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ORIGINAL ARTICLE

Is there more than one way to skin a newt? Convergent toxin resistance in snakes is not due to a common genetic mechanism

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Convergent evolution of tetrodotoxin (TTX) resistance, at both the phenotypic and genetic levels, characterizes coevolutionary arms races