemical cues, while laboratory bioassays show that predatory odonate nymphs exposed to TTX reduce predation rates, mean velocities when striking prey, and mean angular velocities when compared to controls. In the final chapter, male site fidelity and individual TTX levels were tracked in situ over four breeding seasons. Data reveal fluctuations in individual and breeding population TTX levels, suggesting that TTX is not a stable trait. Furthermore, statistical analyses indicate that fidelity to a pool is significantly linked to relative TTX concentrations. Collectively, these results demonstrate a striking ecological role for *T. torosa* and TTX, and imply that the loss of this amphibian in our study system may have wide-reaching ecological consequences.

The dissertation of Gary Bucciarelli is approved.

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University of California, Los Angeles

2015

For those who have dared to think differently.....

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Chapter One is a reproduction of:

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GMB reviewed literature, drafted and revised the manuscript, and created graphics.

ARB, TSG, and LBK helped to conceive subtopics and revise drafts of the manuscript.

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GMB designed experiments, conducted field work, performed statistical analyses, drafted and revised the manuscript, and created graphics. AL helped to design experiments, perform laboratory experiments, and assist with revisions. LBK and DBG helped to design the experiments and revise the manuscript.

Chapter Three is a reproduction of:

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GMB designed experiments, performed field work, laboratory experiments, and statistical analyses, created graphics, and drafted and revised the manuscript. LBK helped design experiments and helped to revise the manuscript.

Chapter Four is a version of a manuscript submitted for publication.

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Bucciarelli, GM & Kats LB. 2015. Effects of newt predator cues on the distribution and foraging of stream macroinvertebrates. Hydrobiologia **749** 69-81.

Bucciarelli, GM, Blaustein AR, Garcia T. & Kats LB. 2014. Invasion complexities: The diverse impacts of invasive species on amphibians. Copeia **4** 611-632.

Bucciarelli, GM, *Li A, Kats LB, & Green DB. 2014. Quantifying tetrodotoxin levels in the California Newt using a non-destructive sampling method. Toxicon **80** 87-93.

Kats, LB, **Bucciarelli GM**, Vandergon TL, Honeycutt RL, *Mattiasen E, *Sanders A, Riley SP, Fisher RN. 2013. Effects of natural flooding and manual trapping on the facilitation of invasive crayfish-native amphibian coexistence in a semi-arid perennial stream. Journal of Arid Environments. **98** 109-112.

Kats, LB, **Bucciarelli GM**, *Schlais DE, Blaustein AR, & Hahn B. 2013. Ultraviolet radiation influences perch selection by a neotropical poison dart frog. ***PLoS ONE***.
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CHAPTER 1

Invasion complexities: the diverse impacts of nonnative species on amphibians

REVIEWS

Copeia 2014, No. 4, 611–632

Invasion Complexities: The Diverse Impacts of Nonnative Species on Amphibians

Gary M. Bucciarelli[1], Andrew R. Blaustein[2], Tiffany S. Garcia[3], and Lee B. Kats[4]

Since the first documented declines of amphibian species, researchers have learned how nonnative species can depress amphibian populations and lead to local extinctions. Here, we explore the dimensions of invasions in the context of evolutionary history, anthropogenic disturbance, and climate change. Recent studies indicate that the nonnative groups that have most negatively affected amphibians are plants, fishes, and other amphibians. We review current work aimed at determining the direct and indirect effects of nonnative species on amphibian health, genotypes, and native ecosystem structure, as well as research examining invasions from a community level perspective. We also describe synergistic effects between abiotic, biotic, and nonnative factors. Recent studies have documented the intricacies of invasions and how numerous aspects of invasions can interact additively and complementarily to the detriment of the native ecosystem. Understanding the complexity of invasions means considering if and how biological, environmental, and ecological processes within ecosystems are being reshaped as a result of introduced species. Assessing the ecology and ecosystem dynamics of invasions at multiple levels, from the genome to the ecosystem, is paramount to the conservation, restoration, and future research of invaded amphibian ecosystems.

AQUATIC species tend to be susceptible to abrupt changes in biological community dynamics and composition (Fisher and Shaffer, 1996; Gamradt and Kats, 1996; Riley et al., 2005; Hamer and Parris, 2013; Pease and Wayne, 2014). Thus, amphibians are known to be particularly sensitive to community changes that occur when nonnative species become established (Kats and Ferrer, 2003; Blaustein et al., 2011). Numerous studies have documented the decline of amphibian populations that occur with the introduction of a nonnative species. Moreover, studies have noted the displacement and extinction of local native species after the introduction and establishment of a nonnative species (Gilpin and Soulé, 1986; Huxel, 1999; Pimm and Raven, 2000; Sax and Gaines, 2008). As the frequency and duration of nonnative species invasions escalates, novel negative effects on amphibians are being documented. Increasingly, studies are delineating the mechanisms that determine whether amphibians will be eliminated with the appearance of nonnative species or whether they will maintain some level of coexistence. What effect nonnative species may have on amphibians can depend heavily on evolutionary histories of both the amphibians and the invasive species (Keller and Taylor, 2008). As such, predicting the rate and future spread of prominent nonnative species and subsequent impacts on amphibians will be critical for management and conservation.

Understanding how nonnative species impact amphibians is especially important because it is one of the factors contributing to amphibian population declines and extinctions (Collins and Storfer, 2003; Kats and Ferrer, 2003; Collins, 2010). One estimate suggests that the extinction rate of amphibians is more than 200 times the background extinction rate (McCallum, 2007). A higher percentage of amphibians are threatened than birds or mammals (Stuart et al., 2004), with many amphibians on the brink of extinction. Nonnative species can be directly responsible for the negative impacts on native amphibian populations via predation and competition, or their effect can be indirect, by introducing a pathogen or altering habitat. Synergistic effects between abiotic, biotic, and nonnative species may also occur. In this review we discuss the numerous ways amphibians are impacted by introduced species (see Fig. 1) through direct threats, such as predation and displacement, as well as indirectly by affecting the genotypes of community members and structure of native ecosystems. We also provide insight into the intricacies of nonnative–native interactions from a community level perspective and how numerous aspects of the process can interact additively and complementarily to the detriment of native amphibians and their ecosystems.

The role of evolutionary history in the complexity of nonnative invasions in amphibian systems.—Some amphibians species may lack the evolutionary history to respond to pressures from nonnative species in ecological time, and in many cases populations decline. Invaders can harm amphibians 1) by predation (Salo et al., 2007), 2) through competition (e.g., Pearson and Goater, 2009), 3) by spreading disease (e.g., Kiesecker et al., 2001a; Blaustein and Kiesecker, 2002),

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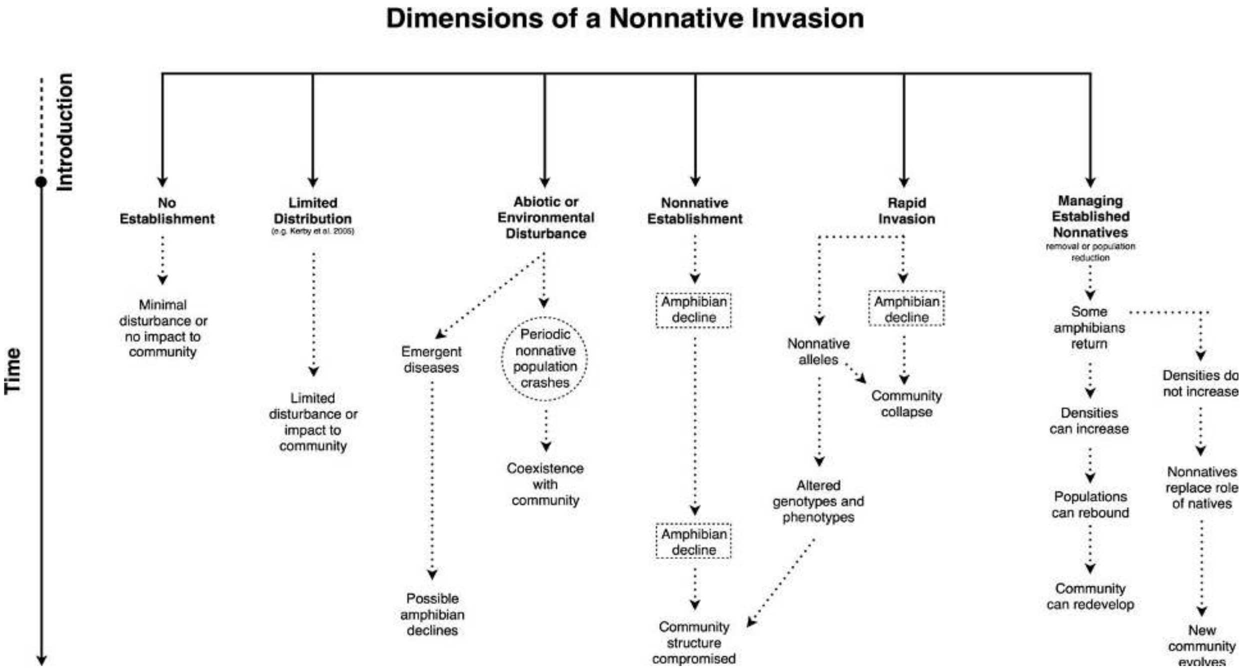

Fig. 1. The potential consequences of a nonnative species being introduced into an ecosystem that includes native amphibian species. Known invasion outcomes are presented across the top of the figure. The long term effects of a nonnative invasion are played out through time under each outcome.

4) by compromising the immune system of native amphibian within communities (Hayes et al., 2010), or 5) by hybridizing with them (e.g., Riley et al., 2003; Storfer et al., 2004; Ryan et al., 2009). Nonnative animal and plant species can also indirectly alter the habitat in ways that affect native amphibians (see Table 1 for an overview).

In many instances, even when amphibians have evolved with a functionally similar predator, they fail to avoid it. Gall and Mathis (2010) found that larval salamanders behaviorally responded to chemical cues from native predatory fish, but showed minimal or no response to chemical cues from ecologically similar, nonnative predatory fish. However, in a recent laboratory study, Davis et al. (2012) found that San Marcos Salamanders (*Eurycea nana*) exposed to chemical cues from a sympatric native sunfish, sympatric introduced sunfish, allopatric sunfish, and sympatric nonnative, non-centrarchid fish all elicited similar behavioral responses. Activity of salamanders was reduced, with no differences in the antipredator behavioral response between cue treatments. The authors reason that adult San Marcos Salamanders generalize fish predator cues within a genus and across a family, likely because they are similar to recognized sympatric predators.

Amphibians are generally more naïve toward introduced predators due to the heterogeneity of predation regimes in freshwater systems compared to the more homogenous predation regimes of terrestrial and marine systems (Cox and Lima, 2006). Terrestrial predators or their ecologically similar equivalents are often widespread across continents. However, in freshwater systems it is not uncommon that prey organisms have evolved without fish predators. For example, Foothill Yellow-Legged Frog (*Rana boylii*) tadpoles have evolved with newt predators and nonpredatory fishes (Paoletti et al., 2011). As a result of this shared evolutionary history with nonpredatory fishes, they show virtually no response to cues from introduced Smallmouth Bass (*Micropterus dolomieu*). In the few examples where amphibians have survived the introduction of a nonnative species, it appears that those species benefitted due to a genetic propensity for induced defenses (Moore et al., 2004).

Pinpointing mechanisms that allow nonnative species to successfully become established and integrated within novel habitat has been challenging. Though it is quite clear that the dispersal and proliferation of nonnative species is significantly influenced by human-mediated changes to hydroperiod (Herwig et al., 2013), waterways (Maret et al., 2006; Johnson et al., 2008; Ficetola et al., 2011; Davies et al., 2013) and urbanization (Riley et al., 2005), researchers have found ecological factors that largely affect the growth of nonnative species populations. As an example, nonnative amphibian success increases in the presence of congeneric species, and appears to be greater on islands than on the mainland (Tingley et al., 2011). Tingley et al. also found that the probability of a nonnative species succeeding in a novel habitat increases when abiotic conditions of the invaded habitat are similar to a nonnative species original habitat. Larger brain size relative to body size has also been suggested to be a successful trait of invaders (Amiel et al., 2011), and has also been considered for the invasion success of birds and mammals (Sol et al., 2005, 2008). Employing molecular genetics tools can also be useful to infer source population and invasion routes, effects of nonnative species upon native species, amounts of genetic diversity throughout nonnative species, and the role of adaptation and admixture. However, there are limitations to the application of molecular genetics tools when applied to biological inva-

Table 1. An overview of the effects that nonnative species have on native amphibian species based on experimental and theoretical research reviewed. Effects on native amphibian species can be negative or positive, and may occur directly (i.e., predation, competition, hybridization, coexistence) or indirectly (i.e., habitat alteration, facilitation, disease vector). Ultimate outcomes may include reduced or increased survival, delayed development, population declines, loss of habitat, modified behavior, increased abundances, decreased diversity, decreased breeding and recruitment, coexistence, decreased densities and distributions, limitations on gene flow, and altered genotypes and phenotypes.

Invasive species	Native species	Effects on native species	Reference
Invasive plants			
Triadica sebifera	*Pseudacris fouquettei*, *Lithobates sphenocephalus, Hyla versicolor*	Reduced survival (*P. fouquettei* and *L. sphenocephalus*) and negatively affected development (*H. versicolor*)	Cotten et al., 2012
	L. sphenocephalus	Negatively affected hatching success	Adams and Saenz, 2012
Phragmites australis	*Rana sylvatica*	Habitat alteration and increased developmental time	Perez et al., 2013
	Bufo fowleri	Population decline, loss of habitat	Greenberg and Green, 2013
	Ambystoma maculatum and *Lithobates palustris*	Negatively affected development and survival	Martin and Blossey, 2013
Rhamnus cathartica	*Pseudacris triseriata*	Negatively affected development and survival	Sacerdote and King, 2013
Lonicera maackii	*Anaxyrus americanus*	Habitat alteration, mortality, and modified behavior	Watling et al., 2011a, 2011b
	Lithobates palustris and *A. americanus*	Habitat alteration (microclimate) and decreased diversity	Watling et al., 2011c
	Lithobates clamitans	Increased abundance	Watling et al., 2011c
Microstegium vimineum	*R. sylvatica, L. palustris,* and *A. americanus*	Additional habitat	Nagy et al., 2011
Elaeis sp.	*Hyla labialis* and *Hyla glandulosa*	Decreased diversity	Faruk et al., 2013
Lythrum salicaria	*B. americanus*	Decreased survival and increased developmental time	Maerz et al., 2005; Brown et al., 2006
Phalaris arundinacea	*R. pretiosa*	Degraded ovipositing habitat	Kapust et al., 2012
	A. americanus, Hyla crysoscelis, L. pilustris, and *L. sylvatica*	Negatively affected survival	Rittenhouse, 2011
P. arundinacea, Phragmites australis, and *Rhamnus frangula*	*L. sylvaticus*	Increased survival and decreased developmental time	Stephens et al., 2013
Invasive invertebrates			
Lumbricus terrestris	*Plethodon cinereus*	Increased survival	Ransom, 2011, 2012a
Amynthas sp.	*P. cinereus*	Habitat alteration and decreased diversity	Maerz et al., 2009
Solenopsis invicta	*Ambystoma opacum* and *A. talpoideum*	Predation	Todd et al., 2008
	Gastrophryne caroleninsis	Coexistence	Deyrup et al., 2013
Procambarus clarkii	*P. regilla, Pseudacris cadaverina, Taricha torosa,* and *Bufo boreas*	Population declines	Riley et al., 2005
	P. regilla	Predation	Pease and Wayne, 2014
	Pleurodeles waltl, Triturus marmoratus, Rana perezi, Hyla arborea, and *Pelodytes punctatus*	Predation	Cruz et al., 2008
	Pelophylax perezi	Predation	Nunes, 2011
	Alytes sisternasii, *Discoglossus galganoi*, *Pelobates cultripes*, *Pelodytes ibericus*, *Bufo calamita*, *Hyla arborea*, *Hyla meridionalis*, *R. perezi*, *P. waltl*, *Salamandra salamandra*, *Triturus boscai*, *T. marmoratus*	Predation	Cruz and Rebelo, 2005
	Bufo calamita	Predation and deterred breeding	Cruz et al., 2006a

Table 1. Continued.

Invasive species	Native species	Effects on native species	Reference
	Alytes cisternasii, *Discoglossus galganoi*, *Pelobates cultripes*, *Pelodytes ibericus*, *Bufo bufo*, *B. calamita*, *Hyla arborea*, *H. meridionalis*, *Rana perezi*, *Pleurodeles waltl*, *Salamandra salamandra*, *Triturus boscai*, *T. marmoratus*	Predation and decreased diversity	Cruz et al., 2006b
	Alytes cisternasii, Discoglossus galganoi, *Pelobates cultripes*, *Pelodytes ibericus*, *Bufo bufo*, *B. calamita*, *Hyla arborea*, *H. meridionalis*, *Pelophylax perezi*	Predation, altered life histories and morphological changes	Nunes et al., 2014
	Pelobates cultripes	Predation	Polo-Cavia and Gomez-Mestre, 2013
Invasive amphibians			
Xenopus laevis	*Discoglossus pictus*, *Hyla intermedia*, and *Pelophylax synklepton esculentus*	Decreased breeding	Lillo et al., 2011
Lithobates catesbeianus	*Rana draytonii*	Predation	D'Amore et al., 2009
	L. sylvaticus	Disease vector for *Batrachochytrium dendrobatidis*	Greenspan et al., 2012
	Ambystoma tigrinum stebbinsi	Predation	Maret et al., 2006
	Eight unnamed frog and toad species	Decreased density and diversity	Li et al., 2011
	P. regilla	Competition	Preston et al., 2012
	Hypsiboas albomarginatus	Competition	Both and Grant, 2012
Ambystoma tigrinum mavortium	*A. tigrinum stebbinsi*	Hybridization	Storfer et al., 2004
	Ambystoma californiense	Hybridization	Riley et al., 2003; Ryan et al., 2009
Osteopilus septentrionalis	*Hyla cinerea* and *Hyla squirella*	Associated with reduced survival and abundance	Rice et al., 2011
Discoglossus pictus	*S. salamandra*, *T. marmoratus*, *Lissotriton helviticus*, *Alytes obstetricans*, *Pelodytes punctatus*, *Pelobates cultripes*, *Bufo calamita*, *B. bufo*, *Hyla meridionalis*, and *P. perezi*	Competition and decreased diversity	Richter-Boix et al., 2013
	B. calamito and *P. punctatus*	Competition	Richter-Boix et al., 2013
Invasive fishes			
Lepomis macrochirus, *L. cyanellus*, and *Ameiurus melas*	*Ambystoma texanum*	Predation	Walston and Mullin, 2007
Salvelinus fontinalis	*Ambystoma gracile*	Associated with decreased recruitment	Hoffman et al., 2004
S. fontinalis and *Oncorhynchus mykiss x O. m. aquabonita* hybrids	*Rana muscosa*	Associated with population declines	Knapp et al., 2007
Salmo trutta	*Salamandrina perspicillata*	Associated with decreased recruitment	Piazzini et al., 2011
Cyprinus carpio, *Perca fluviatilis*, and *Gambusia holbrooki*	*Litoria booroolongensis*	Predation	Hunter et al., 2011
Gambusia affinis, *Lepomis cyanellus*, *Lepomis macrochirus*, *Poloxis annularis*, *Ameiurus melas*, and *Micropterus salmoides*	*A. tigrinum stebbinsi*	Predation	Maret et al., 2006
Carassius auratus	*Ambystoma macrodactylum columbianum*	Predation	Monello and Wright, 2001

Table 1. Continued.

Invasive species	Native species	Effects on native species	Reference
Percichthys colhuapiensis	*Atelognathus patagonicus*	Associated with population declines	Cuello et al., 2009
Gambusia affinis	*Salamandra infraimmaculata*	Predation	Segev et al., 2009
	P. regilla	Disease vector for *Ribeiroia ondatrae*	Orlofske et al., 2012
	P. regilla and *T. torosa*	Predation	Preston et al., 2012
Gambusia holbrooki and *Perca fluviatilis*	*Geocrinia victoriana*, *Limnodynastes dumerilii*, *Limnodynaste peronnii*, *Crinia signifera*	Associated with decreased abundances	Hamer and Parris, 2013
O. mykiss, salvelinus alpinus, S. fontinalis, Carassius auratus, G. affinis, Lepomis gibbosus, and *Phoxinus phoxinus*	*Triturus alpestris* and *Triturus helveticus*	Associated with population declines	Denoël et al., 2005
O. mykiss, S. fontinalis, Tinca tinca, C. carpio, Phoxinus phoxinus, and *Chondrostoma arcasii*	*T. alpestris, T. helveticus, T. marmoratus*	Associated with limited distribution	Orizaola and Braña, 2006
Lepomis gibbosus and *Pseudorasbora parva*	*Triturus cristatus*, *Triturus vulgaris*, *Hyla arborea*, and *Rana temporaria*	Decreased diversity	Hartel et al., 2007
O. mykiss	*Anaxyrus boreas*	Disease vector for *Saprolegnia ferax*	Kiesecker et al., 2001a
O. mykiss and *S. fontinalis*	*Rana cascadae*	Population declines	Pope, 2008
	Rana cascadae	Competition	Joseph et al., 2011
	R. muscosa	Predation	Vredenburg, 2004
O. mykiss and *S. trutta*	*Hadromorphryne natalensis*	Decreased abundances	Karssing et al., 2012
O. mykiss and *P. promelas* Rafinesque	*A. macrodactylum*	Predation, competition	Pearson and Goater, 2009
O. mykiss and *O. m. aquabonita*	*R. muscosa*	Competition	Finlay and Vredenburg, 2007
O. mykiss and *S. trutta*	*Cryptobranchus alleganiensis alleganiensis* and *Cryptobranchus a. bishopi*	Predation	Welsh et al., 2006
O. mykiss, S. trutta, and *S. fontinalis*	*L. sylvaticus*	Coexistence	Schank et al., 2011
Lepomis auritus	*Eurysea nana*	Predation	Davis et al., 2012
Micropterus dolomieu	*Rana boylii*	Predation	Paoletti et al., 2011
Leucaspius delineatus	*Rana temporaria*	Predation	Leu et al., 2009
Gasterosteus aculeatus	*T. alpestris, T. helveticus, T. vulgaris*	Associated with limited distribution	Denoël and Ficetola, 2008
Invasive mammals			
Neovision vison	*R. temporaria*	Decreased densities and distribution	Ahola et al., 2006; Salo et al., 2010
N. vison and *Rattus rattus*	*Salamandra salamandra gallaica*	Predation	Velo-Anton and Cordero-Rivera, 2011
R. rattus	*Leiopelma hochstetteri*	Coexistence	Nájera-Hillman et al., 2009
Herpestes javanicus	*Babina subaspera*	Associated with limited distribution and gene flow	Iwai and Shoda-Kagaya, 2012
Dasypus novemcinctus	*Plethodon angusticlavius*	Predation	Crane et al., 2011

sions, which typically occur at rapid timescales. Fitzpatrick et al. (2012) suggest that population genetics can really only effectively be used to test hypotheses regarding the geographic origin of invasions, bottlenecks, and hybridization events given the assumptions of neutral theory inherent in population genetics analyses.

Interactions between native amphibians and nonnative plants.— The potential consequences of introduced flora upon amphibians is perhaps the least studied of the nonnative–native amphibian interactions currently recognized, although a body of research is beginning to develop (Martin and Murray, 2011). Maerz et al. (2005) first reported that native tadpoles (*Bufo americanus*) appeared to have reduced performance when raised in habitats that contained Purple Loosestrife (*Lythrum salicaria*). They hypothesized that high tannin levels from the leaves of the plant produce potentially toxic environments for developing tadpoles. In a follow up experiment, Brown et al. (2006) showed that native tadpoles had slower development and decreased survivorship when reared in experimental venues with Purple Loosestrife extracts, and the addition of leaf litter of *L. salicaria* exacerbated the effects. The authors found that algal communities differed between Purple Loosestrife venues and other venues, leading them to conclude that food quality and quantity were responsible for reduced tadpole performance. More recently, Maerz et al. (2010) have suggested that nonnative plants can ultimately change the nutrient quality of the detritus that tadpoles feed upon.

Other introduced plants have also shown marked effects on amphibians. Watling et al. (2011a, 2011b) have suggested that toad tadpoles (*Anaxyrus americanus*) reared in extracts from a nonnative shrub (*Lonicera maackii*) were more likely to die than tadpoles in native plant extracts. However, experimental studies showed that *L. maackii* is not fatal to three amphibian species tested: *Ambystoma maculatum, Hyla* sp., and *Lithobates blairi*. A behavioral study looking at the same species of larvae grown in extracts of *L. maackii* noted that larvae made frequent trips to the water surface, suggesting that the extracts may interfere with the respiratory physiology of the tadpoles. Other research by Watling et al. (2011c) demonstrated that amphibian species richness and evenness were lower in plots invaded by *L. maackii.* This also resulted in shifts in species composition. The authors suggest that nonnative plants may change microclimate when they become well established and ultimately negatively affect entire native communities. It also appears that invasions of *L. maackii* can indirectly affect native herpetofauna by shortening the hydroperiod of ephemeral ponds and streams. Boyce et al. (2012) found that transpiration of *L. maackii* was a significant source of habitat water loss, accounting for roughly 10% of stream flow draining in the wetland study area. The authors indicate that this estimate may be at the lower end since basal areas in the study sites were not as large as those in other invaded habitat.

The introduced deciduous tree Chinese tallow (*Triadica sebifera*) has also been found to affect anurans. Cotten et al. (2012) tested extracts from *T. sebifera* and two native tree species on survival and development of larval *Pseudacris fouquettei*, *Lithobates sphenocephalus*, *Hyla versicolor*, and *Incilius nebulifer* from eastern Texas. Early breeding amphibians (*P. fouquettei* and *L. sphenocephalus*) had lower survival and exhibited different growth patterns than later breeding amphibian species when exposed to *T. sebifera*. *Hyla versicolor* showed significant morphological and developmental differences between treatments. Adams and Saenz (2012) demonstrated similar effects of Chinese Tallow on eggs of *Lithobates sphenocephalus*. All eggs exposed to Chinese Tallow leaf litter died, regardless of concentration. The authors observed that greater amounts of Chinese Tallow leaf litter resulted in lower dissolved oxygen and pH levels.

Common Reed (*Phragmites australis*) is another plant nonnative to North America that researchers have determined affects tadpole development and food availability. Perez et al. (2013) used field enclosures with varied densities of the reed and found that survival did not differ between treatments, but tadpoles in medium and high reed density enclosures developed more slowly. Due to this delayed growth, the authors suggest larvae in ponds invaded by Common Reed could be exposed to threats for greater periods of time, including natal pools drying out and predation. Other researchers have found that the spread of Common Reed has contributed to an ongoing loss of amphibian breeding habitat and population declines for Fowler's Toads, *Bufo fowleri* (Greenberg and Green, 2013). Other nonnative reed grasses like the Reed Canary Grass (*Phalaris arundinacea*) appear to disrupt Oregon Spotted Frog (*Rana petiosa*) ovipositing habitat (Kapust et al., 2012). Rittenhouse (2011) had previously found no direct toxic effects of Reed Canary Grass on tadpoles, but did find that the reed reduced survival in native amphibian species and suggests it was due to decomposing reed grass that led to anoxic larval environments.

A recent study of the direct effects of European Buckthorn (*Rhamnus cathartica*) metabolites on amphibians was conducted by Sacerdote and King (2013). European Buckthorn is an aggressive nonnative that produces a secondary metabolite, emodin, which leaches into soil and water. They quantified amounts of emodin at amphibian breeding sites and exposed native Western Chorus Frog (*Pseudacris triseriata*) and African Clawed Frog embryos to an ecologically realistic gradient of emodin concentrations. Both frog species responded negatively to emodin; embryo mortality and malformation occurred, although the effect of the compound was more pronounced in Western Chorus Frog embryos.

Nonnative plants may not always pose immediate negative threats to amphibians (Hayes and Holzmueller, 2012). Nagy et al. (2011) found that a nonnative grass, Japanese Stilt Grass (*Microstegium vimineum*), can offer cover and nesting habitat for native frogs, especially in degraded habitat. Their research shows that in landscapes where White-Tailed Deer (*Odocolileus virginianus*) have overgrazed native flora, which has typically served as cover for native amphibians, *M. vimineum* has been able to provide suitable habitat. The authors later raise an interesting point, which is to consider how removal of a nonnative species may affect native species, especially if a native species has been able to exploit a nonnative species when there is little to no native habitat left. This seems particularly relevant when native habitat has been compromised as a result of land alteration, climate change, or the integration of nonnative species into native ecosystems. Similar questions are bound to arise as native forest plant communities become more disturbed and likely experience drastic changes in species composition.

Stephens et al. (2013) have explored how changes to tree forest composition have resulted in altered leaf litter input. Consequently, it appears that nonnative leaf litter affects the fitness of larval amphibians. They found that litter from native trees caused frogs to grow larger, develop faster, and survive better than larvae that were exposed to nonnative leaf litter treatments. Similar results have also been observed when Wood Frogs (*Lithobates sylvaticus*) were exposed to an array of native and nonnative species specific leaf litter (Stoler and Relyea, 2013). Compounds from chemically distinct tree species affected growth and developmental rate, and produced drastic morphological responses in intestines length and tail muscle depth.

The connection between terrestrial nonnative plants and terrestrial salamanders has not been as clear (Maerz et al., 2009). No significant decrease in woodland salamander species was found with increasing densities of nonnative plants. However, there were subtle interactions occurring between plants, nonnative earthworms, and amount of leaf litter that impacted salamanders. Maerz et al. (2009) concluded that rather than Eastern Red-Backed Salamander (*Plethodon cinereus*) abundance declining in response to the establishment and spread of nonnative plants, abundances were more strongly tied to nonnative earthworms due to shared leaf litter resource base.

Martin and Blossey (2013) present results that suggest there is intraspecific variation in plant compounds between plant populations and that such variation can significantly affect amphibians. In laboratory experiments, they exposed Spotted Salamanders (*Ambystoma maculatum*) to leaf litter extracts from 14 different populations of native and nonnative *Phragmites australis* and observed varied larval survival among plant populations. These results suggest that there is likely considerable intraspecific variation of secondary compound chemistry between plant populations, which managers and scientists may need to consider when preserving habitat and determining the direct and indirect effects of nonnative plants.

Nonnative plants may also have indirect effects upon amphibians, which appear to be remarkably different from native flora. Smith (2013) discussed how leaf phenology of nonnative trees is an important invasion mechanism. Compared to native tree species, the leaves of nonnative trees often emerge earlier in the spring and abscise later in the fall. Amphibians may indirectly be harmed in a number of ways. Cooler water temperatures due to increased shading could affect rates of metamorphosis, and synergistic effects of secondary metabolites or other organic compounds could be deleterious to tadpoles (e.g., Martin and Blossey, 2013). At a much larger scale, variation of native tree communities in forested ecosystems could have even greater consequences, since they often can control forest structure and ecosystem dynamics (Ellison et al., 2005). Amphibian genotypes and phenotypes could ultimately be affected too, with the relative fitness of community members, species composition, and abundances all being influenced by interspecific variation in native ecosystems (e.g., Whitham et al., 2008).

The effects of nonnative terrestrial invertebrates upon native amphibians.—While many current studies focus on the spread of aquatic nonnative species and their impacts upon amphibians, Todd et al. (2008) have noted that human-mediated effects upon the landscape are facilitating the spread of nonnative Fire Ants (*Solenopsis invicta*) to the detriment of terrestrial salamanders. They compared Fire Ant densities in non-harvested woodlands to partially thinned stands and clear-cut stands. They found that more disturbed habitats had higher numbers of Fire Ants and that terrestrial salamanders at these locations (*Ambystoma* spp.) suffered higher predation from Fire Ants. Conversely, Deyrup et al. (2013) found that native Eastern Narrow-Mouthed Toad (*Gastrophyrne carolinensis*) in Florida consumed ants from numerous genera, including ants known to possess toxins or other toxic substances. The authors conclude that *G. carolinensis* could possibly subsist on exotic species of ants such as Fire Ants. Studies in Indonesia show similar results. On the island of Sulawesi, an endemic toad (*Ingerophrynus celebensis*) preys on a nonnative ant species (*Anoplolepis gracilipes*), which ultimately promotes native ant diversity (Wanger et al., 2011).

Introduced earthworms also directly and indirectly affect amphibian ecology (Ransom, 2011, 2012a). As ecosystem engineers, their establishment has altered nutrient regimes, leaf-litter decomposition rates, and soil structure. Greiner et al. (2012) demonstrated that nonnative Asian (*Amynthas hilgendorfi*) and European (*Lumbricus rubellus*) earthworms increased leaf litter decomposition rates and increased concentrations of mineral forms of phosphorous and nitrogen. Research performed by Ransom (2011) shows that the Common Earthworm (*Lumbricus terrestris)* decreases amounts of leaf litter and macroinvertebrates. However, earthworm burrows provided effective refuge for *Plethodon cinereus* during encounters with predators and overwintering habitat. In follow up studies, Ransom (2012b) showed that recent or historical co-occurrence of *P. cinereus* with North American native (*Eisenoides carolinensis* or *Diplocardi* sp.) or nonnative (*L. terrestris*) earthworms affected burrowing time and time to first burrow. *Plethodon cinereus* that evolved with native earthworms (~7,000 years ago) and without did not differentiate between earthworm species as prey, nor did *P. cinereus* demonstrate any preference for species-specific earthworm burrows. However, if populations of *P. cinereus* co-occurred with native earthworms, they attacked worms more quickly, had shorter handling times, and were more likely to consume earthworms. Ransom demonstrates the importance of understanding the various ways in which nonnative species interact with native species and communities. It is thought that coexisting species of earthworms can perhaps facilitate invasional meltdown (Simberloff and Von Holle, 1999), which takes place when a nonnative species acts as a catalyst and increases the possibility of greater numbers of nonnative species invading an ecosystem, thus amplifying the effects of those invaders (Cameron and Bayne, 2011; Cameron et al., 2012). This may result in strong negative impacts upon local native species, especially at the species level and potentially throughout the entire community.

The effect of nonnative aquatic invertebrates.—Often nonnative species reshape communities and ecological associations, in which case the behaviors and antipredatory responses of natives become unfit. Diamond and Case (1986) have suggested that amphibians often cannot coexist with introduced species due to a lack of shared evolutionary history and inexperience with a functionally equivalent native predator. Thus, native amphibian populations are more likely to decline if an introduced species is from a novel functional group. Ricciardi and Atkinson (2004) used a meta-analysis and found that high impact invaders are usually species that belong to a genus not currently found within an ecosystem. As an example, amphibian populations in North America and Europe have been dramatically reduced by the introduction of the Red Swamp Crayfish (*Procambarus clarkii*). Amphibian populations that suddenly must cope with *P. clarkii* do poorly, in part, because these same amphibian species have not encountered voracious aquatic omnivores like *P. clarkii* in their native habitat (Cruz et al., 2008). Native crayfishes have very small geographic ranges (Lodge et al., 2000), and as a result many amphibians have no evolutionary history with them.

Nunes (2011) investigated how coexistence time with *P. clarkii* affected Iberian Waterfrog (*Pelophylax perezi*) antipredatory behavior. In laboratory experiments, tadpoles of *P. perezi* from populations that did not co-occur with *P. clarkii* reduced behavior in the presence of crayfish. However, populations with long-term exposure to *P. clarkii* did not show a similar response, but did exhibit morphological defenses, such as a deeper tail and shorter head-body length. Although the invasion of *P. clarkii* occurred roughly 30 years ago, the authors believe that these predators imposed such strong selection pressure that it has led to a rapid evolution of morphological defenses.

Similar experiments conducted by Gomez-Mestre and Diaz-Paniagua (2011) showed that chemical cues from *P. clarkii* failed to activate morphological defenses in *P. perezi*, even though chemical cues from native predatory dragonfly nymphs did. The authors argue that responding to crayfish chemical cues would be adaptive, but that the observed lack of morphological defenses in *P. perezi* could be due to cue recognition failure and too recent of an invasion for defenses to evolve. It is also possible that the opposing results are due to inherent variation within and between populations, as observed by Nunes (2011) and Nunes et al. (2014), and may be attributable to differences in exposure and thus, selection intensity.

Recent experiments by Nunes et al. (2013, 2014) show that anurans may not respond to chemical cues from *P. clarkii,* but will respond defensively to chemical cues from native predatory macroinvertebrates. In the presence of adult crayfish, a subset of anurans exhibited behavioral defenses, which appear to have been facilitated by chemical cues from injured conspecifics that *P. clarkii* preyed upon (Nunes et al., 2013). Nunes et al. (2014) also tested how larval morphology, growth, and development may differ between nine tadpole species from southern Portugal reared in the presence of *P. clarkii* and native predatory odonate nymphs (*Aeshna* sp.). They found that more species of tadpoles responded with morphological or life history changes when grown with native odonate predators than when grown with predators of *P. clarkii*. The authors suggest that intra- and inter-specific variation in response to *P. clarkii* could lead to a greater risk of those individuals and species becoming extirpated, ultimately reducing local biodiversity throughout invaded communities. Divergence in morphology and behavior has also been observed in North America between naïve Pacific Tree Frog tadpoles (*Pseudacris regilla*) exposed to *P. clarkii* chemical cues and tadpoles from streams with crayfish (Pease and Wayne, 2014).

In other systems, naïve tadpoles of the Western Spadefoot Toad (*Pelobates cultripes*) were not capable of responding to water-borne chemical stimuli from *P. clarkii* unless the stimuli was a combination of *P. clarkii* and injured conspecific chemical compounds (Polo-Cavia and Ivan Gomez-Mestre, 2013). Experimental results showed that conditioned tadpoles experienced greater survivorship than unconditioned tadpoles. Ultimately, the ability of native amphibians to detect, respond to, and learn from introduced species, especially through association with alarm cues, may very well improve recruitment and decrease the vulnerability of native amphibian species to novel predators.

Compromised recruitment and impaired reproduction have also been documented as a direct result of *P. clarkii* expanding into novel habitat. The presence of *Bufo calamita* throughout breeding habitat in the southwest Iberian Peninsula was negatively associated with presence of *P. clarkii* (Cruz et al., 2006a). Surveys indicated that coexistence between *B. calamita* and *P. clarkii* was limited to only one of 31 ponds. Experimental evidence showed that the survivorship of embryos was significantly reduced in the presence of *P. clarkii*. Furthermore, breeding sites disturbed by *P. clarkii* in the southwest Iberian Peninsula also appear to negatively affect breeding activity of urodeles, too. Cruz et al. (2006b) indicate that the probability for all local urodeles to breed is deterred in the presence of *P. clarkii*, regardless of predatory fish. These results imply that communities within which amphibians reside may be permanently altered by the introduction of *P. clarkii* and that overall amphibian species richness may be lowered in these areas, too.

Invasion complexity in amphibian systems promoted by indirect and direct ecological effects of introduced fishes.—One of the greatest threats to native amphibians continues to be introduced fishes (Denoël and Ficetola, 2008; Cuello et al., 2009; Gall and Mathis, 2010; Hunter et al., 2011; Reshetnikov and Ficetola, 2011). Fish introductions can be accidental or intentional when released for game fishing (e.g., Welsh et al., 2006; Barrionuevo and Ponssa, 2008) or as pest control agents (Leu et al., 2009; Segev et al., 2009). While both frogs and salamanders are susceptible to nonnative fish, salamanders appear particularly vulnerable (Denoël et al., 2005; Orizaola and Braña, 2006; Piazzini et al., 2011). Piazzini et al. (2011) found that the presence of introduced trout and crayfish reduced the probability of Spectacled Salamander (*Salamandrina perspicillata)* egg occurrence from a mean probability of 0.90 to 0.12. Interestingly, native crayfish (*Austropotamobius fulcisianus*) had no detectable effect. These studies underscore the importance of disentangling the complexities of native and nonnative pressures on amphibian populations.

Though most studies demonstrate that introduced game fishes reduce native amphibian populations by directly consuming adult or larval amphibians (Pope, 2008), recent studies indicate that even fishes not traditionally thought of as predators will consume native amphibian larvae, hence reducing frog and salamander populations (Monello and Wright, 2001; Leu et al., 2009). Furthermore, introduced fishes have numerous indirect effects on native amphibians too, affecting organisms at numerous scales ranging from the genome to the ecosystem (Cucherousset and Olden, 2011). Joseph et al. (2011) found that when adult Cascades Frog (*Rana cascadae*) co-occurred with introduced trout they had a smaller proportion of adult aquatic insects in their diet relative to those not found with trout. Fish gut content analyses showed that trout were feeding heavily on the aquatic stages of numerous insects. Frogs co-occurring with trout fed more heavily on terrestrial insects such as grasshoppers. The authors concluded that introduced trout affect native amphibians directly through predation and indirectly through resource competition (Finlay and Vredenburg, 2007), and it appears that these indirect effects in freshwater systems often have far reaching ecological consequences (Strayer, 2010).

Introduced fishes have can significantly alter trophic webs and resource partitioning by disrupting reciprocal prey subsidies. Benjamin et al. (2013) found indirect effects of nonnative Brook Trout (*Salvelinus fontinalis*) that have replaced native Cutthroat Trout (*Oncorhynchus clarkii*). The nonnative Brook Trout reduced the flux of emerging insects by greater than 50%, which they predicted would reduce spider abundance by 20% and the birds that prey on them. The consequences of these introductions may not only have negative biological effects on native amphibians in these communities, but also negative effects at the ecosystem level as well (Dunham et al., 2004).

The global spread of nonnative amphibians.—The topic of nonnative species has become one of great concern in the scientific community and has increasingly gained the attention of policy makers and the general public. There is no sign that the spread of nonnative species to new habitats is slowing, and it is often amphibians as a taxonomic group that are most vulnerable to invaders. However, nonnative amphibian species have successfully invaded native amphibian ecosystems, too. The American Bullfrog (*Lithobates catesbeianus*) is an example of a nonnative species that has had profound effects on native amphibians where it has been introduced (Adams and Pearl, 2007). This species, native to eastern North America, is now globally widespread and continues to establish new populations outside of its native range (Ficetola et al., 2007a; Akmentins and Cardozo, 2010), including South America, where it is predicted to invade biodiversity hotspots (Nori et al., 2011), and in the peninsula region of Baja California (USA), where the number of invaded sites doubled over an eight-year period (Luja and Rodríguez-Estrella, 2010a).

Lithobates catesbeianus is known to reduce native amphibian populations by preying upon natives and competing for resources (Adams and Pearl, 2007; D'amore et al., 2009). Although there are patterns where native amphibians and the presence of bullfrogs are negatively correlated, the underlying mechanisms of displacement of native amphibian species remains complex (Blaustein and Kiesecker, 2002; Kiesecker et al., 2001a; Pearl et al., 2004). For example, in the Willamette Valley of Oregon (USA), the impact of *L. catesbeianus* on native Red-Legged Frogs (*Rana aurora*) appears to be a mixture of direct and indirect effects compounded by habitat modifications, which intensify interactions. The presence of larval and adult bullfrogs results in alterations of microhabitat used by Red-Legged Frogs that make them more susceptible to predation by fishes (Kiesecker and Blaustein, 1998). Laboratory experiments have shown that tadpoles that were syntopic with bullfrogs display antipredator behavior when presented with chemical cues of larval or adult bullfrogs, but tadpoles from populations that were allotopic to bullfrogs did not (Kiesecker and Blaustein, 1997). In the field and in the laboratory, these behaviors resulted in higher rates of predation in tadpoles from allotopic populations (Kiesecker and Blaustein, 1997). However, further complexity was illustrated in this system because in field experiments, Red-Legged Frog tadpoles that altered their microhabitat use in the presence of bullfrogs had decreased growth and increased predation by fishes (Kiesecker and Blaustein, 1998). Thus, modified use of habitat appear to play a major role in the interactions of bullfrogs with native amphibians (Kiesecker, 2003 and references therein).

Understanding habitat conditions that allow *L. catesbeianus* to thrive in nonnative ecosystems is also being explored, as are patterns of native species distributions when ecosystems are invaded (Fuller et al., 2010). Others have tested the limits of the capacity of *L. catesbeianus* to adapt to local environmental conditions. Cook et al. (2013) determined that larvae of *L. catesbeianus* in the Willamette Valley, Oregon (USA) cannot survive changing hydroperiods because they seem to lack developmental plasticity. The authors suggest that artificially manipulating hydroperiod as a means to control nonnative American Bullfrogs could successfully help manage bullfrog invasions since it will not induce rapid metamorphosis.

In some cases, climate and land-use models can predict where American Bullfrogs might spread next (Ficetola et al., 2007b), and environmental DNA (eDNA) is also being used to detect *L. catesbeianus*, as well as other nonnative species, before they become established (Dejean et al., 2012). In field studies, the sensitivity of eDNA was compared to traditional field methods of call and visual surveys. Using eDNA, *L. catesbeianus* was detected in 38 sites while it was detected in only seven sites using survey methods. The results of this study imply that traditional survey methods have probably underestimated the presence of nonnative species, but it also shows great promise for detecting nonnative species early, when densities are low and manageable, and across all life stages. eDNA can also be used to detect threatened or cryptic species, or species whose numbers have been diminished due to the detriment of a nonnative species.

Another amphibian that has now spread to several continents and continues to show signs of invading new geographic regions is the African Clawed Frog, *Xenopus laevis* (e.g., Faraone et al., 2008; Measey et al., 2012). According to Lillo et al. (2011) African Clawed Frogs reduce the occurrence of native amphibian species from breeding sites in certain Italian populations. Like bullfrogs, African Clawed Frogs can potentially transmit the emerging infectious disease agent Bd (*Batrachochytrium dendrobatidis*) to native amphibians (Solís et al., 2010). Researchers are attempting to predict the spread of this nonnative amphibian to mediate further impacts on native species and their habitat (Fouquet and Measey, 2006; Rebelo et al., 2010; Lobos et al., 2013).

One area of research in invasion biology that remains truly understudied is how vocalizing nonnative species may potentially displace native vocalizing species. Strauss et al. (2006) asked if the deafening mating chorus of introduced Coqui Frogs affects the acoustic landscape of calling native species. Though much research has been conducted on the response of birds increasing song pitch to compete with noise due to urbanization (Slabbekoorn and Peet, 2003; Nemeth and Brumm, 2009), little has focused on how introduced bird species, as well as introduced frog species, affect calling. Frogs demonstrate higher pitched calls in urban settings (Parris et al., 2009), and to compensate for the extremely broad range of acoustic space that waterfalls occupy, some frogs have evolved calls that contain ultrasonic harmonics (Narins et al., 2004). But do the calls of frogs change in response to a vocalizing nonnative species? A particularly interesting study conducted by Both and Grant (2012) demonstrates how nonnative amphibians can displace native amphibians from their acoustic niche. In their experiments, native male White-Banded Tree Frogs (*Hypsiboas albomarinatus*) shifted their calls to significantly higher frequencies when researchers played back recordings of nonnative American Bullfrogs (*L. catesbeianus*) vocalizing. The tree frogs continued to use higher frequencies even after the bullfrog calls were terminated; however, tree frog call duration did decrease. Rate of call or inter-call interval did not change. Because the American Bullfrog call occupies a broad frequency band, the researchers expect the effects of bullfrog vocalization to be especially severe to communities of vocalizing species by disrupting numerous acoustic niches.

Hybridization between native and nonnative amphibians.—Community wide consequences have been observed when native genotypes are replaced by nonnative genotypes. When such evolutionary events occur, communities and ecosystems can become statistically distinct. Nonnative species that introgress through hybridization with native species not only change native species genotypes, but can also alter phenotypes and have the potential to largely affect ecological processes (e.g., Ryan et al., 2009). Ryan et al. (2009) found that hybridization between native, federally endangered California Tiger Salamanders (*Ambystoma californiense*) and introduced Barred Tiger Salamanders (*A. tigrinum mavortium*) produces offspring that significantly reduce survival of native amphibian community members. Furthermore, hybrid larvae reduced the size at metamorphosis of native larvae and prolonged the time to metamorphosis. These researchers also observed Mendelian dominance on size, time of metamorphosis, and predation rate of hybrids. Their results demonstrate how the displacement of native genotypes can potentially compromise ecosystems by generating novel genotypes and phenotypes.

Ryan et al. (2013) also found that salamanders with hybrid genotypes were able to survive major environmental stress while native genotypes could not. All native genotype salamanders died off unless they had already metamorphosed prior to die-off events. Throughout the range of native California Tiger Salamanders, human-mediated landscape modification, pesticide use, and climate change continue to pose a threat. These results imply that native genotype salamanders could be rapidly displaced by nonnative genotypes as a result of these anthropogenically induced environmental stressors.

The genetics of native amphibian species may also be disrupted indirectly by nonnative species impeding gene flow. On the Amami Islands of Japan, an introduced mongoose has created a large habitat gap between a native population of Otton Frog (*Babina subaspera*). Researchers have tested for genetic structure, gene flow, and genetic diversity and discovered very little gene flow between the disjunct population (Iwai and Shoda-Kagaya, 2012). The researchers also found that the disruption of gene flow potentially caused by mongoose has additionally led to relatively recent genetic differentiation.

Invasions complicated by the spread of pathogens.—Nonnative species may also be a major carrier of pathogens that infect native amphibians. The globally distributed water mold *Saprolegnia* (Wood and Willoughby, 1986; Blaustein et al., 1994; Kiesecker and Blaustein, 1997) has become widespread due to movement of hatchery-raised fishes (Blaustein et al., 1994) and has shown to negatively affect Western Toad (*Bufo boreas*). In laboratory experiments, mortality induced by *Saprolegnia* was greater in Western Toad embryos exposed directly to hatchery-reared Rainbow Trout (*Oncorhynchus mykiss*) experimentally infected with *Saprolegnia* than in control embryos (Kiesecker et al., 2001a). Embryos also developed significant *Saprolegnia* infections when raised on soil that was experimentally exposed to trout infected with *Saprolegnia* (Kiesecker et al., 2001a). It also appears that different strains of *Saprolegnia* may have different virulence (Kiesecker et al., 2001a), and as a result, introduced fishes may transmit strains of *Saprolegnia* that are more virulent to native amphibians. Furthermore, variation in susceptibility to *Saprolegnia* may contribute to pathogen-induced changes in species interactions and community structure. Kiesecker and Blaustein (1999) observed that the differential effects of *Saprolegnia* on larval recruitment of Pacific Tree Frog (*Hyla regilla*) and Cascades Frog (*Rana cascadae*) reversed the outcome of competitive interactions between the two species (Kiesecker and Blaustein, 1999). Larvae are potentially affected by *Saprolegnia* if the mold infects eggs, which may cause early hatching and increase larval susceptibility to predation (Gomez-Mestre et al., 2006). Moreover, the effects of *Saprolegnia* infections on amphibian larval recruitment are moderated by the spatial distribution of egg masses and their exposure to sunlight (Kiesecker and Blaustein, 1995, 1997). Kiesecker et al. (2001b) and Kiesecker and Blaustein (1995) reported that periodic mass mortality of embryos of *B. boreas* in Oregon resulted from a synergism between ultraviolet-B (UV-B) radiation and infection with *Saprolegnia*. UV-B exposure was in large part determined by water depth at oviposition sites. Kiesecker et al. (2001b) linked El Niño/Southern Oscillation (ENSO) events with decreased winter precipitation in the Oregon Cascade Range and suggested that less winter snow pack resulted in lower water levels when toads breed in early spring. Toad embryos developing in shallower water exposed to higher levels of UV-B radiation experienced increased mortality from infection with *Saprolegnia*. In this example, global events and nonnative fish species combine in a complex series of interactions that clearly affect local amphibian populations. Given the widespread practice of introducing hatchery-reared fishes and climate-induced changes in snow pack levels, we suggest that fishes used in stocking programs could be an important vector for diseases responsible for amphibian losses.

Lithobates catesbeianus may also be a carrier of the chytrid fungus (*Batrachochytrium dendrobatidis*, Bd), that causes chytridiomycosis, a disease that negatively affects amphibian populations and is associated with global amphibian declines (Daszak et al., 2004; Briggs et al., 2010; Duffus and Cunningham, 2010; Kilpatrick et al., 2010; Gervasi et al., 2013a; Olson et al., 2013). The chytrid fungus has a long evolutionary history, which predates its recent outbreak (Rosenblum et al., 2013). It is hypervirulent to some species (Blaustein et al., 2005; Searle et al., 2011) and has emerged across at least five continents (Farrer et al., 2011). American Bullfrogs are widely reported to be a tolerant host and a carrier of Bd, and can often spread the pathogen to less tolerant hosts (Greenspan et al., 2012). As an example of how bullfrogs may spread the chytrid fungus, recent South American studies have noted the fungus in frog species where it had not previously been found, and this infection corresponds closely with the recent invasion of the American Bullfrog (e.g., Arellano et al., 2009; Barrasso et al., 2009).

Although *L. catesbeianus* may be carriers of Bd in some situations, they may be quite susceptible to Bd in others. It appears that susceptibility depends upon the strain of Bd in question and the ecological circumstances (Gervasi et al., 2013b). Moreover, since *L. catesbeianus* do not occupy all regions where Bd is found they cannot be responsible for transmitting the fungus to many native species, especially for example, amphibians that inhabit high elevation habitats. Gervasi et al. (2013b) showed that the dynamics of transmitting Bd from *L. catesbeianus* to native species may be more complex than previously thought. They exposed laboratory-reared metamorphs of *L. catesbeianus* to one Bd strain isolated from western toads and another strain isolated from *L. catesbeianus* to examine whether metamorphs were differentially susceptible. Bullfrogs were susceptible to the strain isolated from the western toads and not the other. In both experiments, infection load detected in the skin decreased over time, suggesting that bullfrog metamorphs from some populations may be inefficient long-term carriers of Bd. *Lithobates catesbeianus* also appears to be a carrier of a novel species of *Chlamydiales* bacteria, *Candidatus Amphibiichlamydia ranarum*, which causes diseases in amphibians and possibly harbors emerging amphibian pathogens such as ranavirosis (Fard et al., 2011; Martel et al., 2013).

The community level effects of nonnative species.—As concern grows regarding the effects nonnative species can have upon native species, there is an increasing emphasis to test whether their impacts upon amphibians reverberate throughout communities. Blaustein et al. (2011) argued the importance of researching amphibian declines at the community level and investigating synergistic effects of multiple stressors instead of focusing on single factors. In recent studies, Richter-Boix et al. (2013) compared invaded and non-invaded amphibian populations on the Iberian Peninsula to test whether the introduction of Painted Frog (*Discoglossus pictus*) altered communities. They found that the presence of *D. pictus* compromised species co-occurrence patterns at the regional scale. In non-invaded areas, the community was statistically structured, but in the invaded areas, community structure appeared to be random. Li et al. (2011) looked at frogs on island ponds off the coast of China and found that the higher the density of nonnative American Bullfrogs (*L. catesbeianus*) the lower the richness and density of native frog species. These negative impacts on the native frog community were proportional to the density of American Bullfrogs.

On Pacific islands, the Coqui Frog (*Eleutherodactylus coqui*) has demonstrated negative effects at the community level since its introduction in the late 1980s. This frog reaches high densities, feeds heavily on invertebrates, and appears to cause community-level changes by altering invertebrate communities (Choi and Beard, 2012). Coqui frogs were associated with a decrease in flying or foliage invertebrate communities, reduced the total number of leaf-litter invertebrates by 27%, and increased dipterans by 19%. These results demonstrate a significant effect on the macroinvertebrate community at the landscape level.

The African Clawed Frog (*Xenopus laevis*) has compromised communities as well. Lillo et al. (2011) looked at the impacts of African Clawed Frogs on native amphibian populations in Italy. Three species of the native frog community were extirpated quickly after the establishment of African Clawed Frogs. Only populations of *Bufo bufo* did not seem to be impacted by *X. laevis*. Lobos and Measey (2002) demonstrated that nonnative African Clawed Frogs in Chile prey heavily on zooplankton and aquatic invertebrates, implying that African Clawed Frogs can have widespread negative effects on aquatic systems.

Introduced fishes are also known to compromise aquatic systems. Hamer and Parris (2013) found that nonnative fishes negatively affect larval amphibian communities in urban wetlands. The authors suggest that draining wetlands throughout the year and preserving or restoring natural ephemeral wetland habitat is necessary to conserve amphibian assemblages in urban areas. Others have found that the diversity of pond breeding assemblages—that includes amphibians—increases after nonnative predatory fishes are removed from pond habitats (Knapp et al., 2007; Walston and Mullin, 2007).

Aquatic amphibian communities can also be affected simultaneously by multiple nonnative species. Preston et al. (2012) showed the ways in which multiple invaders can directly and indirectly affect such communities. Across 139 wetlands, they determined that introduced fishes and bullfrogs (*L. catesbeianus*) decrease the probability of occupancy of native Pacific Tree Frogs (*Pseudacris regilla*) using occupancy models and experimental venues. In the mesocosm experiments they found that introduced fish reduce zooplankton and palatable amphibian larvae, which increased nutrient load and phytoplankton. Because unpalatable bullfrog larvae had little competition, they were able to grow rapidly. Bullfrog larvae did not reduce the survival of native amphibian larvae, but did reduce native larvae growth rates. Apparently, the combined effects of these introduced species interact additively.

Nonnative species can also negatively affect native amphibians by altering host-parasite interactions. Orlofske et al. (2012) studied whether alternative hosts and predators of parasites effect trematode (*Ribeiroia ondatrae*) infection in a native frog (*Pseudacris regilla*). They found that native amphibians could remove up to 93% of infectious stages and thereby reduce the infection rate in *P. regilla* by half, but that introduced predatory fishes did not reduce transmission. Though other amphibians in this system had similar infection intensities as *P. regilla*, introduced fishes had much lower infection intensities. These results suggest that native amphibians can serve as alternative hosts, while nonnative species do not. Similarly, native Australian Green Tree Frog (*Litoria caerulea*) were shown to be unaffected by an introduced parasite, the lungworm *Rhabdias pseudosphaerocephala*, which arrived with introduced cane toads (*B. marinus*; Pizzatto and Shine, 2011). *Litoria caerulea* can harbor very high numbers of the parasite, but host-switching occurs between *L. caerulea* and an allied frog species, *Litoria splendida*. When infected, *L. splendida* survivorship is significantly reduced, yet the fitness of *L. caerulea* is not affected by the parasite.

Nonnative species may also affect communities by dampening diversity. Cruz and Rebelo (2005) studied the effect of nonnative crayfish on amphibian species native to the Iberian Peninsula. Using mesocosm experiments they examined the survival rates of embryos and larvae from 13 native amphibian species when exposed to *Procambarus clarkii*. Only survivorship of *Bufo bufo* was not reduced by crayfish. As a follow up study, Cruz et al. (2008) also used field surveys to demonstrate the same phenomenon. Most native amphibian populations were greatly reduced or extinct within eight years of the crayfish introduction. Crayfish invasions pose similar threats in areas of the Colorado River Basin. In conjunction with nonnative fishes, Martinez (2012) suggests that nonnative crayfish (*Orconectes virils*) invasions may have been overlooked and severely underestimated in the Upper Colorado River Basin, and as a result create a most challenging recovery and preservation of the native community.

Anthropogenic disturbance as a dimension of invasions.—Current studies of nonnative species and amphibians include how future disturbances and anthropogenic induced climate change may further negatively affect native amphibians and convolute nonnative–native amphibian interactions. Much of this literature links modified habitat to the loss of amphibian species or shows an overall negative trend in amphibian species abundances and distributions due to anthropogenic disturbance (Trombulak and Frissell, 2000; Ficetola and De Bernardi, 2004; Ernst et al., 2006; McKinney, 2008). Specifically, Herwig et al. (2013) showed that human-modified landscapes associated with the installation of drainage networks, wetland consolidation, and agricultural networks alter hydroperiod, which negatively affect amphibian assemblages, and depress amphibian breeding success in Minnesota (USA) by increasing distributions of native and nonnative fishes. In other studies, maintaining viable populations of salamanders (*Ambystoma tigrinum*) was dependent upon spatial connectivity of wetland habitat (Cosentino et al., 2011), and the preservation of waterfalls in other systems has allowed native amphibians to coexist with nonnative predatory trout species (Karssing et al., 2012). Ficetola et al. (2011) also found that land use affects distribution of *Salamandra salamandra*, which was related to landscape, hydrological, and water characteristics. Heavy sedimentation and siltation of streams from human-modified lands has affected populations of the critically endangered Chile Mountains False Toad (*Telmatobufo venustus*) and congeners, and *ex-situ* assurance colonies have been implemented to mitigate the effects of modified and lost habitat (Fenolio et al., 2011). Other studies show that human-modified tropical forests negatively affect anuran community composition and diversity (Faruk et al., 2013), and human intervention in freshwater ecosystems has been shown to fuel disease emergence (Peeler and Feist, 2011). Furthermore, Hof et al. (2011) have shown how land modification in conjunction with pathogens and climate change will negatively affect amphibian diversity. A recent experimental study has also documented negative synergistic effects between commonly applied insecticides and nonnative species. Kerby and Sih (unpubl. data) show that a carbamate compound, carbaryl, and nonnative aquatic predators can interact to greatly reduce survivorship of a federal species of concern, the Foothill Yellow Legged Frog (*Rana boylii*).

Human-modified landscapes can not only harm amphibian populations directly, but such altered environments are often ground zero for introduced species. Riley et al. (2005) noted that stream habitats that had considerable urbanization nearby also had higher water flows than nearby streams with less urbanization. The increased water flow allowed nonnative crayfishes and fishes to persist versus less urbanized streams where they appeared to have difficulty surviving low summer water flow. Subsequently, areas that contained nonnative species contained fewer native amphibians.

Although there are many ways in which nonnative introductions occur (e.g., Christy et al., 2007), Johnson et al. (2008) have suggested that human-altered bodies of water spur the establishment and proliferation of nonnative species. As they inhabit increasing numbers of water bodies, a stepping stone effect takes place whereby nonnative species gradually radiate from one habitat to a nearby habitat. Dams have been shown to promote this phenomenon (Davies et al., 2013) and commercial livestock facilities also seem to assist nonnative species dispersal (Gonzalez-Bernal et al., 2012). Similarly, where nonnative species previously could not survive the summer drought, cattle ponds have replaced seasonal marshes in Arizona (Maret et al., 2006), inadvertently enabling the survivorship of nonnative predators. Again, the persistence of nonnative species in this system has been harmful to local amphibians. Griffis-Kyle et al. (2011) observed that earthen livestock-watering tanks influence breeding populations of native amphibians in Chihuahuan Desert grasslands in New Mexico (USA) by significantly increasing their breeding range. Although tanks could expand breeding opportunities, the authors speculate that artificial bodies of water in this landscape could serve as sinks for populations and potentially lead to a loss of genetic diversity by eroding local adaptation. To understand the extent that man-made modifications have on the spread of nonnative species, Ficetola et al. (2010) used land-use change models to test their efficacy to predict the spread of the nonnative American Bullfrog (*R. catesbeianus*) from the 1950s to present day in northern Italy. Models that took into account changes in human land-use patterns accurately predicted the spread of the nonnative frog versus models that assumed constant land-use patterns.

In general, climate change is predicted to globally disrupt species distributions and abundances (Bellard et al., 2013). Climate change models indicate that nonnative species will gain greater advantages as a result of warmer water temperatures, shorter winters, less winter hypoxia, and for the most part, improved conditions to facilitate their spread (Brook et al., 2008; Hellman et al., 2008; Rahel and Olden, 2008). Loyola et al. (2012) estimated whether climate change could drive the invasion of American Bullfrogs (*L. catesbeianus*) into established reserves in the Atlantic Forest Biodiversity Hotspot. Employing distribution and climate models, they found that the American Bullfrogs will likely invade reserves as a result of climate change. These results obviously raise concerns, since the goal of establishing preserves is to protect species, diversity, and native habitat. Should climate change improve conditions for nonnative species to spread and become established, then preserves may be jeopardized.

How climate change will affect amphibians and invading species at the organismal level, as well as ecological and evolutionary processes (see Shine, 2012) may become an even greater concern in the future (Lawler et al., 2009). New invasion prevention and control strategies will need to be considered to protect and preserve native amphibian populations from further decline (Beachy et al., 2011; Snow and Witmer, 2011; Cabrera-Guzmán et al., 2013). Models may need to incorporate interspecific dispersal patterns and species interactions to more accurately determine species responses to climate change (Urban et al., 2013). It has also been suggested that fitting models with data from a species pooled range and not just mean dispersal range will assist in better predicting the extent of invasions and biotic responses to climate change (Broennimann and Guisan, 2008; Urban et al., 2013).

Amphibian–nonnative coexistence and ecosystem restoration.—More than two decades of studies have documented amphibian population declines, and many more studies are now attempting to understand the relationship between nonnative species and amphibian population status and conservation. The questions being asked are: Can nonnative species be removed and habitat restored to pre-invasion conditions? Will amphibian population numbers improve when nonnative species are removed? Are there situations where native amphibians can persist with nonnative predators? Rice et al. (2011) showed that an introduced frog species, *Osteopilus septentrionalis*, that had invaded natural protected areas in the Everglades, Florida (USA) and preys on larval and adult species of native tree frogs (*Hyla cinerea* and *Hyla squirella*), could have significant population level effects on native amphibians. After a 12-month effort to capture and remove *O. septentrionalis*, estimated abundances of native tree frog species increased. Within a similarly short time frame, Cascade Frog (*Rana cascadae*) densities, survival, and recruitment increased in the Klamath Mountains of California (USA) when introduced fishes were experimentally removed from lakes (Pope, 2008). Predatory introduced trout from mountain lakes have compromised amphibian assemblages in other systems too, but when removed, native frogs and salamanders were able to recover (Hoffman et al., 2004; Vredenburg, 2004; Knapp et al., 2007). Other studies have shown that when nonnative predatory mink are removed from islands, native amphibian populations begin to increase (Ahola et al., 2006; Salo et al., 2010). Velo-Anton and Cordero-Rivera (2011) documented an island population of nonnative mammals preying upon a native salamander (*Salamandra salamandra*). They suggest that eradication could positively affect the salamander population, but may be extremely difficult given the topography and dense vegetation of the island.

Armadillos (*Dasypus novemcinctus*), which have recently expanded their range in North America, pose similar threats to native salamanders (*Plethodon angusticlavius*; Crane et al., 2011), as do introduced feral swine that have invaded southern parts of the United States (Hartley et al., 2012). Research conducted by Crane et al. (2011) indicates that salamanders are able to detect armadillo chemical cues and that such cues cause *P. angusticlavius* to exhibit antipredator behavior. Removal of these mammals could also benefit native ecosystems, but will be extremely challenging given the lack of predators, high fecundity, and survival of these species.

In many systems, total removal of an alien species may not be possible, and Hartel et al. (2007) suggest that some amphibian populations might persist with nonnative predators where habitat complexity exists. Nájera-Hillman et al. (2009) and Salo et al. (2010) found that native frog species were able to persist with nonnative predatory mammals in sites with complex habitat. Habitats that had cover in the form of boulders and vegetation (shrubs and grasses) allowed native amphibians to persist better than habitats with less cover. Other studies have concluded that complexity provides benefits for urban herpetofauna (Banville and Bateman, 2012). Schank et al. (2011) also found that complexity of habitat positively affected coexistence of native frogs (*Lithobates sylvaticus)* in lakes with stocked trout. The presence of native fish, lake trophic status, and the length of larval period of *L. sylvaticus* also seem to promote amphibian persistence in this system.

Seasonal water conditions can also encourage coexistence between natives and introduced species. Pagnucco et al. (2011) hypothesized that the observed coexistence of introduced lake chub (*Couesius plumbeus*) and native salamanders (*A. macrodactylum)* in Linnet Lake, Alberta (Canada) may be due to winter hypoxic conditions that lead to major lake chub die offs, relaxing predation pressure on salamanders during breeding. Abiotic factors can also aid the persistence of the federally endangered California Tiger Salamander (*Ambystoma californiense*). Because of ongoing hybridization between an introduced congeneric, native populations of *A. tigrinum* have been genetically compromised. Johnson et al. (2013) demonstrate that native genotypes are better able to survive in rapidly drying mesocosms relative to other genotypes. They suggest that management of aquatic habitat could minimize the spread of nonnative genotypes.

Kats et al. (2013) report that above average rainfall combined with the trapping of nonnative crayfishes during dry years promotes coexistence between native amphibians and invaders. Above average rainfall results in flooding events, and in streams with steep gradients, nonnative crayfishes that are more adapted to swamp habitats are washed away, allowing native amphibians bursts of successful breeding and recruitment. In years where rainfall is below average, traps are set to bring down crayfish populations and give native amphibians an opportunity to breed. Similarly, Luja and Rodríguez-Estrella (2010b) found that tropical cyclones produce heavy water flow that periodically washed out nonnative American Bullfrogs. Endemic Baja California Tree Frogs (*Pseudacris hypochondriaca curta*) have evolved with cyclonic conditions and their populations are not impacted in the same way as bullfrogs. These periodic tropical storms facilitate the coexistence of the native tree frogs and the nonnative bullfrogs. Walls et al. (2013) modeled the effect that extreme variation in precipitation can have on amphibians, and stressed the importance of considering how precipitation patterns, and not just total amounts of rainfall, will affect amphibians and ecological interactions with native and introduced species.

Coexistence between nonnative and native species may also be possible when native species are capable of regulating the abundances of a nonnative species. Competition by native Green Tree Frogs (*Litoria caerulea)* significantly reduced the growth rate of nonnative Cane Toads (*Bufo marinus)* and increased their larval period in natural Australian waterbodies (Cabrera-Guzmán et al., 2013), as well as significantly decreased the body length and mass of Cane Toad metamorphs. There was no significant negative effect on tree frog tadpoles from Cane Toad tadpoles. However, there was strong intraspecific competition between tree frogs, which led to an increased larval period and reduced survival, growth rate, or size at metamorphosis. The authors suggest that native tree frogs could be used as part of an integrated regime to control nonnative species and make coexistence possible, but at what cost to the native species remains unclear. Follow up studies in the same system indicate that *B. marinus* could also be controlled, and possibly eradicated, by using intraspecific chemical stimuli (Crossland et al., 2012).

Currently, the number of documented restoration projects that have eradicated nonnative species without having negative consequences to the native ecosystem remains low (however, see Hobbs and Cramer, 2008 for an overview). Nonnative removal experiments have demonstrated that the negative effects of nonnative species throughout communities can be reversed if nonnative species are removed (Hartman and McCarthy, 2004; Vredenburg, 2004; Gratton and Denno, 2006; Knapp et al., 2007; Bay and Sher, 2008; Marchante et al., 2009). Yet, there are few, if any, examples where nonnative species are successfully removed and amphibian populations return to pre-invasion population numbers, or where native amphibians can successfully coexist with nonnative predators (Kats and Ferrer, 2003). As has been detailed, the complexity of invasions has diverse impacts that resonate throughout the entire community. As a result, the task of conserving and preserving habitat to prevent the introduction and proliferation of nonnative species has been overwhelming.

A major question with regard to invasions is whether nonnative species are eliciting behavioral responses in native species that may affect communities, and as a result reshape biological, environmental, and ecological processes within ecosystems. Native species already show shifted behavioral responses in the presence of nonnative species, and as a result demonstrate remarkably different feeding ecology, breeding habits, communication systems, or use of resources. As we work to understand how amphibians are responding to nonnative species, it seems opportune to identify their responses to the removal or reduction of nonnative species. Furthermore, if a nonnative species has led to the displacement of native species and altered their ecology, we should perhaps begin to wonder how reintroduced native species will respond to one another, and to question whether the effect of nonnative species has been so extreme as to have reduced native species ability to coexist as they once did prior to the establishment and proliferation of an introduced species.

Given the multitude of challenges facing amphibian populations, ongoing projects aimed at restoring native populations of frogs and salmonids seem paradoxical. In the Santa Monica Mountains (Los Angeles, CA, USA), native amphibians have experienced population declines associated with the individual and synergistic effects of nonnative crayfish (*Procambarus clarkii*), predatory fish (*Gambusia affinis*), and siltation of streams due to land modification and altered fire regimes, which has resulted in remarkably less deep pools in many streams (Bucciarelli and Kats, unpubl. data). Consequently, suitable habitat for the reintroduction of the threatened Red-Legged Frog (*Rana boylii*) is limited to sites that are occupied by native amphibians such as the California Newt (*Taricha torosa*), a Species of Special Concern in the southern part of its range (Jennings and Hayes, 1994). Translocating egg masses of *R. boylii* to sites where *T. torosa* breed may be problematic for subsequent generations given that *T. torosa* prey on *R. boylii*. Coexistence of *R. boylii* and *T. torosa* occurs in more northerly localities where greater population numbers of *R. boylii* mitigate predation pressure by *Taricha*. However, in the Santa Monica Mountains, populations of *R. boylii* are nearly nonexistent and thus, predation by *Taricha* may overwhelm populations of *R. boylii*. In a system attenuated by predatory nonnative species, as well as abiotic stressors, how will the reintroduction of this native ranid into limited and sympatric habitat occupied by a native predatory amphibian pan out?

The challenges facing conservation and restoration programs is not limited to native amphibian populations, and programs focused on the restoration of other native taxa may further threaten amphibian populations. Also in the Santa Monica Mountains, restoring native trout habitat to facilitate their return to local streams is an even more complex restoration project. As mentioned, amphibian populations in the Santa Monica Mountains have declined over the last ten years. As a result, it is questionable if amphibian populations can persist in the presence of reintroduced native fish, while simultaneously experiencing continued habitat loss, increased fire frequency, drought, and land modification. Essentially, the ability of native species to coexist has been greatly compromised as a result of the direct and indirect effects of nonnative species. Effectively restoring the ecosystem by reintroducing native species is thus intricately entwined in the role and history of nonnative species in the system. Even after their removal, the long-term effects of invasions throughout ecosystems will likely linger. That is not to say the work should not occur, but instead to realize that ecosystems will likely be haunted by the ghosts of nonnative species and in effect, play a major part in recovery and restoration projects, possibly making it more complex and longer than expected, or impractical due to the interaction of multiple stressors that cannot be mitigated and limited logistical resources. Undoubtedly, similar nonnative–native restoration scenarios lie on the horizon and exploring the complexity of not only invasions, but restorations too, will surely provide many exciting projects.

Conclusion.—The negative effects that invasions may generate throughout ecosystems make it difficult to imagine a positive role for nonnative species in ecosystems. However, Schlaepfer et al. (2011) have speculated that nonnative species may contribute to conservation objectives. Where the effects of climate change and habitat modification are depleting native species abundances and biodiversity, they suggest nonnative species may persist, provide some beneficial ecosystem services, and may ultimately evolve into new, endemic taxa. The authors also propose that nonnative species may provide resources (food or habitat) for rare native species, functionally replace extinct taxa, and perform beneficial ecosystem functions. In contrast though, it seems that numerous native species and overall biodiversity have been compromised as a result of nonnative species (Simberloff et al., 2013), and that nonnative species will likely impair more than aid conservation efforts. As a result, when eradication is not opted for, land managers will need to revise conservation and restoration practices (Hobbs et al., 2009).

Given the relatively short period of time since widespread biological invasions have occurred in communities where amphibians reside, the long-term consequences are unclear (Crooks, 2011). It is apparent that not all nonnative species will have an immediate negative impact upon native species. However, long-term studies are necessary to assess the true effects of nonnative species on native species (Strayer et al., 2006). Time lags may obscure initially observed neutral or positive immediate effects of nonnative species (Crooks, 2011). Some nonnative species may take a much greater amount of time from the period of introduction to a point of integration within the native ecosystem before they produce a negative effect (Crooks and Soulé, 1999). Maintaining nonnative species at low densities could dampen, mitigate, or prevent the immediate negative effects of nonnative species and preclude potentially much greater negative effects associated with time lags.

Understanding and predicting when the population of a nonnative species will increase and cause major changes in an ecosystem is a critical component of conservation biology. Metrics used to ascertain the effects of nonnative species must be augmented, and be capable of providing quantitative evidence of the ways in which native communities are impacted by nonnative species (Vilà et al., 2009; Simberloff et al., 2013). Methods used to elucidate the effects of nonnative species should be able to measure both their direct and indirect effects upon an ecosystem. Developing some metric to quantify how integrated a nonnative species is within a native community may be a valuable gauge for scientists, land managers, and policy makers. Understanding how native species, communities, and ecosystem functioning are affected by nonnative species and sharing pertinent data with policy makers, land managers, and the public is crucial to achieving conservation goals.

As globalization continues, massive trade and travel will likely accelerate the frequency of introduced species to nonnative habitats (Vitule et al., 2012), and it appears that less developed countries with rapidly growing economies will be more prone to nonnative species introductions (Lin et al., 2007; Vitule et al., 2012). In the coming decades, invasion biologists may want to quantify how native communities throughout less developed countries respond to nonnative species and compare those initial responses to previously invaded communities throughout more developed countries. Doing so may provide a valuable framework for building predictions about biological invasions. Furthermore, results gleaned from nonnative founder populations may offer scientists and land managers insight into the ways ecosystem impacts develop. Researchers could work to determine how nonnative species affect nutrient regimes, test whether structural change due to nonnative species affects ecosystem processes, measure the strength of selection upon native species, or assess population growth of a nonnative species and the direct effect of such an expansion upon the ecology of native species.

Though most introductions of nonnative species do not have an impact at the level of the ecosystem (Simberloff, 2011), it is clear that interactions of nonnative and native species in many systems need to be further studied. It is difficult to estimate how ecosystems and native species have been, are, or will be, affected by the introduction, establishment, and integration of nonnative species as exemplified by the complex interactions discussed in this review. The rate of amphibian population declines coupled with the increased rate of introduced nonnative species into communities with amphibians should catalyze research efforts to reveal the role of nonnative species in community dynamics.

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CHAPTER 2

Quantifying tetrodotoxin levels in the California newt using a non-destructive sampling method

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Quantifying tetrodotoxin levels in the California newt using a non-destructive sampling method

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ABSTRACT

Toxic or noxious substances often serve as a means of chemical defense for numerous taxa. However, such compounds may also facilitate ecological or evolutionary processes. The neurotoxin, tetrodotoxin (TTX), which is found in newts of the genus *Taricha*, acts as a selection pressure upon predatory garter snakes, is a chemical cue to conspecific larvae, which elicits antipredator behavior, and may also affect macroinvertebrate foraging behavior. To understand selection patterns and how potential variation might affect ecological and evolutionary processes, it is necessary to quantify TTX levels within individuals and populations. To do so has often required that animals be destructively sampled or removed from breeding habitats and brought into the laboratory. Here we demonstrate a non-destructive method of sampling adult *Taricha* that obviates the need to capture and collect individuals. We also show that embryos from oviposited California newt (*Taricha torosa*) egg masses can be individually sampled and TTX quantified from embryos. We employed three different extraction techniques to isolate TTX. Using a custom fabricated high performance liquid chromatography (HPLC) system we quantified recovery of TTX. We found that a newly developed micro-extraction technique significantly improved recovery compared to previously used methods. Results also indicate our improvements to the HPLC method have high repeatability and increased sensitivity, with a detection limit of 48 pg (0.15 pmol) TTX. The quantified amounts of TTX in adult newts suggest fine geographic variation in toxin levels between sampling localities isolated by as little as 3 km.

1. Introduction

An impressive diversity of taxa possess unique toxic or noxious substances (Yokoo, 1950; Noguchi and Hashimoto, 1973; Daly et al., 1987; Noguchi et al., 1982, 1986; Hwang et al., 1989; Berenbaum, 1995; Daly, 1995; Kubanek et al., 1995; Cimino and Ghiselin, 1998; Hartmann and Ober, 2000; Dumbacher et al., 1992, 2000; Fahey and Garson, 2002; Wood et al., 2012; Savitzky et al., 2012). Often these compounds serve as a means of chemical defense. However, they may also facilitate ecological and evolutionary processes (Elliott et al., 1993; Zimmer et al., 2006; Brodie and Brodie, 1990; Bucciarelli and Kats, in review). Prerequisite to determining how these compounds affect such processes, is the need to quantify their abundance within individuals and populations. Doing so provides the necessary foundation to understand their potential effect upon ecosystems at varied levels and time scales.

Newts in the family Salamandridae possess an extremely powerful neurotoxin, tetrodotoxin (TTX). Amounts of TTX found in newts have been quantified over

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the last 80 years to understand its occurrence and distribution across taxa (Twitty and Johnson, 1934, Mosher et al., 1964; Wakely et al., 1966; Shimizu and Kobayashi, 1983). Recently, broad geographical sampling and population estimates of TTX levels in newts of the genus *Taricha* have revealed its role as an agent of selection upon predatory garter snakes (Brodie et al., 2005; Hanifin et al., 2008). In other systems, larvae of the California newt, *Taricha torosa*, detect TTX, which elicits an antipredatory response. Larvae utilize TTX as a chemical cue to avoid cannibalistic adult *T. torosa* that will prey upon them when resources are scarce (Elliott et al., 1993; Zimmer et al., 2006). TTX from *T. torosa* may also affect macroinvertebrate foraging behavior (Bucciarelli and Kats, in review).

In order to quantify amounts of TTX in *Taricha*, whole newts have often been collected and sacrificed, or captured and brought into captivity to be sampled. Though populations of *Taricha* in central and northern California have historically been rather large, with upwards of 5000 newts found in breeding localities (Coates et al., 1970), not all populations are as substantial. Populations of *T. torosa* in southern California experience drastically different environmental selective pressures compared to congeners in northern locations, which has likely contributed to the decrease in breeding adults and low recruitment observed in their southern range (Jennings and Hayes, 1994). *T. torosa* found in the southern coastal areas of California (Santa Monica Mountains, Los Angeles, CA) breed in streams that are often ephemeral, in a heavily modified landscape that is arid, mountainous, and warmer than other parts of its range. Long term monitoring of amphibian populations across this landscape shows a negative trend in *T. torosa* populations. Currently, *T. torosa* is listed at the state level as a species of special concern (Jennings and Hayes, 1994; Thomson et al. in review).

In order to minimally disturb and help preserve *T. torosa* populations, we speculated that a smaller tissue sample than previously used to quantify TTX in *Taricha* (e. g. Hanifin et al., 2002) could be collected from adult newts in the wild. This would obviate the need to 1) collect animals, 2) disrupt breeding, 3) sacrifice animals, and 4) inflict a large wound. Few methods designed to sample TTX from animals have used non-lethal protocols (Khor et al., 2013; Hanifin et al., 2002), however these methods still required animals to be removed from natural habitat. As previous studies have documented low to non-existent amounts of TTX in *Taricha* populations (Hanifin et al., 1999), we improved upon an existing high performance liquid chromatography (HPLC) method to increase sensitivity and detect lesser amounts of TTX, should *T. torosa* in southern California have extremely low levels of TTX. Finally, we sought to detect and quantify TTX from wild embryos collected from egg masses oviposited late in the breeding season.

2. Methods

2.1. Materials

TTX solutions were prepared using commercial TTX (citrate salt, Fisher Scientific) and stored at 2–4 °C. All other reagents were ACS reagent grade or better (Fisher Scientific). Water was purified to >16.5 MΩ-cm and filtered through a 0.22 μm nylon membrane filter (Barnstead Nanopure II).

2.2. Instrumentation

2.2.1. High performance liquid chromatography (HPLC) system

A high performance liquid chromatography system coupled with fluorescence detection (HPLC-FLD) was adapted from a previous design (Yasumoto and Michishita, 1985). The chromatography system consisted of a Thermo Separation Products pump (P4000), a manual injector with a 50 μL loop (Rheodyne 7125), and a fluorescence detector (Waters model 474) equipped with a 16 μL flow cell. Fluorescence was observed at 505 nm with 381 nm excitation. The analytical column was a Sphericlone ODS 5 μm d_p 4.6 × 150 mm (Phenomenex, Torrance, CA, USA) protected with a SecurityGuard™ C18 guard cartridge (Phenomenex). The mobile phase consisted of 2 mM heptanesulfonic acid in 50 mM phosphate buffer, pH 7. The optimal flow rate was 0.35 mL/min. All components were connected via PEEK tubing and fittings.

2.2.2. Post-column derivatization

A second HPLC pump, identical to the analytical pump, supplied 4 M NaOH at 0.35 mL/min to facilitate post-column derivatization. We utilized a post-column reactor (Analytical Scientific Instruments, model 310) equipped with a 500 μL reactor cartridge thermostatted to 120 ± 0.5 °C. Eluant from the analytical column was mixed with 4 M NaOH at a PEEK mixing-Tee then passed through the post-column reactor (PCR). The analytical mobile phase and NaOH solution were continuously helium-degassed during analysis.

To provide sufficient backpressure to inhibit cavitation and cool the mixture prior to entering the detector, the eluant was passed through a 2 m coil of PEEK tubing (0.13 mm i.d.), which provided about 20 bar backpressure and acted as a heat exchanger to cool the eluant. A 75 psi (5 bar) backpressure regulator was placed on the detector waste outlet to further inhibit cavitation in the flow cell.

2.2.3. High performance liquid chromatography optimization

To test the optimization of this system, we ran TTX standards ranging from 5 to 200 ng/mL at temperatures from 100° to 140 °C. Furthermore, we tested the repeatability of our system by completing serial injections of a standard. At least 20 injections per standard were performed. An ANOVA was used to test injection to injection repeatability.

2.3. Animal sampling and intra-individual variation

Adult male California newts (*T. torosa*) were collected by hand or dip net from watersheds throughout the Santa Monica Mountains (Los Angeles, CA, USA). We improved a non-lethal sampling technique adapted from Hanifin et al. (1999) whereby a 2 mm skin biopsy tool (Acu-Punch, Acuderm Inc. Fort Lauderdale, FL) is used to collect tissue from wild caught individuals. This method requires approximately 84% less tissue from animals.

Given the reduced tissue sample size, we tested variation within an individual by collecting three dorsal skin samples from adult male newts during the breeding season. Prior to collecting tissue, newts were anesthetized in a 300 ppm methanesulfonic acid (MS222) solution. Dorsal skin is known to have a uniform distribution of toxin glands (Hanifin et al., 2004), but collecting tissue too close to the vertebrae risks harming animals. Therefore, the sample was collected 1 cm away from the vertebrae, near the posterior dorsolateral area. Tissue was collected from the same area of an adult for each newt, followed by two subsequent samples collected randomly in the same proximity on either side of the newt. Sampling tools were sterilized between individuals. All samples were immediately placed in 300 μL of 0.1 M aqueous acetic acid and stored on dry ice before being transported to the laboratory. Anesthesia usually wore off within 5 min and newts were returned to their original habitat.

Embryos were collected from late-season oviposited egg masses found in local watersheds of the Santa Monica Mountains. Only egg masses that were detached from vegetation and found in shallow, drying pools were sampled. In the field, a scalpel was used to dissect an egg within the mass. The embryo was collected, rinsed with water, placed in 0.1 M aqueous acetic acid, and stored on dry ice. Entire egg masses were not compromised during collection, and the remaining egg mass was returned to the pool from where it was collected. A mixed model with a random intercept was used to test intra-individual variation of TTX levels between physical sampling locations on an individual newt.

2.4. *Toxin extraction*

2.4.1. *Original method*

To extract TTX from newt skin and embryo samples, we followed the method outlined by Hanifin et al. (1999). Briefly, an entire skin sample or embryo was weighed to the nearest 0.1 mg and macerated with the 300 μL of 0.1 M acetic acid in a glass tissue grinder. Suspensions were heated in a boiling water bath for 5 min then cooled in an ice bath for 5 min. Samples were sedimented and separated at 13,000 × *g* for 20 min. Supernatant was then collected and placed in centrifugal filters (Amicon Ultra, 10,000 MWCO) and centrifuged for 20 min. The remaining filtrate was diluted to 1 mL with 0.1 M acetic acid.

2.4.2. *Modified method*

Following the published method described in *2.4.1*, we performed a simple modification to the procedure. Prior to the final dilution to 1 mL, the supernatant was transferred to the centrifugal filter a second time and centrifuged for 20 min at 13,000 × *g* to determine if a second pass over the pellet bed in the centrifugal filter would wash remaining TTX from any solids.

2.4.3. *Micro-extraction method*

We speculated that polar and ionic surfaces on proteins from newt tissue could be binding TTX through the centrifuging process and as a result reduce amounts of TTX recovered. To test this, we developed an experimental procedure, optimized for the 2 mm skin sample, whereby extractions were macerated, boiled, and cooled as described in *2.4.1*. Samples were suspended in centrifugal filters and sedimented once for 20 min. Any solids remaining in the centrifugal filter were then washed by adding 100 μL of 0.1 M acetic acid to each filter and centrifuging for 20 min. Samples were diluted to 1 mL with 0.1 M acetic acid. For each extraction method used, extracts were immediately analyzed by HPLC or stored at −80 °C for later analysis.

2.5. *Recovery studies of TTX from newt skin samples and embryos*

To evaluate the recovery of the toxin from samples by the different extraction methods, a sample was macerated in a glass tissue grinder and separated into equal volumes in two vials. One vial was spiked with standard TTX and 100 μL of 0.1 M acetic acid was added to the other vial as a control. Spiked samples and the corresponding controls were then prepared following one of the extraction methods. Because of the limited number of embryos collected, embryos were prepared following only the modified and micro-extraction methods.

3. Results

3.1. *Temperature optimization and injection to injection repeatability*

Chromatograms in Fig. 1 show the separation of TTX from other epimers in two different adult skin samples. TTX epimers are clearly separated. We tested the sensitivity of the HPLC system by running TTX standards (5–200 ng/mL) at a range of PCR temperatures (100 °C–140 °C at 10° intervals). We observed an increase in sensitivity at 130 °C, but beyond 130 °C a loss of sensitivity was observed. The effect of PCR temperature upon sensitivity is presented in

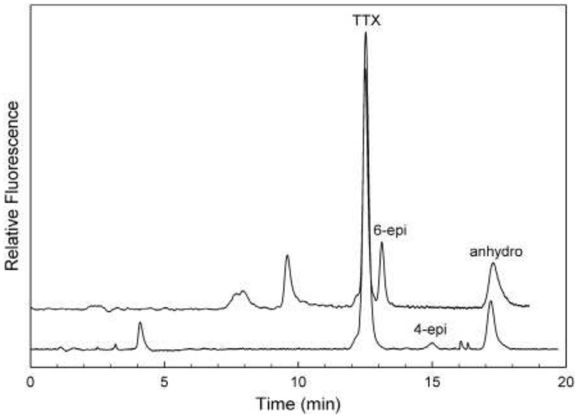

Fig. 1. HPLC-FLD chromatograms from adult male newts show the separation of TTX from other epimers. Observed epimers included 4-epi-, 6-epi-, and anhydro- TTX.

calibration curves in Fig. 2. The linearity of each calibration curve was high ($r^2 > 0.98$).

At a PCR temperature of 100 °C a detection limit of approximately 1 ng (3 pmol) TTX (S/N > 3) was measured, indicating no significant improvement in detection limit over other previous reports utilizing HPLC-FLD (Jen et al., 2008). At 120 °C we measured a detection limit of 48 pg (0.15 pmol) TTX (S/N > 3) and at 130 °C 41 pg (0.13 pmol). Operating the PCR at 120 °C offered the optimal compromise between stability, sensitivity, and detection limit.

Greater than 20 injections per standard, from a range of 5–200 ng TTX per mL, were used to test our HPLC method. Detected amounts of TTX from serial injections of standards was not significantly different (*ANOVA*, $p = 0.94$, $F = 0.059$, $df = 90$).

3.2. Intra-individual variation

Individual amounts of TTX in each of the three skin samples from ten adult male newts were derived using the peak area from HPLC chromatograms. TTX in all three skin samples from an individual are presented in Fig. 3. Results from the analysis showed no significant variation from injection to injection ($p = 0.613$) and no significant difference in variation of TTX levels between pairs of sampling locations on a newt ($p > 0.1$).

3.3. Toxin recovery from skin and embryo samples

Percentages of recovered TTX differed between extraction methods in both adult skin and embryo samples (Fig. 4). These differences were significant for embryo (*t test*, $p = 0.01$, $df = 6$, $t = 3.409$) and adult skin samples (*ANOVA*, $p < 0.00$, $t = 3.4$, $df = 23$). A post hoc analysis using a Tukey HSD test showed that recovered amounts of TTX were significantly different between the micro-extraction method and the original and modified methods ($p < 0.05$), but recovered amounts were not significantly different between the original and modified methods. TTX extracted from embryos using the modified method showed an average recovery of 73.4% ($n = 4$, percentage recovery SD = 22.1%, range = 62.5%–80.9%), whereas TTX extracted from embryos using the micro-extraction method showed considerably greater recovery at an average of 92.9% ($n = 4$, percentage recovery SD = 12.8%, range = 81%–99.4%). Skin samples extracted using the original method recovered on average 74.9% of TTX ($n = 4$, percentage recovery SD = 21.7%, range = 8.4%–90.5%). The modified method recovered on average 60.4% of TTX ($n = 17$, percentage recovery SD = 11.9%, range = 25.3%–96.1%). However, samples prepared with the micro-extraction methods recovered 95.9% of TTX ($n = 5$, percentage recovery SD = 8.9%, range = 84.3%–99.8%).

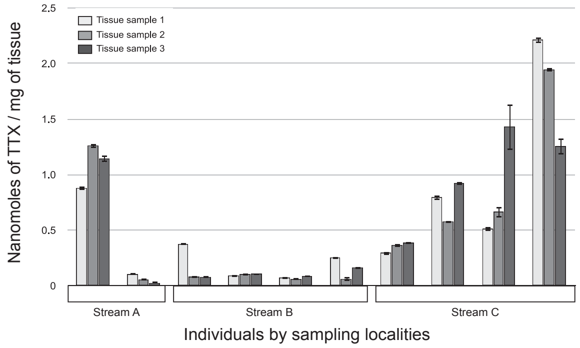

Fig. 3. Intra-individual variation of TTX levels of ten adult newts from three different watersheds. TTX levels between pairs of sampling locations on a newt were not significantly different.

4. Discussion

We modified a non-lethal biopsy method to collect smaller dorsal skin samples from wild-caught newts to quantify amounts of TTX in adult tissue. Because of the current state-level conservation status of *T. torosa*, this method mitigates any negative consequences that previous tissue sampling methods could have upon already greatly reduced populations in the Santa Monica Mountains. Unlike previous methods where newts were collected (e.g. Hanifin et al., 1999, 2002, 2004), this method ensures that

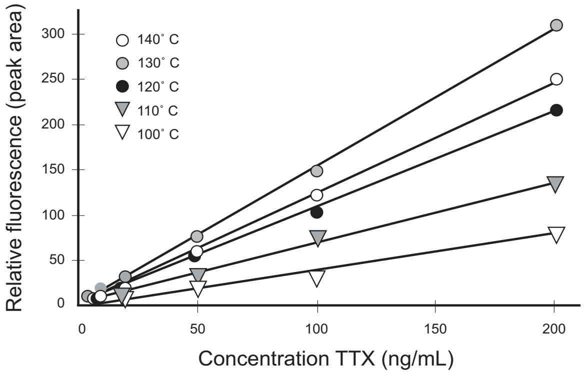

Fig. 2. Calibration curves of increasing concentrations of TTX at five temperatures. Sensitivity increases with increasing post column reactor (PCR) temperature, but decreases beyond 130 °C.

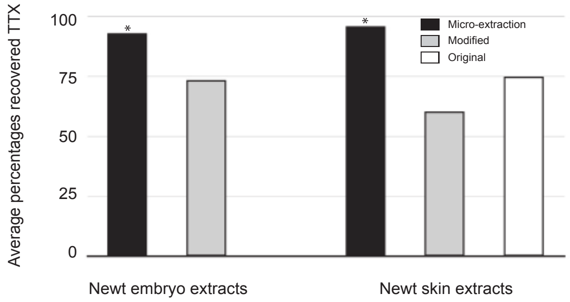

Fig. 4. Average recovered amounts of TTX from newt embryo extracts and newt skin extracts using micro, modified, or original extraction methods. Significantly greater amounts of TTX were recovered for both newt embryo and newt skin extracts using the micro-extraction method. Amounts of TTX recovered between original and modified methods were not significantly different.

live animals are not sacrificed nor removed from breeding localities. No fatalities occurred during the sampling of newts, and in subsequent sampling events, adult newts previously sampled were recaptured and showed signs of nearly complete recovery in as little as 14 days.

The variability of our non-lethal sampling method was tested by taking additional tissue samples from adult males in relatively the same region of the body immediately after an initial sample was collected. Though variation in amounts of TTX detected between sampling locations on the dorsal region of a newt was observed, the overall variation in amounts of TTX within an individual was generally consistently high or consistently low relative to other individuals. It does not appear that sampling causes animals to immediately release more toxin. Furthermore, our data show that skin samples taken along the length of a newt in relatively the same dorsal area can be used to describe amounts of TTX in the skin of adult newts. Given the small tissue size, individuals could be sampled repeatedly through time at fine temporal scales to better understand the chemical ecology of *Taricha*, but how repeated sampling of wild newts at such scales would affect individuals and TTX levels is unclear. Previous studies show that TTX levels increase in captive individuals, yet these individuals were only sampled three times throughout a year (Hanifin et al., 2002). How TTX levels change within populations throughout a regular breeding season remains undetermined.

Toxin was also successfully extracted from embryos and quantified. Because embryos were rinsed, it is not likely that embryonic fluid or egg mass material contributed to the detection of TTX in the embryo samples. Larvae and eggs are known to have TTX, but to our knowledge this is the first experiment to isolate embryos in the wild and extract and quantify amounts of TTX from them. Over the last five breeding seasons, the frequency of observing adults in streams has gradually declined and the breeding window has significantly decreased (Bucciarelli and Kats personal observations), but egg masses can often be found in streams later in the season. Though congeneric TTX levels appear to correlate with female TTX levels (Hanifin et al., 2003), whether TTX levels in embryos correlates with adult male TTX levels remains untested. In this study, only male *T. torosa* were sampled since females are almost always gravid when encountered during the breeding season. It is entirely possible that embryos could provide a snap shot of population level adult TTX levels, and describing and comparing patterns of TTX in embryos and adults could clarify whether TTX in *Taricha* is genetically controlled or the result of endosymbionts (Lehman et al., 2004).

Recovery experiments indicate that the original and modified TTX extraction methods did not recover considerable amounts of TTX, however, we should note the original method was for approximately six times more tissue, and this could affect recovery. The results of our recovery experiments show that washing the centrifugal filter with acetic acid can greatly increase the percentage of TTX recovered, leaving on average less than 10% TTX returned, as opposed to nearly 26%–40% with the other methods. These results suggest that the efficiency of a single extraction is relatively poor and is only marginally improved, if at all, by a second pass of the original extractant across the tissue bed in the centrifugal filter. Performing a second extraction by washing the tissue bed with fresh acetic acid improves the extraction yield. In addition, a small amount of TTX may be retained in the tissue bed in the centrifugal filter when only a single extraction is performed, even when washed with the original extractant, thereby contributing to the lower extraction yield. The micro-extraction method appears to be extremely reliable with improved yield and high precision. Inadequate recovery in earlier studies utilizing the original extraction method could be a partial explanation of previously observed levels of phenotypic mismatch between *Taricha* predator resistance and *Taricha* toxicity. As a result, overall toxicity of some populations may be underestimated, and at evolutionary scales contribute to the degree of mismatch between less toxic newt populations and predator populations with disproportionately greater resistance.

Interestingly, the patterns of TTX variation between watersheds (Fig. 3) suggest that *T. torosa* throughout the Santa Monica Mountains may exhibit fine scale geographic variation in TTX levels. Given the minimal amount of sampling to describe the chemical ecology of *Taricha*, our sampling methods allow researchers to collect data on populations of newts in the wild and across broad or fine scale geographical ranges. Other species known to possess TTX could also be sampled at similar scales using these methods. A relationship between concentrations of TTX in skin samples and total amounts of TTX in newt skin does exist (Hanifin et al., 2004). A correlation between concentrations of TTX in organs of an invertebrate and overall TTX concentrations has also been observed in an invertebrate (Khor et al., 2013). Future studies could quantitate overall amounts of TTX in *T. torosa*, different organs, or blood.

Use of fluorescence spectroscopy to detect TTX has become one the most common methods of analysis (Yasumoto and Michishita, 1985; Hanifin et al., 1999; Asakawa et al., 2000; O'Leary et al., 2004; Mebs et al., 2012; Sup) and though a number of other methods are also in use (Alcaraz et al., 1999; Kawatsu et al., 1999; Ito et al., 2006; Diener et al., 2007; Jen et al., 2008; Huang et al., 2008; Man et al., 2010; McNabb et al., 2010, Fong et al., 2011; Kudo et al., 2012; Cho et al., 2012; Stokes et al., 2012; Suprasert, 2013), each with its distinct advantages, HPLC with fluorescence detection remains popular, fast, and economical because of its simple and flexible sample preparation. Furthermore, our developed HPLC system demonstrates high repeatability, obviating repeated injections of samples. Thus, a small single tissue sample from an individual and a single HPLC injection can reliably be used to characterize TTX, thus conserving specimens, time, and funds.

Various TTX stereoisomers and epimers occur in TTX laden newts, and in some populations of *Taricha*, newts possess the epimer 6-epi TTX. When performing chromatography this epimer can be problematic because its peak is very close to TTX and separation of the two peaks does not always occur. Furthermore, HPLC-FLD systems can be approximately 20 times more sensitive to this epimer (Yasumoto and Michishita, 1985). Therefore, separation of

6-epi TTX from TTX is essential to determining actual amounts of TTX in samples. From the population of newts we sampled, 4-epi-, 6-epi-, and anhydro- TTX were observed and successfully separated from the TTX peak (Fig. 1). Other epimers did not occur in sufficient concentrations to be unequivocally identified.

To quantitate much smaller amounts of TTX and improve detection limits, our system was operated at temperatures above the commonly maintained 100 °C (Yasumoto and Michishita, 1985; O'Leary et al., 2004; Yotsu-Yamashita et al., 2012). Although the system is relatively stable for analysis up to 130 °C, we operated at 120 °C to achieve a higher level of stability. As a result, the mobile phase solutions needed to be continuously sparged with helium during analysis to prevent outgassing and cavitation. This made it necessary to have backpressure regulation before and after the detector to inhibit bubble formation.

Our results broadly contribute to the ongoing efforts to quantitate TTX for human health purposes, but also begin to characterize the chemical ecology of newts. Relative to the current understanding of how newt predator resistance plays out at evolutionary scales, an understanding of *Taricha* and their chemical ecology across landscapes is absent. Our sampling methods and the developed HPLC system allow for such a study. Given the current status of *T. torosa* in southern California, quantifying TTX levels in wild populations may also have long term implications for their conservation. Currently, no TTX-laden organisms is known to produce TTX without endosymbionts, but if the *Taricha* TTX phenotype is uniquely genetically controlled, then determining the landscape genetics of *Taricha*, and *T. torosa* specifically, may be used by land managers to conserve *Taricha* populations, as well as explain patterns of TTX variation at both fine and broad geographical scales.

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Conflict of interest

None declared.

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CHAPTER 3

Effects of newt chemical cues on the distribution and foraging behavior of stream macroinvertebrates

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PRIMARY RESEARCH PAPER

Effects of newt chemical cues on the distribution and foraging behavior of stream macroinvertebrates

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Abstract Many amphibians possess noxious or toxic substances for self defense. These compounds have been characterized largely as chemical defenses, but may promote ecological and evolutionary processes. The California newt, *Taricha torosa*, possesses a potent neurotoxin, tetrodotoxin (TTX), which serves as a chemical defense, chemical cue to conspecifics, and selection pressure that has selected for evolved resistance in a predator. However, the potential effects of TTX upon the broader community and on behavior, in general, have been overlooked. Field assays conducted during the newt breeding season indicate that the macroinvertebrate community responds to adult newt chemical cues by altering foraging behavior. In these assays, significantly fewer macroinvertebrates were found in experimental areas with enclosed newts relative to enclosures with a non-predatory amphibian. Laboratory bioassays showed that dragonfly nymphs (*Anax junius*) reduced predatory behavior and moved less in the presence of adult newt chemical cues. When exposed to TTX, nymph mean angular velocities were reduced four fold and mean velocity magnitude was reduced threefold relative to controls. Overall, these results support the hypothesis that chemical stimuli from predators, and TTX specifically, can shape species interactions at lower trophic levels and potentially affect community organization.

Keywords Chemical cues · Macroinvertebrates · Tetrodotoxin · Newts · *Taricha torosa* · *Anax* · Ephemeroptera · Santa Monica Mountains · Kairomone · Predator–prey

Introduction

Predation can have strong influences on prey populations, community organization, and ecosystem functions (Paine, 1966; Menge, 2000). Predators often affect communities directly via consumption (Sih et al., 1985), but they may also indirectly do so by inducing non-lethal effects (Lima & Dill, 1990) and alternative behavioral characteristics (Werner & Anholt, 1996; Kats & Dill, 1998). Such altered traits may include defensive posturing, initiating an evasive response, feigning death, hiding, and reducing activity (Endler, 1986; Lima & Dill, 1990; Scrimgeour & Culp, 1994; Relyea, 2000; Trussell et al., 2003; Ferrari et al., 2010). Modified traits may further affect the composition and structure of communities via

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changed species interactions (Abrams, 1983), resulting in significantly different feeding ecologies (Skelly, 1992; Wissinger & McGrady, 1993; Schmitz et al., 1997). Ultimately, ecologists have learned that direct and indirect effects of predator and prey interactions can profoundly influence community composition and diversity.

Historically, the effect that predators have upon communities has been assessed by quantifying the rates at which they consume prey. Though these studies have produced valuable concepts, such as trophic cascades (Carpenter et al., 1985), they have asymmetrically represented the predator perspective in predator–prey relationships. How prey respond to predators remains unclear in many systems. Furthermore, the indirect effects that predators may have upon the community remain vague, even though it is apparent that the indirect effects of some predators may exceed or match their direct effects (Trussell et al., 2003). The plasticity of prey phenotypes in response to predators, whether developmental, morphological, physiological, or behavioral, may have consequences upon species interactions at multiple trophic levels (Abrams, 1995; Werner & Peacor, 2003).

Chemical compounds released by predators can influence the behavior of prey species, as well as other organisms within the community (Petranka et al., 1987; Dahl, 1998; Turner et al., 2000; Hay & Kubanek, 2002; Pohnert et al., 2007; Hay, 2009), and facilitate numerous ecological processes (Pawlik, 1992; Hadfield & Paul, 2001; Roberts & Uetz, 2005; Chivers et al., 1996; Tamburri et al., 1996). Macroinvertebrates have demonstrated vast behavioral responses to predator chemical cues (Burks & Lodge, 2002; Dodson et al., 1994), whereby the indirect effects of these compounds elicit decreased browsing, foraging, and reproductive activity (Juliano & Gravel, 2002; Koch et al., 2007; Schneider et al., 2014), reduced predatory behavior (Wisenden et al., 1997), altered habitat use (Camacho & Thacker, 2013), and increased attachment strength to substrates and aggregation rate (Naddafi & Rudstam, 2013).

The unique chemical defenses that numerous taxa have evolved may elicit similar behavioral responses, potentially promoting ecological, as well as evolutionary processes beyond just the survival of those individuals (Elliott et al., 1993; Brodie et al., 2005). Larval Lepidopterans utilize pyrrolizidine alkaloids as a chemical defense, but it serves as a sexual attractant for adults (Weller et al., 1999). Amphibian noxious or toxic skin substances, which have long been viewed as poisons or feeding deterrents, may also facilitate intraspecific competitive interactions (Summers, 1999; Crossland et al., 2012) and influence the selection of larval rearing sites (Schulte et al., 2011). Given the strong role of these substances in predator–prey relationships (Daly, 1995), chemical defense molecules may not only affect prey directly, but may also broadly impact prey populations and community organization indirectly.

The California newt (*Taricha torosa*) and all congeners possess a potent neurotoxin, tetrodotoxin (TTX) that is considered to serve foremost as a chemical defense (Twitty, 1966). However, TTX also acts at other ecological and evolutionary scales. During aquatic life history stages, adult *T. torosa* are potentially cannibalistic. The neurotoxin, which is continuously given off by adults as a byproduct of chemical defense, elicits an antipredator behavior in *T. torosa* larvae (Elliott et al., 1993; Zimmer et al., 2006). For another *Taricha* species, the rough-skinned newt, *T. granulosa*, the toxin has acted as a selection pressure for resistance in predatory garter snakes (Brodie et al., 2002). These studies demonstrate the immense selective pressure TTX can exert within systems. However, what effect newt chemical cues, and specifically TTX, have upon organisms at lower trophic levels remains largely undetermined.

The hypothetical effects newt chemical cues and TTX may have on lower trophic levels can be investigated by testing whether sympatric macroinvertebrate species within lotic habitat respond to it. Macroinvertebrates are fully capable of perceiving and responding to chemical cues from predators (Krieger & Breer, 1999) and the modalities for such responses can be morphological, physiological, or behavioral (Tollrian & Harvell, 1999). Though the sclerotized cuticle of many macroinvertebrates traditionally is thought to function as an antipredator mechanism and physical barrier to mitigate external selective pressures (Wood et al., 2010; Castellanos et al., 2011), it also links external stimuli to internal neuronal pathways (Casas & Dangles, 2010). Newt chemical cues, and specifically the highly potent neurotoxin TTX that is found in these cues, may affect macroinvertebrates behavior, either as a result of initiating an antipredator response or by sub-lethal poisoning.

Stream habitats in southern California (USA) provide an ideal system to evaluate our hypotheses as macroinvertebrates and *T. torosa* co-occur during the breeding season of newts. As generalist consumers, adult *T. torosa* predominantly feed upon the macroinvertebrate community (Kerby & Kats, 1998), with the majority of their diet comprised of Ephemeroptera (Bucciarelli and Kats, unpublished data). In this study, we investigate if chemical cues from *T. torosa* affect the behavior of the macroinvertebrate community by altering foraging behavior and if TTX has any effect on invertebrate predator behavior. Our study is composed of three specific questions: (1) Does exposure to chemical cues from captive newts influence habitat selection by aquatic macroinvertebrates; (2) do newt chemical cues affect macroinvertebrate predator foraging behavior; and (3) does exposure to waterborne TTX affect macroinvertebrate locomotor activity.

Methods and materials

Field behavioral bioassay of newt chemical cues

Field behavioral bioassays of macroinvertebrate responses to newt chemical cues were conducted in Cold Creek Preserve (Los Angeles County, California, USA) using prefabricated transparent acrylic enclosures (18 cm long × 10 cm wide × 14 cm high). Each enclosure was ventilated by drilling 1-cm hole into the top. An acrylic grated bottom (18 cm long × 10 cm wide; grate size = 1.25 cm × 1.25 cm) allowed chemical cues from an animal to diffuse into pools.

Adult male *T. torosa* (mean $SVL \pm \mathrm{SD} = 77.5$ mm ± 0.14 mm, $n = 10$) and adult Pacific tree frogs, *Pseudacris regilla* (mean $SVL \pm SD = 31.1$ mm ± 0.88, $n = 10$) were collected by hand immediately before the start of a trial. This frog species was chosen as a biological control to provide an additional source of amphibian chemical cues because it is a habitat generalist that coexists in riparian habitats with *T. torosa,* is abundant, breeds in streams, and is often observed in stream pools. We also chose *P. regilla* because adults do not prey upon aquatic invertebrates. Enclosures were oriented with the long axis parallel to the direction of the stream current, grate side down, and approximately 12-cm deep in a pool. Each enclosure rested on a small frame to allow for approximately 3 cm between the bottom of the enclosure and the bed of the pool. An airspace of 2 cm remained at the top of the enclosure to allow animals to surface. Selected pools were devoid of newts and frogs, and no newts or frogs were observed 50 m upstream or downstream from experimental pools.

In total, we performed 10 replicates over a two-week period. We used 10 pools and no more than one trial was conducted in a pool at a time. A trial consisted of two paired enclosures, one housing an adult *T. torosa* and the other a single *P. regilla*. Each enclosure was separated by at least 80 cm. A distance of approximately 30 cm remained between each enclosure and the bank. The location of enclosures in a pool was varied for each trial. Enclosures were anchored and left until the following morning (~12 h). Upon returning, a 20-cm-diameter ring was placed around each enclosure to establish a search area. We chose a 20-cm diameter because it allowed for at least three diameters distance between each paired enclosure and because compounds released from newts in similar flow regimes (~ 1–$2\ \mathrm{cm}^{-1}$) are detectable at close proximity (1–9 cm from the animal, D. Schar, unpublished data). A distance of at least 100 m was maintained between experimental pools when multiple trials were conducted simultaneously.

At the end of trials, the location of each enclosed animal was noted. All animals were released by hand into the original pool they occupied. Rocks, vegetation, and cobble were collected to enumerate and identify macroinvertebrates within each search area. For each replicate, the number of macroinvertebrates was totaled by order for a frog area and a newt area. Count data from the ten frog areas were then merged and a final tally of macroinvertebrates for each order within all frog areas produced. This was repeated for newt areas. To test the effect of newt chemical cues on macroinvertebrate habitat selection, we compared tallies of macroinvertebrates by order between frog and newt areas using a Chi-square test. Thus, each row of the matrix in the 5 × 2 contingency table represented a macroinvertebrate order (Ephemeroptera, Trichoptera, Coleoptera, Plecoptera, and Odonata).

Collection of egg masses and larval culture for laboratory assays

A total of five *T. torosa* egg masses were collected from Cold Creek Preserve and transported in stream water to the laboratory. Egg masses were selected based on embryo developmental stage to ensure larvae hatched at similar times. Egg masses were incubated in 5-µm-filtered, dechlorinated tap water in a walk-in cooler at stream temperature (11.1–12.6°C) and maintained on a 12:12 h light:dark cycle. Larvae were fed ad libitum crushed or whole black worms (*Lumbriculus variegates*). Approximately 30 days post-hatch, larvae were collected for use in macroinvertebrate behavioral bioassays.

Preparation of newt chemical cues

Adult male newts (mean *SVL* ± *SD* = 73.6 mm ± 2.05 mm, *n* = 15) were collected by hand from Cold Creek Preserve. Newt chemical cue solutions were prepared in the field by placing five newts in ~1 l of 5 µm-filtered, dechlorinated tap water for 1 h. The dorsum of each newt was massaged for 1 min at the start of the hour to stimulate the release of skin secretions (Zimmer et al., 2006). At the end of the hour, newts were removed and released. Three batches were prepared, combined, and then transported back to the laboratory. The solution was brought to a final volume of 4 l, filtered to 0.45 µm, and then held at stream temperature before being used in laboratory behavioral bioassays. The entire solution was used within 3 h of being prepared. A 1-ml aliquot was taken from the final 4 l solution, and high-performance liquid chromatography coupled with fluorescence detection (HPLC–FLD) was used to confirm the presence and concentration of TTX. Calibration curves attained from analyzed standard TTX solutions were used to derive the concentration of TTX in our sample. Details of the chromatography methods are outlined in Bucciarelli et al. (2014).

Laboratory behavioral bioassay of newt chemical cues

Antipredator behavior in response to chemical compounds is observable in numerous taxa (Kats & Dill, 1998). However, it is unclear whether *T. torosa* chemical cues mediate similar behaviors in the macroinvertebrate community. One of the predatory odonate nymphs in this community, the green darner (Aeshnidae: *Anax junius*), was selected to test behavioral responses to *T. torosa* chemical cues and TTX. This odonate is an abundant and common predatory macroinvertebrate whose aquatic life history stages overlap *T. torosa* breeding and larval development periods. Additionally, green darners feed on *T. torosa* larvae and adult *T. torosa* feed on *A. junius* (Kerby & Kats, 1998). Nymphs were collected from Cold Creek Preserve 24 h before the start of experiments at sites upstream from our experimental pools. All nymphs were collected on the same day and transported in stream water to the laboratory. Individuals were housed in semi-opaque acrylic chambers (33 cm long × 20 cm wide × 12 cm high) filled with 250 l of 5-µm-filtered, dechlorinated tap water at stream temperature and maintained on a 12 h:12 h light:dark cycle.

Preliminary feeding experiments were conducted to determine *A. junius* feeding behavior. Nymphs were starved for 24 h and then fed ad libitum *L. variegates*. *Anax junius* was continuously fed and demonstrated predator behavior up to 140 min. Laboratory behavioral experiments were therefore terminated at 150 min.

Dragonfly nymph behavioral bioassays in response to newt solutions were performed in non flow-through semi-opaque acrylic chambers (33 cm long × 20 cm wide × 12 cm high). Dechlorinated, 5-µm-filtered, tap water at stream temperature (12–15°C) was used to bring each chamber to a volume of 750 ml. A semi-opaque acrylic glass plate (8 cm long × 8 cm wide) with four metal legs rested horizontally 2 cm from the bottom of each chamber to mimic stream refuge.

Before the start of trials, a nymph (mean length ± *SD* = 37.9 mm ± 3.8 mm, *n* = 36) was transferred to a chamber and allowed to acclimate for 5 min. At the start of each trial, three *T. torosa* larvae (mean length ± *SD* = 18.68 mm ± 0.41 mm, *n* = 99) were placed in a chamber with the nymph. A total of 250 ml of the newt solution was added to each of half of the chambers. Stream water (5-µm-filtered) was used as a control and added to the remaining chambers.

Nymph movements, use of refuge, and number of surviving larvae were noted every 10 min for 150 min. Nymphs were considered to move if they were observed crawling, swimming, striking at prey, or using limbs. The proportion of observations a nymph moved was calculated and percentages arcsine transformed. All data were normally distributed. A *t* test was used to detect if *T. torosa* chemical cues affected nymph movement.

The number of surviving larvae in each replicate at every 10 min interval was used to evaluate if newt chemical cues affected predatory behavior in *Anax junius*. Data were shaped to long format and treatment, replicate, time, and number of surviving larvae encoded as variables in STATA/SE (v 12.0). A Poisson regression tested the effect of time, treatment, and the interaction of both variables upon number of surviving larvae. Replicate was used as the random intercept variable to control for repeated measures across time.

Laboratory behavioral bioassay of TTX

Bioassays were performed in flow-through transparent acrylic chambers (7 cm long × 3 cm wide) with a 1-cm water depth to test the behavioral response of nymphs to TTX. Nymphs were collected and housed as previously described. Dechlorinated, 5-µm-filtered, tap water at stream temperature (12–15°C) was pulled into chambers using gravity lines (internal diameter = 0.16 cm). A foam diffuser (pore diameter = 100 µm) was mounted at the input of the chamber to create a uniform distribution of TTX as it moved toward nymphs. The velocity of water in chambers was similar to observed stream velocities when nymphs were collected (~ 1–$2\ \mathrm{cm}^{-1}$). Outputs on the flow-through chambers prevented overfilling and maintained a consistent 1-cm water depth. The test solution of TTX was prepared at a concentration of $1.0 \times 10^{-8}\ \mathrm{mol\ l}^{-1}$ using dechlorinated, 5-µm-filtered tap water and TTX powder (Sigma Aldrich). This concentration was chosen based on HPLC–FLD analysis of amounts of TTX present in the prepared newt solution. Dechlorinated, 5-µm-filtered, tap water was used as the control.

All bioassays were recorded at 30 frames s^{-1} using a digital camera with video recording capabilities (Nikon, D90, 18–200 mm lens). The camera was mounted 50 cm directly above a chamber to record nymph behavioral responses. The flow-through chamber and camera were placed within a blind to minimize visual disturbance. White parchment was placed underneath chambers to create contrast. Each sheet had a 5-cm marker placed on it to create calibration points for video analyses. Chambers were rinsed for 20 min with a 1:10 solution of hydrochloric acid and dechlorinated, 5-µm-filtered, tap water at the end of each trial to remove any remaining compounds.

For each experiment, a nymph (mean length $\pm SD =$ 192 mm ± 21 mm) was placed in a chamber and oriented toward the input. Nymphs were allowed to acclimate for 5 min, during which filtered tap water passed through the chamber. At the start of a trial, the input for the chamber was either switched to the control ($n = 3$) or TTX solution ($n = 5$). Approximately 1 min was provided for the control or TTX solution to reach the nymph before offering a prey item (*L. variegates*). Prey were placed at the top of the chamber, directly in front of the diffuser, approximately 7 cm from the nymph. Behavioral reactions were recorded from the start of the 5 min acclimation period to 5 min post consumption of the prey item and ranged from 63 to 509 s.

Recorded video of the bioassays was categorized into three segments: (1) the acclimation period, (2) introduction of prey to first strike, and (3) post consumption of prey. Video of nymph bioassays was then transferred to a computer, played back, and movement noted during each second of each segment. We used the same methods outlined in the behavioral bioassay of newt chemical cues to quantify nymph movement. Data were arcsine transformed and normally distributed. An effect of TTX upon percentage of observations animals moved, latency to strike at prey, and time required to consume prey was evaluated using *t* tests.

Both angular velocity and velocity magnitude were analyzed in bioassays to determine the dynamics of nymph predatory behavior and locomotor activity in the presence of TTX. We considered angular velocity to be an indicator of the speed at which nymphs changed directions when stalking prey and velocity magnitude a measurement of the speed at which nymphs strike at prey. Vector quantities derived from Tracker software (version 4.8) were used to calculate mean angular and mean velocity magnitudes for each nymph. We used Tracker data from when a nymph began stalking prey up to the start of a strike to produce individual mean angular velocity, and the data from the duration of each strike to derive individual mean velocity magnitude values. For each vector quantity, a *t* test was used to statistically compare individual means between treatments.

Results

Field behavioral bioassay of amphibian chemical cues

A significantly lower number of macroinvertebrates were found within newt areas relative to frog areas

($\chi^2 = 24.14$, $P < 0.001$, $df = 4$, Fig. 1). Collected macroinvertebrates consisted of six orders: Ephemeroptera, Trichoptera, Coleoptera, Plecoptera, Odonata, and Megaloptera, listed from most abundant to least. Because only two Megaloptera specimens were found within all search areas, they were excluded from our analysis. Prior to removing enclosures, we noted the location of individual frogs and newts. Frogs were always found at the water surface, while newts were either at the bottom or at the water surface of an enclosure.

Laboratory behavioral bioassay of newt chemical cues

Nymph behavior was affected by chemical cues released from newts. We found that nymphs moved significantly less when exposed to newt chemical cues relative to control solutions (t test, $t = 5.3204$, $P < 0.001$, $df = 31$, Fig. 2). Poisson regression indicated that the percentage of surviving larvae was affected by the presence of newt chemical cues (Fig. 3). The interaction term between time and treatment in the Poisson regression was significant ($P = 0.049$), indicating a time effect across treatments. Because the interaction term was significant, we tested if the effect of time was significant in each treatment using predicted values from the previously fit model. The effect of time was significant ($P = 0.040$) and it was twice as strong in the control treatment. Additionally, the number of replicates with all larvae surviving by the end of the experiment totaled 7 of 16 in the newt chemical cue treatment relative to 2 of 17 in the control.

Percentage of instances nymphs used refuge did not differ between treatments (mean percentage of time in refuge ± SD: cue = 33.69 ± 7.14, $n = 16$; control = 32.06 ± 7.36, $n = 17$; t test, $t = 0.1585$, $P = 0.8751$, $df = 31$). Nymphs were observed under and on the side of refuge in both treatments.

HPLC–FLD was used to determine if newt solutions contained TTX. Chromatograms indicated the presence of TTX in newt solutions at concentrations comparable to amounts observed in previous studies (Zimmer et al., 2006; Bucciarelli et al., 2014). The actual amount of TTX detected from the aliquot of the 4 l newt chemical cue solution was approximately 1×10^{-9} mol l^{-1} of TTX. Because the analyzed solution was diluted when it was brought to a final volume of 4 l, behavioral bioassays of TTX were conducted using a concentration of TTX a magnitude of order greater than what was measured with HPLC–FLD.

Laboratory behavioral bioassay of TTX

Behavioral responses of nymphs significantly differed between treatments in only one segment of the

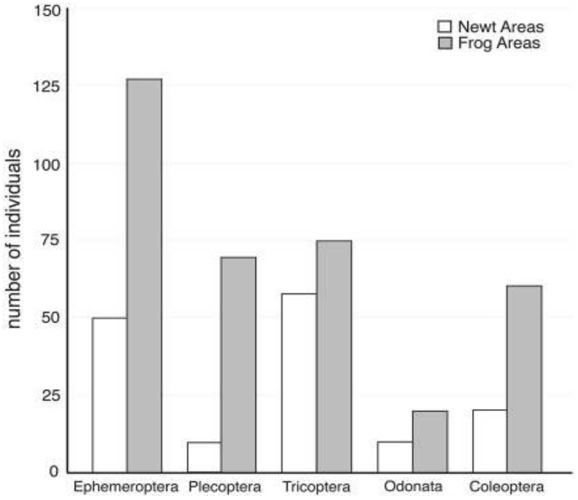


Fig. 1 Cumulative number of macroinvertebrates by order from either newt or frog areas in field behavioral bioassays. Significantly fewer macroinvertebrates were found in newt areas relative to frog areas

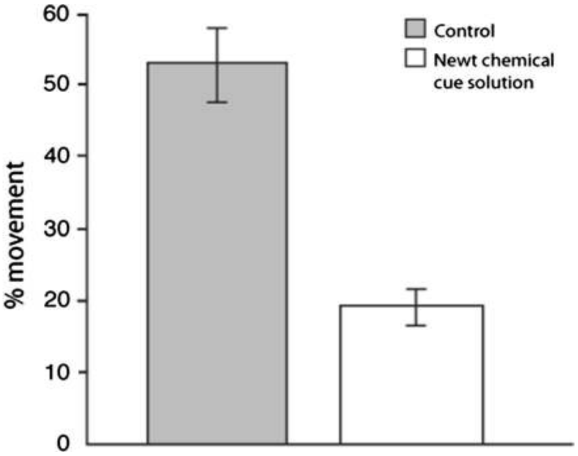
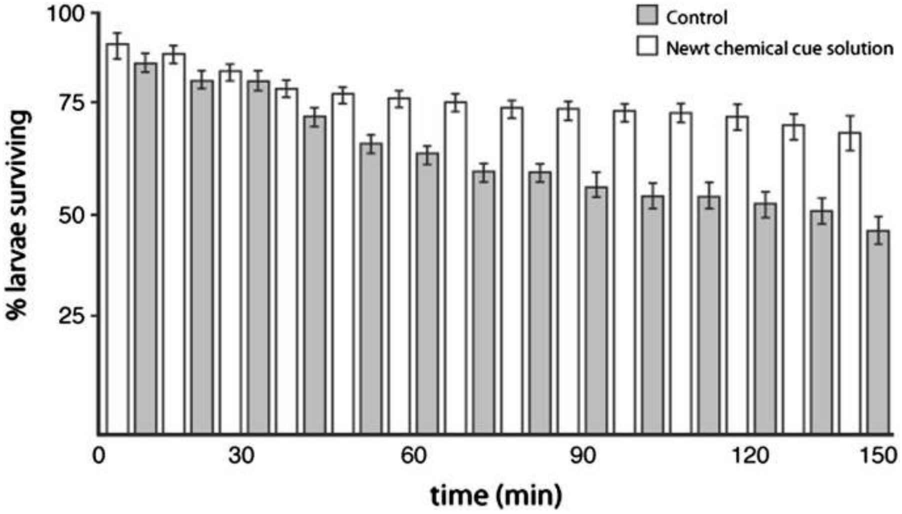


Fig. 2 Behavioral response of *Anax junius* nymphs to prepared newt chemical cue solutions in laboratory behavioral bioassays. The percentage of instances nymphs moved over the 150-min period was significantly less in the presence of newt chemical cue solutions

Fig. 3 Percentage of surviving *T. torosa* larvae through time in the presence of predatory *Anax junius* nymphs. *Open bars* indicate overall percentage of surviving larvae in experimental chambers where *A. junius* was exposed to newt chemical cue solutions. *Solid bars* show the same data for controls



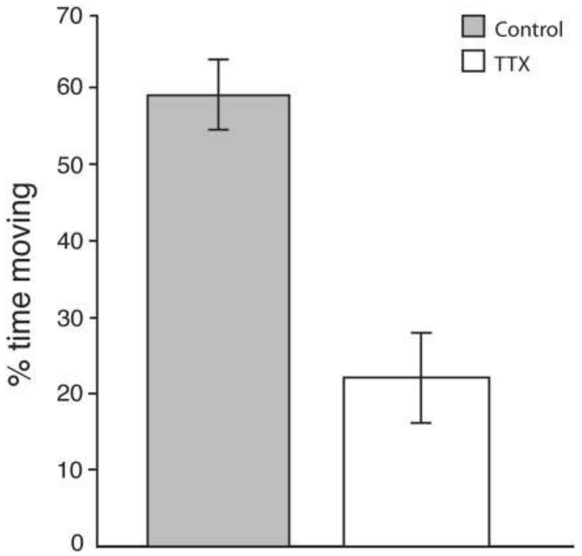


Fig. 4 Behavioral response of *Anax junius* nymphs to TTX in laboratory behavioral bioassays. The mean percentage of time nymphs moved, from when prey was introduced to the first strike, was significantly less when nymphs were exposed to TTX

bioassay, which was during the introduction of prey to first strike segment. Nymphs exposed to TTX moved on an average nearly three times less during the time leading up to first strike relative to nymphs in control chambers (t test, $t = 4.11$, $P = 0.006$, $df = 6$, Fig. 4). Nymph movement did not differ between treatments during the 5-min acclimation segment or the 5-min post-feeding period (acclimation: t test, $t = 0.75$, $P = 0.48$, $df = 6$; post-feeding: t test, $t = 1.07$, $P = 0.32$, $df = 6$). No significant difference in time to strike at food (t test, $t = 1.09$, $P = 0.316$; mean: TTX = 69.6 s; control = 24.0 s) or time to consume food (t test, $t = 2.18$, $P = 0.072$; mean: TTX = 229.0 s; control = 38.3 s) was detected.

Mean velocity magnitude and angular velocities significantly differed between treatments (Fig. 5). Nymph mean velocity magnitude was significantly reduced in TTX treatments (t test, $t = 2.33$, $P = 0.049$, $df = 6$). Similarly, nymph mean angular velocity was significantly reduced when nymphs were exposed to TTX (t test, $t = 2.51$, $P = 0.045$, $df = 6$; Fig. 6).

Discussion

Chemical compounds from animals are capable of altering predator and prey behavior (Stowe et al., 1987; Hare & Eisner, 1993; Flowers & Graves, 1997; Nyström & Åbjörnsson, 2000; Turner et al., 2000), deter or poison predators (Kats et al., 1988; Daly, 1995), indicate reproductive status (Roelofs et al., 2002), aid in foraging (Weissburg & Zimmer-Faust, 1993), or signal an impending threat (Sullivan et al., 2011). It is also clear that chemical cues from predators affect foraging behavior and trophic relationships (Abrams, 1983; Juliano & Gravel, 2002; Koch et al., 2007; Camacho & Thacker, 2013; Naddafi & Rudstam, 2013; Schneider et al., 2014). The idea that defensive compounds can affect community organization, trophic webs, and biogeochemical processes has been proposed (McClintock & Baker, 2001; Pohnert et al., 2007; Hay, 2009), and in marine

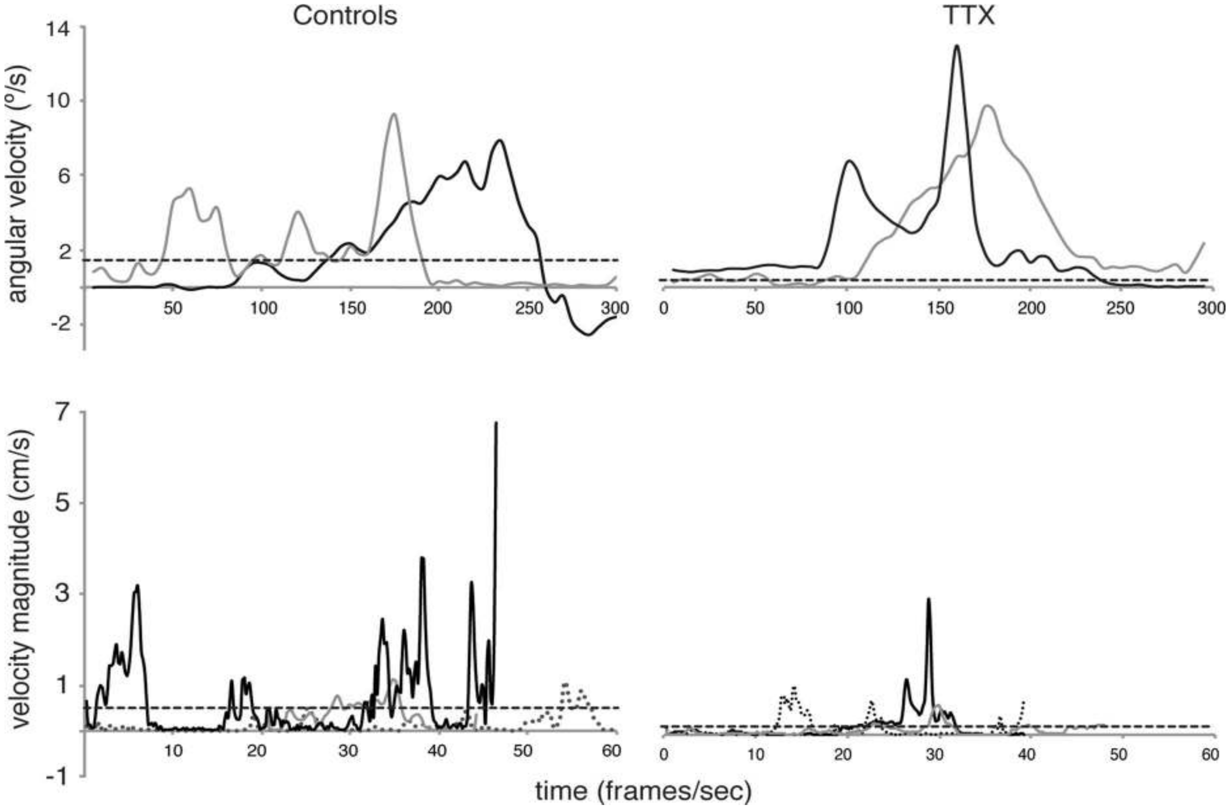


Fig. 5 Examples of angular velocities and velocity magnitudes of *Anax junius* nymphs recorded in laboratory behavioral bioassays. Each line represents an example of *A. junius* angular velocities or velocity magnitudes from TTX or control bioassays. *Solid* and *gray lines* show the angular and magnitude velocities of the same nymph in a replicate. The velocity magnitude of additional nymphs is represented by *dotted lines*. *Horizontal dashed lines* show mean values

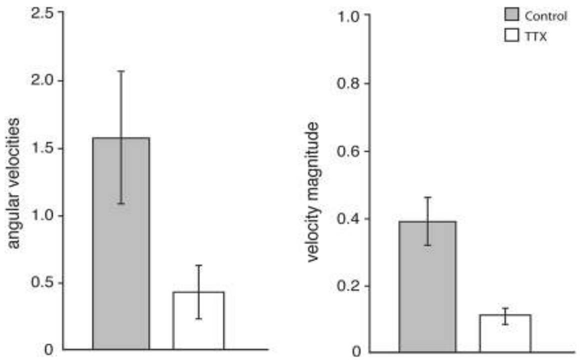


Fig. 6 Mean angular velocities and velocity magnitudes of *Anax junius* nymphs in laboratory behavioral bioassays. Relative to nymphs in the control, nymphs exposed to TTX in flow-through chambers showed significantly decreased mean angular velocities in the period leading up to the first strike at prey. When striking at prey, nymph velocities were significantly less when TTX was present

systems these effects can reverberate throughout ecosystems, ultimately affecting structure and function (Hay & Kubanek, 2002). However, the capacity of chemical defense compounds to alter communities, affect trophic relationships, and prey populations in freshwater systems remains understudied.

Our results lend support to the idea that amphibian toxins may have ecological significance at lower trophic levels. In-stream behavioral bioassays showed that a greater number of individuals in the macroinvertebrate community foraged away from point sources of *T. torosa* chemical compounds (Fig. 1). This implies that newt chemical cues elicit a behavioral response similar to those observed in other macroinvertebrates (Peckarsky, 1980; Petranka & Fakhoury, 1991; Kiesecker et al., 1996; Wisenden et al., 1997; Jacobsen & Stabell, 2004; Gall & Brodie, 2009). Remaining in close proximity to newts would likely be maladaptive since adult *T. torosa* are opportunistic consumers, feeding preferentially on macroinvertebrates (Kerby & Kats, 1998), and primarily on Ephemeroptera. It is possible that macroinvertebrates were attracted to frog chemical cues. However, over the course of our field experiment, we sampled three random areas in pools, each 20 cm in diameter and found that the mean cumulative number of macroinvertebrates in these areas (mean = 36.67 ± 6.33 SE, $n = 3$) did not differ from frog areas (mean = 35.40 ± 4.86 SE, $n = 10$). Given the feeding ecology of *T. torosa*, compounds from newts seem likely to influence the macroinvertebrate community, whereas macroinvertebrates would likely forego responding to *P. regilla* chemical cues since they pose no threat (Sih, 1987; Chivers & Smith, 1998). Ultimately, when *T. torosa* breed in streams, TTX as a byproduct of chemical defense may indirectly affect trophic relationships and community organization.

Macroinvertebrate behavioral responses to *T. torosa* chemical cues appear to be an antipredator response to a waterborne signal. Particularly interesting in our study is the behavioral response of Ephemeroptera, which seem to be highly responsive to newt chemical cues (Fig. 1). Aquatic mayflies have demonstrated a remarkable ability to detect chemical stimuli and modify behavior in order to avoid predation (McIntosh & Peckarsky, 1996; Scrimgeour et al., 1994). Scrimgeour et al., (1994) found that chemical cues alone will increase drift rates and reduce the use of upper substratum surfaces. Similar antipredator responses to *T. torosa* compounds would seem adaptive given that Ephemeroptera larvae compose approximately 50 % of the aquatic newt diet (Bucciarelli, unpublished data) and that densities are highest in March through June, considerably overlapping the *T. torosa* breeding season (R. Ferrer, unpublished data).

In our laboratory bioassays, dragonfly nymphs exposed to newt solutions on average consumed fewer *T. torosa* larvae than controls (Fig. 3). The results of Poisson regression showed that the effect of time was twice as strong in the control group, indicating that larvae in the presence of nymphs without TTX were much more likely to be consumed as time progressed. Ultimately, this may be due to the significantly reduced percentage of time nymphs spent moving. An underlying cause of reduced nymph activity could be the synergistic effect of exposure to waterborne chemical stimuli and TTX in larvae, whereby cues from newt solutions induced antipredator behavior and TTX from ingested larvae sub-lethally poisoned nymphs. However, nymphs observed in bioassays using newt solutions and pure TTX spent relatively similar amounts of time moving (Figs. 2, 4). Although time scales varied, the pattern suggests that TTX in larvae does not poison nymphs. While no larvae were observed to be unpalatable, we cannot conclude if larvae were devoid of TTX due to ontogeny (Mathis & Vincent, 2000; Zimmer et al., 2006) or if nymphs are resistant to TTX, as observed in other macroinvertebrate orders (Gall et al., 2011). Attempts to understand community resistance and *Taricha* larval TTX levels are ongoing.

Collectively, our laboratory bioassays show that compounds from *T. torosa* affect *A. junius* nymphs. In our control treatments, nymph mean angular velocities were nearly four times greater in control bioassays compared to TTX bioassays, while mean striking velocities in controls were more than three times greater relative to mean velocities of nymphs exposed to TTX (Fig. 6). In other studies, quantitative estimates of velocities have successfully been used to understand escape behavior of shrimp (*Crangon crangon*) and how varied kinematics may affect predation risk (Arnott et al., 1998). Other researchers have determined that slugs (*Deroceras reticulatum*) alter velocities and turning rates in response to predator chemical cues (Armsworth et al., 2005). Moreover, a recent study of grasshopper (*Melanoplus femurrubrum*) jumping biomechanics measured angular and takeoff velocities and found that escape performance and endurance are positively associated with spider predators (Hawlena et al., 2011). What effect these modified locomotor behaviors have on fitness is undetermined, but for *A. junius* nymphs such changes may likely be an additional component to effective antipredator behavior (Hopkins et al., 2011).

Although the mechanism is unidentified, the observed reductions in nymph movement and velocity when exposed to TTX suggest that it can elicit behavioral responses, which may not be akin to the antipredatory behavioral response observed in other community members (Zimmer et al., 2006). In previous studies of odonates (Wisenden et al., 1997; Baker et al., 1999; Elkin & Baker, 2000) and other macroinvertebrates (Juliano & Gravel, 2002), Zygopterans responded to predator chemical stimuli by reducing the frequency of feeding strikes, head turns, and walking movements (Wisenden et al., 1997). Additionally, Juliano & Gravel (2002) observed that larval mosquitoes reduce browsing activity when waterborne chemical stimuli from predatory macroinvertebrates are present. Thus, it is likely that costs and benefits of behavioral and physiological strategies are balanced by natural selection. Specifically, TTX may simultaneously deter predators and warn prey.

It is also possible that TTX physiologically affects nymphs, and as a result inhibits locomotor activity. Voltage-gated sodium channels, which are the target of TTX, control action potentials (Catterall, 1980). These channels are also the targets of numerous poisons known to affect macroinvertebrate physiology (Catterall, 1980; Catterall et al., 1981; Gusovsky et al., 1988; Olivera et al., 1990; Narahashi et al., 1992; Song et al., 1996; Cestele et al., 1998; Dechraoui et al., 1999; Li et al., 2001; Yotsu-Yamashita et al., 2004; Du et al., 2013). Some taxa buffer the effects of such poisons by investing energetic resources into cuticle deposition, thereby decreasing the need to upregulate transcription of more enzymes to catalyze the metabolism of poisons (Wood et al., 2010). Potentially, *A. junius* buffers the effects of waterborne TTX by both cuticle deposition and enzyme activity, although the amounts of TTX entering their system may still have consequences, especially when ingested. One such effect may include reduced locomotor activity. In behavioral bioassays to TTX, time to consume prey did not significantly differ between nymphs exposed to waterborne TTX and controls. As such, it is not likely that waterborne TTX poisons nymphs. However, the observed behavioral responses of odonate nymphs to waterborne TTX suggest that interacting with this compound is costly. How exactly other macroinvertebrates in this study system behaviorally respond to TTX is not known.

Unlike *Taricha* in more northern localities, newts in the Santa Monica Mountains occupy streams with low volumes of water and slow flow (G. Bucciarelli & L. Kats, personal observations). Contrary to conventional wisdom (e.g., Stebbins & McGinnis, 2013), a small number of adult newts stay in these streams far after the breeding period has ended. Given the unique environmental conditions in this part of their distribution, and the existing roles of TTX in mediating ecological and evolutionary processes, it appears that community members at lower trophic levels detect TTX-laden chemical cues from *T. torosa* and that these chemical cues can affect macroinvertebrate behavior. Selection for a response to newt chemical stimuli could have evolved because of the slow flowing and low volume pools found throughout the Santa Monica Mountains relative to breeding localities with greater volumes of water and faster flow regimes. Given the community response observed in field behavioral bioassays (Fig. 1), TTX given off by *T. torosa* likely functions as a chemical cue and not a poison that induces paralysis or sub-lethal affects. If the results of our field and laboratory bioassays are an accurate indication of the ways in which TTX affects foraging and predatory behavior of the macroinvertebrate community, as well as the stream community at a broader scale (Elliott et al., 1993), then we speculate that the loss of *T. torosa* could impact trophic relationships at multiple scales. Future studies to assess the indirect role of *T. torosa*, as well as TTX in other systems, are of great promise considering the diverse roles of chemical signals and the ubiquity of the neurotoxin TTX.

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CHAPTER 4

Individual fluctuations in toxin levels affect breeding site fidelity in a chemically defended amphibian

Abstract

Behaviors that influence habitat selection strongly determine species movement patterns. One component of animal behavior that largely influences movement patterns and habitat choice is site fidelity. California newts (family Salamandridae) demonstrate remarkable site fidelity, typically homing to the same pool of a stream each breeding season. Individuals often occupy a specific pool throughout the breeding season, but some males shift among breeding pools, altering their set of potential mates, competitors, and predators. In this study, we measured dermal concentrations of the chemical defense compound tetrodotoxin (TTX) in recaptured male California newts (*Taricha torosa*) over four breeding seasons to evaluate whether relative TTX concentrations are associated with breeding site fidelity in the field. Tree regression and random forest models showed that an individual's relative TTX concentration best explains fidelity to a breeding pool and suggest that newts may be able to assess both their own concentrations of TTX and that of conspecifics to make decisions about remaining in or abandoning a breeding pool. In addition, our four years of field sampling indicate that TTX concentrations of individuals and group means fluctuate tremendously, implying that TTX is not a stable phenotypic trait. This observation adds a novel dimension to our understanding of TTX in nature, potentially requiring a reevaluation of the co-evolutionary dynamics of predation and this potent toxin.

INTRODUCTION

The constraints and mechanisms by which species select their breeding habitat is fundamental to their ecology, evolution, and conservation. The behaviors that influence habitat selection, for example, strongly determine species movement patterns, thereby affecting interactions with other organisms and the environment [1], fitness and local density [2,3], the spatial scale at which populations are regulated [4], metapopulation dynamics [5,6], and the evolutionary units within a population [7,8]. One behavior that largely influences movement patterns and habitat choice is site fidelity.

Site fidelity, whereby individuals tend to return to a previously occupied site, is a life history strategy that occurs in many species of at least three phyla [9-11]. The behavior may increase survival by allowing animals to efficiently relocate and utilize previous sites for breeding, feeding and/or overwintering [12], as well as reduce energetic costs [13], vulnerability to predators [14], and risk of mortality [15,16]. Though site fidelity is a behavior that links individual and population level processes [17] with demonstrated ecological and evolutionary consequences, the mechanisms by which it occurs remain poorly understood in most systems.

Some amphibians show remarkable site fidelity and a considerable research effort spanning the last half-century has characterized amphibian social behavior with regard to breeding site fidelity [18-24]. Some amphibian species are known to return year after year to the same pool or physical structures at a breeding site, with individuals often remaining in that limited microhabitat during the entire breeding period [19,25]. However, other individuals may inexplicably abandon a breeding site [19,20,26], complicating explanations for the evolution and maintenance of site fidelity more generally.

Olfaction, as an orientation mechanism, is critical in guiding site fidelity [18,27]. Olfactory cues influence numerous amphibian behaviors [28] and often play an important role in the recognition of individuals, kin, and mates [29-33]. Salamanders use chemical cues to recognize and select habitat [34-37], mark territories [38], and detect oviposition site quality [39-41]. Scent marking, which typically is associated with territorial behavior [34], may also offer intruders the means to assess the competitive quality of other males [42,43]. Although olfaction can guide amphibian site fidelity, the mechanistic underpinnings of movement behavior once individuals reach breeding sites remain vague. Determining the processes that facilitate individuals returning to and maintaining a breeding locality, and why they may abandon these sites, will help model more generally the phenomenon of site fidelity.

Newts of the genus *Taricha* are iteroparous, returning over multiple years each spring to the same pond or reach of a stream to breed [18,44-46]. Although terrestrial most of the year, stream-breeding *Taricha* navigate 250 m - 500 m or more between terrestrial habitat and breeding sites [47,48] with pinpoint precision, often entering their stream within 15 m of previous years [18,19,49,50]. Throughout the breeding season, individuals generally remain within a few meters of their breeding pool [19], and often do not migrate after entering the stream. Olfactory cues aid terrestrial migration to breeding sites [49], and once there, chemical cues remain critical components for sexual and feeding behavior [49,51].

All four currently recognized species of *Taricha* possess a potent neurotoxin, tetrodotoxin (TTX), that is considered to have evolved primarily as a chemical defense. Population variation of *Taricha* TTX concentrations has been observed across broad geographic scales [52], a pattern that is presumed to be the result of a coevolutionary process between newts and predatory *Thamnophis* garter snakes [53]. The prevailing theory, whereby *Taricha* TTX levels escalate in response to *Thamnophis* resistance to TTX, rests on the assumption that the TTX phenotype is genetically based and evolutionarily capable of responding to natural selection. However, the means by which *Taricha* produce and regulate TTX is unknown. Because tetrodotoxin is an alkaloid, it is presumably not the direct product of a specific gene or gene family. The TTX concentrations of the sister taxon of *Taricha*, *Notophthalmus* newts from eastern North America, also vary across broad geographic ranges, but with no apparent relationship to a predator [54]. Furthermore, it appears that other tetrodotoxic taxa produce TTX via endosymbiotic bacteria rather than an endogenous, genetically-controlled process [55-60].

Given the extensive amount of variation observed between individuals and populations [60,61], the role of tetrodotoxin may well be more complex and nuanced than that predicted purely by the coevolutionary predator-prey narrative [62-67]. Tetrodotoxin unquestionably selects for increased resistance in *Thamnophis* snakes [68], but it also appears to facilitate a diverse set of ecological processes. Various taxa rely on TTX as a feeding stimulant [69], sexual attractant [70], or antipredator chemical cue [51,71,72]. Specific to our study system, TTX from potentially cannibalistic adult California newts (*Taricha torosa*) causes conspecific larvae to flee and seek refuge [51,71]. Tetrodotoxin also affects the foraging behavior of stream macroinvertebrate community members, and reduces movement and strike velocities of at least one macroinvertebrate predator [72].

In this study, we quantified TTX levels within and among individuals of a wild newt population (*T. torosa*), and explored the relationship between TTX and site fidelity. We conducted longitudinal sampling of individual newt TTX levels and used a multi-year mark-recapture study to track each individual's chemical defense concentrations and movement patterns within a stream across multiple years. We place these data into the context of an ongoing, multi-decadal study of newt population biology in southern California streams during a severe drought and a time of precipitous amphibian declines. Overall, our results show that site fidelity at the stream level is exceptionally strong, with no marked individual recaptured in an adjacent stream over 20 years of intensive study and hundreds of marked newts. However, consistent with other studies, we have observed that some individuals will move up or down stream among breeding pools or occupy different pools across years, and we analyze these temporal movement patterns as a consequence of newt morphology and an individual's relative TTX concentrations. Given that TTX is both a defensive chemical and conspecific chemical cue, we designed our study to determine whether individual TTX concentrations influence site fidelity patterns in breeding male California newts. Our results indicate that i) adult male TTX levels are linked to patterns of fidelity at breeding sites, and ii) that TTX levels of wild individuals not only fluctuate, but mean TTX levels of our single breeding population span the full range of *T. torosa* chemical defense levels previously measured across the entire species range.

METHODS

Study site: The Santa Monica Mountains are a partially isolated range adjacent to Los Angeles, California (USA). They span approximately 76 km west to east and are bounded on the south by the Pacific Ocean. Our study stream is located in the Santa Monica Mountains within Cold Creek Preserve (N 34.091892, W -118.64754). Cold Creek flows intermittently for 7 kilometers and its upper half constitutes a set of *T. torosa* breeding sites with wide, deep, unsilted pools that are devoid of introduced predators [73]. Ten pools within this stretch exceed 2 meters in diameter and 1 meter in depth and are regular breeding sites for *T. torosa*.

***Individual identification of* T. torosa:** As part of a long-term study of *T. torosa* in the Santa Monica Mountains, adult newts (snout-vent length (SVL) > 60 mm) have been marked with passive integrated transponder (PIT) tags since 1991 to track breeding activity and movement patterns. During our study, individuals were captured by hand or dip net and individually scanned with a portable microchip reader (AVID 1002, Avid Identification Systems, Inc, Norco, CA, USA) to determine if they were previously PIT tagged. Adults without a microchip were anesthetized in a 300 ppm solution of methanesulfonic acid (MS222), rinsed with stream water, and a 12 mm bio-glass microchip (AVID 2023) was injected intraperitoneally with a dedicated injector needle (AVID 3001) for permanent individual identification [50]. Injector needles and forceps were sterilized between each use and newts were retained until the effect of MS222 wore off (~ 3 minutes). To reduce stress, no newt was marked and sampled for TTX at the same time. Thus, the first capture of each animal was solely for marking, and during subsequent encounters, TTX tissue samples were collected.

Recapture and animal sampling: Over the majority of this study, the entire length of Cold Creek was surveyed every 5 - 10 days during the breeding season from March 1 to June 28, 2011 - 2014 to collect tissue samples from newts to measure individuals' TTX concentrations through time. During this sampling period, there were 484 total encounters with adult newts. This total is partially composed of 77 unique individuals, 21 of which were sampled more than once and 56 of which were sampled only once. Hereafter, we analyze these data as two separate sets, the first composed of the 21 individuals recaptured and sampled 2 - 4 times and the other formed with the remaining 56 individuals recaptured and sampled only once.

When completing field surveys, each individual we encountered and sampled was sexed, weighed, and SVL and tail height measured. GPS-locality data were collected at each location a newt was sampled, as well as the stream morphology (run, riffle, or one of the ten main breeding pools) where that individual was encountered.

Tissue samples were obtained from the dorsal region with a 2 mm skin biopsy tool [74]. *Taricha torosa* is recognized as a Species of Special Concern in California. Given the limited number of females encountered and the protected status of *T. torosa*, gravid females were not sampled. No newts were ever taken into captivity to be sampled and we also never sampled terrestrial-phase newts.

Quantitation of TTX: Tissue samples were transported on dry ice and stored at - 80 °C. All samples were processed to extract TTX within 30 days following methods described in Bucciarelli et al. [74]. Tetrodotoxin extracted from individual tissue was quantitated with a high performance liquid chromatography system coupled with fluorescence detection (HPLC-FLD) [74]. The HPLC-FLD system was calibrated with TTX standard solutions prepared using commercial TTX (citrate salt, Fisher Scientific). All solutions were stored at 2 - 4 °C.

***Individual and group mean TTX levels*:** Amounts of TTX within a sample were derived from peak area measurements of chromatograms made using the HPLC-FLD system. Each skin sample was weighed and peak area measurements converted to a concentration of nanomoles TTX/mg skin (nmol TTX/mg). We used these data from recaptured or unmarked males sampled on the same day to calculate daily mean TTX values. We considered these daily mean values to be a representation of the overall TTX level of males in Cold Creek on that day. We then derived how much an individuals TTX level deviated from that day's mean TTX value by taking the difference between an individual's TTX concentration and the mean TTX concentration of newts sampled on that same day. Thus, individuals with below-mean TTX values had a negative deviation while those above-average had a positive deviation. These values were used as a continuous variable in subsequent analyses.

Temporal patterns of individual breeding site fidelity: The breeding site history and fidelity of newts was reconstructed from mark-recapture data. For the 21 individuals recaptured and sampled for TTX more than once during 2011 - 2014, we tracked the pool(s) in which an individual was recaptured during this four-year sampling period. Recaptured individuals were divided into two groups and these two groups were used as the categorical response variable in all models. Individuals were coded as a "0" if they were always recaptured in the same pool or as a "1" if they were ever recaptured in different pools. For example, an individual recaptured in the same pool in 2011 and 2012 would be coded as a "0", but an individual that was recaptured in the same pool in 2011 but a different pool in 2012 would be coded as a "1."

Our recapture data also included 56 marked individuals that were only encountered and sampled once during our 2011 - 2014 study period. We assessed the site fidelity of these males by comparing their breeding site when sampled for TTX to their previous recapture location during surveys performed from 1991 - 2014. These individuals were similarly coded as a "1" or "0" to indicate whether they were recaptured in the same pool or not. These individuals do not provide the same information on temporal variation in TTX levels and patterns of site fidelity as do individuals sampled two or more times, but they do allow us to use a much larger sample size to evaluate the relationship between movement, morphological predictors, and deviation in TTX concentration from the daily mean value.

Finally, to better understand breeding site fidelity at the population level, we evaluated the recapture patterns of ~ 1,500 encountered newts during surveys using our long-term data set . Beginning with recapture data from 1991, we calculated the percentage of individuals repeatedly recaptured in the same pool as compared to those that were recaptured in different pools when surveying. These data assist us in placing our four years of movement data into a much larger temporal context and allow us to compare whether observed movement among pools from 2011 - 2014 was extraordinary relative to movement over the previous two decades.

Statistical methods: We performed a series of analyses with classification models, first on our data set composed of 21 individuals recaptured and sampled 2 - 4 times, and then repeated the analysis with the data from the 56 individuals sampled only once. In general, the modeling approach evaluated the relationships between *T. torosa* breeding pool fidelity, individual morphological and TTX measurements, and temporal variables. The first model implemented tree regression (*TREE* v. 1.0-29) [75] in R [76] to identify predictors associated with breeding pool fidelity at Cold Creek. Tree regression uses a binary recursive partitioning method to construct a classification tree, hierarchically ranking predictor variables according to how much and at what level they explain variance in the response variable. Tree regression is attractive because it is easily interpretable and indicates directionality and strength of a response, but is inadequate as a predictor given the limited statement it can make between response and predictor relationships [77].

To better understand which variables predict male fidelity to a breeding pool, we constructed a second model that applied tree regressions (n=10000) using the *RANDOMFOREST* framework (v. 4.6-6) [78] in R. This algorithm iteratively and randomly removes variables to evaluate relationships between predictor and response variables [78]. We chose the random forest model rather than more standard linear regression because it is a non-parametric procedure that does not assume an underlying distribution of the data. It has also been shown to outperform regression [79], to adequately handle clustered data sets with repeated measures [80,81], and to describe the role of continuous variables across a landscape [82]. Another benefit of random forest models is their ability to handle complex interactions between variables [83].

The predictor variables used in tree regression and random forest models were obtained from individual morphological measurements and derived TTX deviation values on the day of each recapture event. We also included temporal variables, including year, month of sampling, time between sampling events, and a variable that we refer to as "measure." The variable measure indicated the cumulative number of samples collected from an individual during the four years and was used to detect a potential effect of repeated sampling on TTX levels. Time between measurements was incorporated to account for potential lag effects.

To assess the robustness of our models, we made predictions from our data set of 21 individuals using the *predict* function in R. Predicted and observed values were statistically tested using a chi-square test in a 2x2 contingency table. In addition, we estimated p-values for each predictor by producing a null distribution of 1,000 permutation replicates of the random forest model in R with the package *rfPermute* (v. 1.6.2).

Finally, we computed matrices of Spearman's rank correlations coefficients using the *Hmisc* framework (v. 3.9-3) [84] in R to test for correlations between TTX concentrations and measured individual body condition traits, which included mass, SVL, and tail height. We performed this analysis using our data set of 21 individuals to determine whether greater individual TTX deviation values were associated with temporal patterns of males with greater mass, size, or tail height.

RESULTS

Mark and recapture: Our collections were heavily male-biased, composed of 409 encounters with males and 75 encounters with females. This result is consistent with other studies of *T. torosa* [49], as well as other salamandrids [85, 86]. As previously stated, we performed separate analyses on the 2011 - 2014 data set, one set which was based on the 21 individuals recaptured and sampled 2 - 4 times and the other set composed of 56 individuals sampled only once. However, we report the results of each analysis together in the following sections.

Individual and group mean TTX concentrations**:** For this study, a total of 166 tissue samples were collected to quantitate TTX concentrations of males within Cold Creek. Individual and group mean TTX concentrations through time are shown in Figure 4.1. Individual male TTX concentrations ranged from 0.00 to 5.72 nmol TTX/mg skin (Table 4.2). Group means varied within a season and between years, fluctuating from a high of 2.22 nmol TTX/mg on May 15, 2012, to a low of 0.03 nmol TTX/mg on April 24, 2013 (Table 4.3). Overall, the greatest and least TTX deviation values were from individuals sampled in March 2011 (+3.60) and May 2012 (-2.04).

Temporal patterns of individual breeding site fidelity: All of the 21 individuals that were sampled multiple times for TTX were found in the 10 largest pools within Cold Creek; none were found in the intervening runs and riffles. Fidelity to a pool was common year after year for the majority of individuals. Of the 21 individuals that were recaptured, 7 males were found in different pools and the remaining 14 males (~ 64%) were repeatedly recaptured in the same pool (Figure 4.2). Similarly, our evaluation of the 56 individuals sampled only once showed that 35 individuals (~ 62%) were recaptured in the same pool. Finally, using the survey data collected from 1991 - 2014, we found that nearly 72% of newts were recaptured in the same pool year after year.

Movement among pools: Tree regression indicated that fidelity to a breeding pool is best explained by individual TTX deviation values and morphology (Figures 4.3a, 4.4a). The most important correlate was an individual's relative TTX level, such that recaptures repeatedly occupying the same pool were associated with TTX concentrations above the mean TTX concentration of all males in the stream at that time of sampling. Mass and SVL were the next most important predictors of movement. Overall, smaller, low-mass individuals with below average TTX concentrations tended to be recaptured in different breeding pools over time.

The randomly permuted tree regressions and resulting random forest model showed that individual TTX deviation values and morphological measurements consistently explained fidelity to a breeding pool more than time related predictors (Figures 4.3b, 4.4b). As with the tree regression, TTX deviation value was the most important predictor, followed by mass and SVL. In addition, tail height was identified as a strong predictor in this model, but not in tree regression. This could be due to cross-correlation with other morphological predictors, such as mass and SVL because larger males tend to have taller tails.

Predicted versus observed values did not significantly differ from one another (*chi-square*, *df*=1, *p*=0.84). All estimated p-values generated in *rfPermute* for model predictors exceeded the 0.05 α level except TTX deviation. The estimated p-values for each predictor in the random forest model are shown in Table 4.3. The permuted null model and observed values for relative TTX levels are shown in Figure 4.5.

Test statistics from Spearman's rank analyses indicated that no morphological trait was significantly correlated with TTX concentrations (*mass*: *p*=0.88; *SVL*: *p*=0.89; *tail height*: *p*=0.68).

DISCUSSION

Although breeding site fidelity is a well-documented phenomenon in a number of amphibian species, a proximate understanding of why some individuals repeatedly use the same site and others do not remains obscure. In a few cases where it has been observed, individual attributes have been identified as a potential causal explanation. For example, Kopecky et al. [88] observed that male Alpine newts (*Mesotrition alpestris*) with high body condition tended to move among adjacent, human-created breeding "rut" sites, perhaps because such movement enhances the most fit male's ability to gain increased access to mates. However, most studies simply document the fraction of individuals that move among breeding sites (e.g. [89]). If we are to understand why some individuals have strong site fidelity and others do not, and use that information to better understand metapopulation dynamics and conserve declining species, a more mechanistic grasp of site fidelity is necessary.

In our system, an individual's relative TTX concentrations best explained site fidelity patterns, such that males with low TTX levels relative to other males concurrently occupying the stream tended to be recaptured in different pools. Roughly two thirds of newts were repeatedly recaptured in the same pool during our 2011 - 2014 study, while the remaining third moved among pools separated by more than 20 meters. From our analyses, these movement patterns appear to be strongly related to relative TTX concentrations. Tree regression and random forest models on both sets of recapture data indicate that temporal patterns do not explain these differences (Figure 4.3, Figure 4.4). Rather, relative TTX concentrations and morphological features best explain fidelity to a breeding pool. We consider the similarity in model results for the two different data sets, which cover different temporal periods to be a strong indication of the importance of TTX in site selection and breeding pool fidelity for *T. torosa*. Furthermore, the percentage of newts recaptured in the same pool during our 20-year mark-recapture study (~ 72 %) is similar to the percentages of newts that exhibit site fidelity in both analyzed data sets, further supporting the interpretation that our focal set of 21 individuals recaptured and sampled during 2011 - 2014 are a reasonable sample of the population.

Surprisingly, greater TTX concentrations do not appear to be a correlate of male body condition, and larger males do not appear to have greater concentrations of TTX. Although individual mass, SVL, and tail height were important predictors in the models, the correlation across 21 individuals between TTX concentrations and body condition was low and non-significant. This suggests that morphological traits may be important not only for mate choice [90], but together with TTX concentrations, may affect male intraspecific interactions.

Animal communication and numerous behavioral processes are shaped by olfactory cues [34-41]. Relevant to our study, chemical cues from male wall lizards (*Podarcis hispanica*) afford conspecifics an opportunity to evaluate competitors [91], allowing competitively inferior males to avoid areas occupied by competitively superior males. In *Taricha*, some adults will remain in pools months after breeding has ceased and these adults can be cannibalistic. Tetrodotoxic chemical cues from nearby adults are sensed by larvae and elicit an antipredatory response [51, 71], and it would not be surprising if TTX is used as an olfactory cue at other life history stages. Adult *T. torosa* engage in complex courtship rituals, and males may be able to gauge competitive or other biological attributes of resident conspecifics via tetrodotoxic cues in pools from rivals, using such information to determine whether to remain in or move out of a pool.

An additional striking result of this study is the amount of TTX variation observed within an individual (Figure 4.2) and one breeding population (Figure 4.1). In principle, broad geographic population variation in *Taricha* TTX levels has been explained as a result of a coevolved relationship with TTX-resistant predatory garter snakes [92] and to a lesser extent, as a response to selection pressure from other predators [64-67]. However, mismatch between garter snake resistance and newt chemical defenses has been observed in over a third of sampled populations [93]. While such geographic patterns may be accurate, they are all based on newts sampled at a single point in time. Certainly the population mean is important, but based on our results it appears that an individual's TTX concentrations, and that of an entire breeding population fluctuate over space and time under natural conditions. The documented individual variation of TTX levels we have observed nearly spans the entire range measured in populations across much of California [93]. Thus, it seems possible that fluctuating TTX levels may be an important component in explaining the observed mismatch between newt TTX levels and garter snake resistance to the toxin. Ultimately, variation in other populations should be assessed across time to better understand what variables drive fluctuations and to more thoroughly evaluate the ecological and evolutionary role of *Taricha* and TTX.

In the general context of a predator-prey model, the complexities of *Taricha* and TTX may have been obscured in part by assuming that newt chemical defenses genetically respond to selection pressure from predators. Such scenarios require that there is a genetic component to *Taricha* chemical defenses, but there is no conclusive evidence for a pathway by which *Taricha*, or any other animal biosynthesizes or regulates TTX (although one has been proposed [94]). The toxin could be the product of endosymbionts, as it is in many other taxa [56], or it may be endogenously biosynthesized uniquely in newts [94]. Enzymes responsible for TTX biosynthesis have not been found in *Taricha* and there is no compelling evidence of bacterial production of TTX in newts [95]. Researchers over the last several decades have tried to determine the proximate mechanisms controlling *Taricha* TTX, but with no definitive conclusions, and more recent efforts evaluating the maintenance of TTX in newts under captive conditions have reach contradictory results. Studies of *Taricha* demonstrate that individual's TTX concentrations are sustained and vary in captivity regardless of diet [62, 96], suggesting a de novo source for TTX production, but TTX is not present in other tetrodotoxic newts reared in captivity from egg [97]. Thus, it remains possible that individual fluctuations and population variation of *Taricha* TTX levels are entirely or largely environmentally induced, or that TTX is a metabolic byproduct with little direct evolutionary significance.

One possible explanation we offer for the observed variation in TTX levels within and between populations is that there may be within-species automimicry at the TTX level. If a pool consists of newts with varying concentrations of TTX, then those with low concentrations may be benefitting from conspecifics without paying an assumed physiological price of maintaining higher TTX levels. While plausible, such a scenario predicts that individuals with low TTX concentrations should stay in a pool, benefiting from the safety in numbers afforded by higher-TTX conspecifics [98,99]. However, we found the opposite, which suggests either i) local predation pressure is not as strong in this population as it is in others [100], and as a result is not driving the relationship between TTX and movement frequency; ii) that cheating is not tolerated, especially in the relatively small breeding pools of southern California; or iii) it may be that males repeatedly moving between pools expend more energy, and as a result cannot pay the cost of both chemical defense and the energetic demands of migration. However, no data detail the cost of TTX production and maintenance in *Taricha*.

Even though *T. torosa* is protected as a Species of Special Concern in southern California [101] it continues to face challenges ranging from habitat loss to urbanization to invasive crayfish predators, stream siltification, wildfires and drought. Strong site fidelity is thought to have evolved as an effective life history strategy, but if newts home to degraded habitat, then populations may face adverse effects [102-104]. Given that individuals with below-average TTX concentrations frequently move greater distances than those with above average TTX concentrations, and that ongoing drought conditions in southern California almost certainly make migration ecologically costly in the xeric southern California landscape, our results point to TTX as a potentially important variable in the conservation of this declining species. Whether TTX can be used as a management tool requires that we determine the causes and consequences of its variation, including its relationship to site fidelity and breeding success. However, if TTX is genetically controlled at some level, and an important component of migratory behavior and survival, then conservation strategies involving assisted migration may benefit from translocating animals from populations where TTX levels of newts are well characterized.

In general, our results indicate that relative TTX levels are strongly associated with breeding site fidelity, such that individuals with below average relative TTX levels were often recaptured in new breeding sites much more than those with above average relative TTX levels. Thus, the social context of TTX appears to be an important contributor to the migration probability and breeding biology of newts, rather than TTX purely serving an antipredator function. Understanding the interaction between TTX variation, breeding site fidelity, and antipredator behavior should assist evolutionary ecologists and conservation managers working to attenuate amphibian declines in California. Whatever the final answers, it does not appear that the *Taricha* TTX phenotype is stable, nor that selection from predators fully explains TTX variation or its ecological role in the evolution of newts.

Figure 4.1. Individual TTX concentrations and daily mean TTX values derived from recaptured adult male *T. torosa* in Cold Creek Preserve from 2011 through 2014. Solid diamonds represent mean male TTX concentrations from all individuals sampled on that date and include standard error bars. Solid circles depict individuals that were repeatedly recaptured throughout the study and are linked to their temporally appropriate mean with dashed vertical lines. Concentrations are presented in units of nanomoles TTX/mg skin and milligrams TTX/g skin for comparative purposes. Note the break in the scale of TTX concentration.

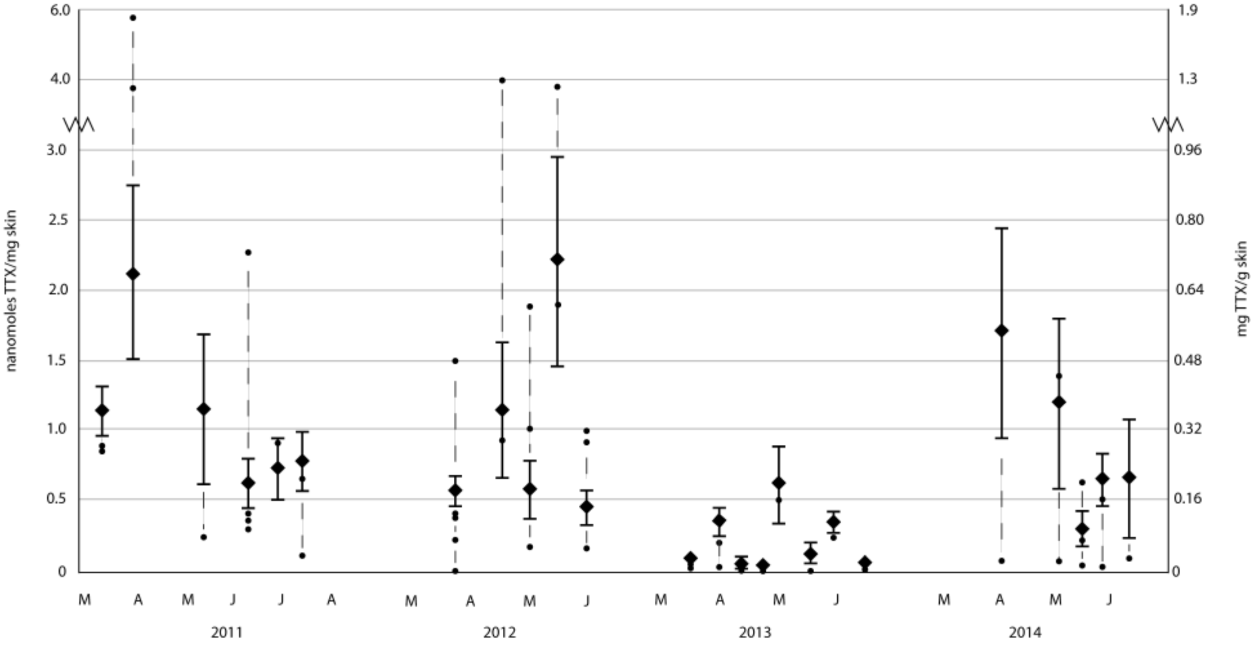

Figure 4.2. Breeding pool fidelity and TTX chronologies of 21 adult *T. torosa* recaptured over a four-year period in a 430 m stretch of Cold Creek. The vertical bar represents the length of the study site in Cold Creek, with each alternating segment scaled to a 20 m section of the stream (note the break in scale). Grey circles along the vertical bar represent the 10 breeding pools. Each entire horizontal dashed line represents an individual newt and the four years of sampling for that individual. Arrows point to the breeding pools where an individual has been recaptured. All individuals depicted on the left were recaptured each time in the same pool, while those shown on the right were repeatedly recaptured in different pools. Each colored circle represents an individuals TTX levels at a point in time. The inner color of a circle shows an individual's TTX concentration when sampled on that day, while the stroke of each circle illustrates the mean TTX concentration of all males in the stream on that same sampling day. The legend shows toxin levels based on previous measurement values [94] and are provided in units of mg TTX as well as nanomoles TTX for comparative purposes between studies.

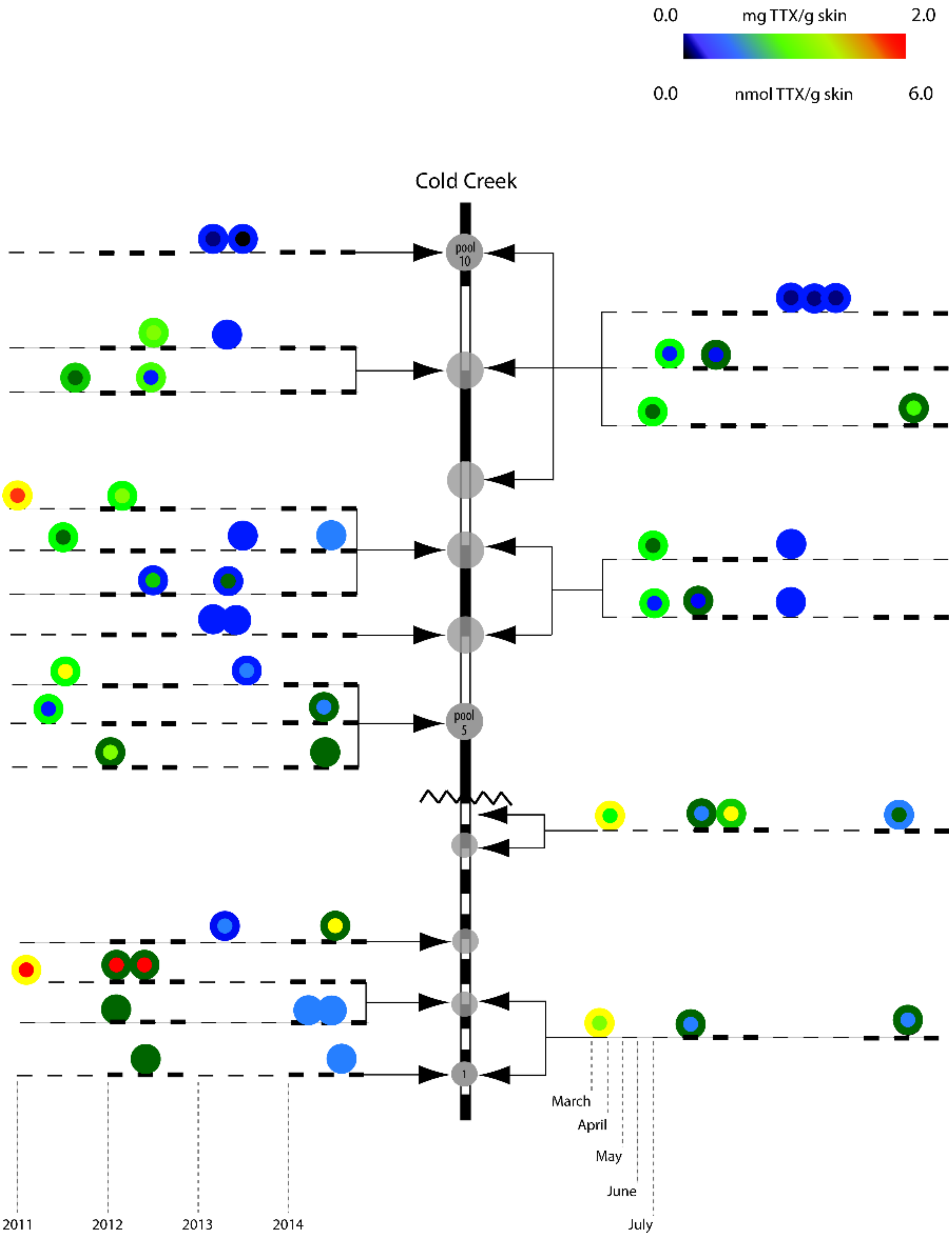
0.0
mg TTX/g skin
2.0
0.0
nmol TTX/g skin
6.0
Cold Creek
pool 10
pool 5
1
March
April
May
June
July
2011
2012
2013
2014

Figure 4.3. a) Tree regression results depicting the relationship between breeding pool fidelity and individual's relative TTX concentrations and morphology for the 21 newts recaptured more than once in Cold Creek. Each node shows which variable hierarchically explained fidelity. Values at the right side of a branch indicate where a split in that variable explains fidelity to a breeding site. The length of a branch illustrates the amount of deviance a variable can explain. b) Importance scores for each variable used in the random forest model as measured by Gini coefficients. Each variable is ordered top-to-bottom from most to least important in explaining fidelity. Gini coefficients represent a measure of node impurity in the classification tree. Predictors with a higher mean decrease in accuracy score are more important variables in explaining fidelity within the random forest model**.**

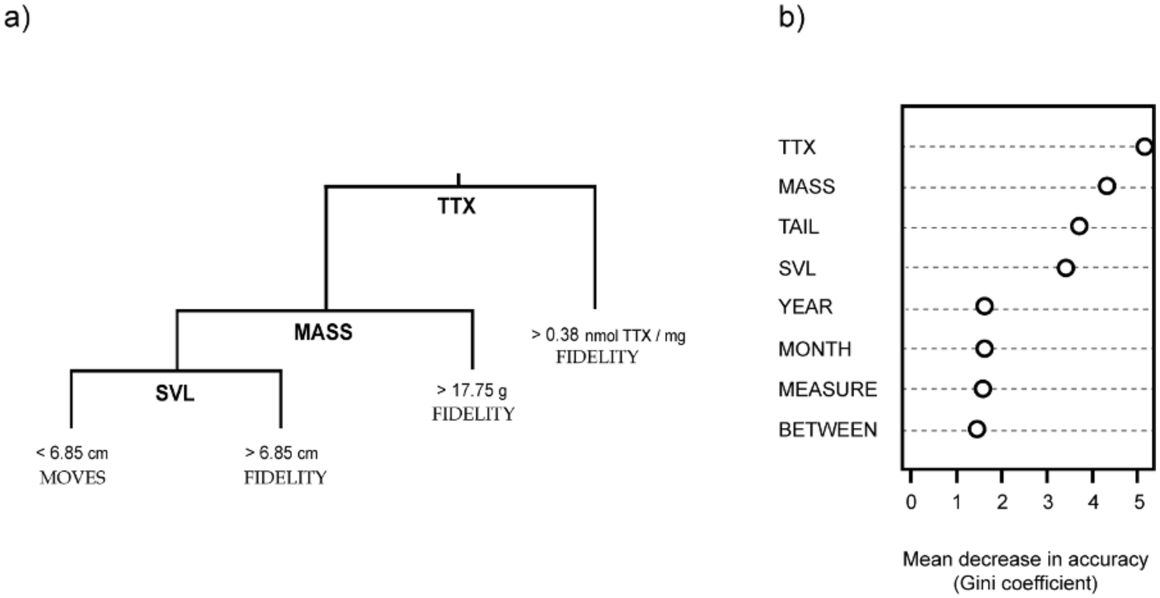

Figure 4.4. a) Tree regression results using site fidelity, relative TTX, and morphology data from 56 individuals sampled only once in Cold Creek. Individuals that tend to be recaptured in the same pool have relative TTX concentrations greater than 0.12 nmol or greater than - 0.16 nmol TTX/mg from the average. Individuals with SVL measurements less than 7.38 and with lower relative TTX concentrations tend to be recaptured in different pools ("moves"). b) Importance scores for each variable used in the random forest model as measured by Gini coefficients. Both models indicate TTX is the most important predictor in explaining breeding site fidelity, followed by morphological predictors, which corresponds to the results observed in the analysis of the 21 individuals recaptured and sampled multiple times.

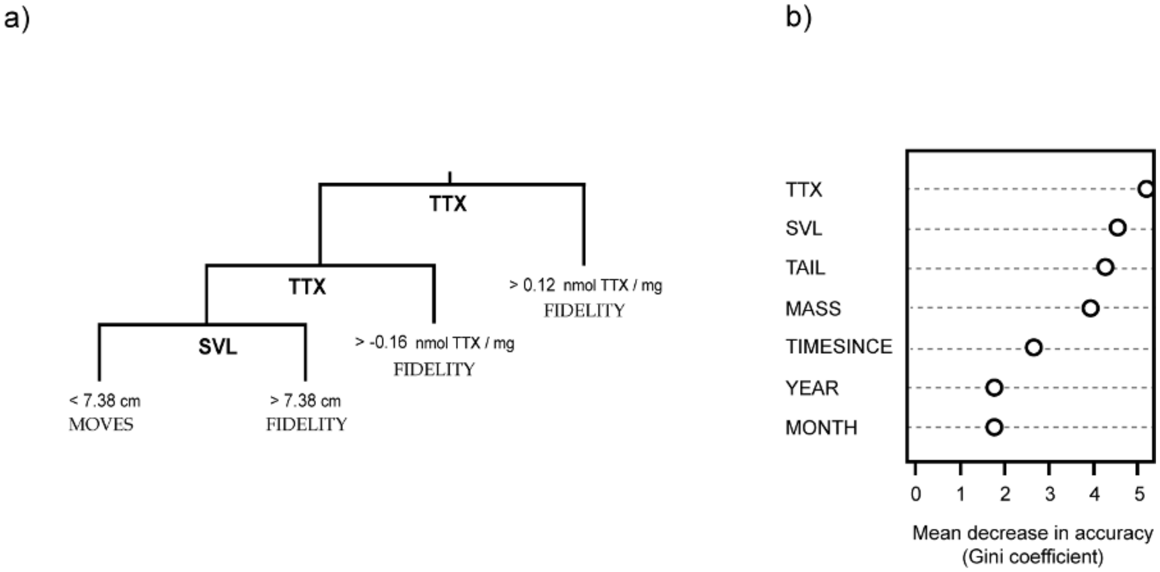

Figure 4.5. A null distribution of permuted mean decrease in accuracy importance scores of the relative TTX predictor used in the random forest model. The Y-axis shows the kernel density of the null distribution, such that the area under the curve = 1. The X-axis represents permuted values of the predictor variable for relative TTX values. The vertical line shows the observed value from the random forest model. The null distribution was used to estimate the p-value of the predictor (p = 0.02). All other p-values for model predictors were not significant.

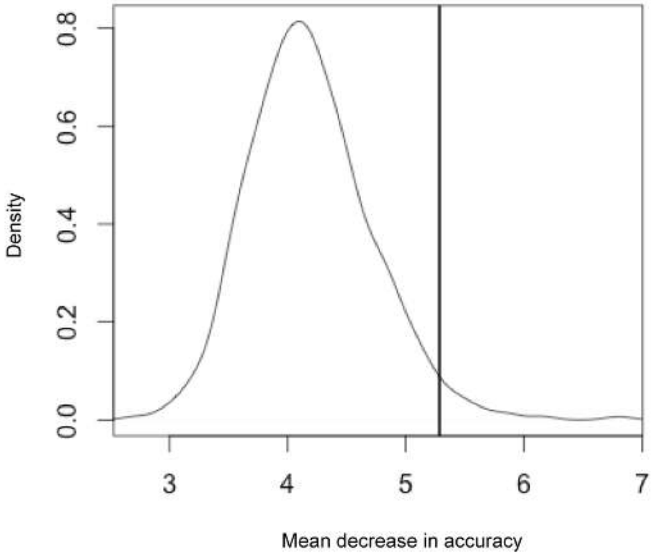

Table 4.1. Individual TTX measurements of recaptured male *T. torosa* over the four-year study period, starting in 2011. Concentrations range from 0.000 – 5.719 nanomoles per mg of tissue.

Individual	Recapture date	TTX (nmol/mg)
a	3/26/11	0.837
a	3/28/12	0.188
a	5/12/12	1.900
a	5/15/14	0.676
b	3/28/11	5.719
b	4/21/12	3.948
b	5/12/12	3.714
c	3/28/11	0.883
c	3/28/12	0.387
c	5/2/14	0.039
d	3/28/11	3.571
d	4/26/12	1.027
e	6/20/11	2.200
e	5/31/13	0.268
f	6/20/11	0.378
f	3/23/13	0.061
g	6/20/11	0.304
g	3/28/12	0.000
g	3/23/13	0.096
h	6/20/11	0.341
h	6/12/13	0.059
h	6/5/14	0.085
i	7/7/11	0.125
i	4/26/12	0.180
j	7/7/11	0.643
j	6/1/12	0.185
k	6/1/12	0.841
k	5/31/13	0.527
l	6/1/12	0.930
l	5/24/13	0.091
m	4/10/13	0.033
m	4/24/13	0.057
n	4/18/13	0.035
n	5/24/13	0.000
o	4/29/13	0.028
o	5/24/13	0.053
o	6/12/13	0.097
p	3/28/12	0.403
p	4/10/14	0.052
p	5/15/14	0.032
q	6/22/11	0.802
q	5/2/14	1.438
r	4/21/12	0.913
r	5/15/14	0.215
s	5/3/11	0.232
s	5/22/14	0.067
t	3/28/12	1.564
t	5/22/14	0.499
u	4/10/13	0.223
u	5/2/14	0.562

Table 4.2. Mean TTX concentrations from 166 adult male *T. torosa* sampled on the same day as a recapture. The mean for each date was calculated using all individuals sampled on the same day as a recapture. The standard deviation (SD) and standard error (SE) are provided to describe amounts of variation in newt chemical defenses within the breeding sites across time.

Date	n	Mean TTX (nmol/mg)	SD	SE
3/26/11	6	1.137	0.376	0.188
3/28/11	9	2.123	1.887	0.629
5/03/11	5	1.149	1.220	0.546
6/02/11	5	0.444	0.296	0.133
6/20/11	7	0.617	0.468	0.191
6/22/11	5	0.718	0.456	0.228
6/27/11	3	4.476	5.795	3.346
7/07/11	5	0.773	0.490	0.219
3/28/12	15	0.563	0.457	0.118
4/21/12	7	1.147	1.304	0.493
4/26/12	7	0.572	0.579	0.219
5/12/12	3	2.225	1.355	0.782
6/01/12	5	0.444	0.296	0.133
3/23/13	5	0.079	0.047	0.021
4/10/13	5	0.344	0.231	0.103
4/18/13	5	0.053	0.038	0.017
4/24/13	7	0.035	0.022	0.010
4/29/13	7	0.610	0.755	0.285
5/24/13	7	0.122	0.218	0.083
5/31/13	5	0.344	0.193	0.086
6/12/13	7	0.061	0.022	0.009
4/10/14	7	1.705	1.869	0.763
5/02/14	5	1.195	1.239	0.620
5/22/14	5	0.645	0.446	0.199
5/15/14	10	0.301	0.432	0.137
6/05/14	9	0.659	1.314	0.438

Table 4.3. Predictors, mean decrease in accuracy Gini scores, and the estimated p-values from permuted null distributions. The only significant model predictor from the null models was relative TTX values.

Predictor	Mean Decrease Gini	Estimated p-value
Relative TTX	5.28	**0.02**
Mass	4.23	0.35
Tail height	3.92	0.59
SVL	3.26	0.98
Year	1.62	0.26
Month	1.64	0.78
Measure	1.37	0.33
Time between measurement	1.46	0.44

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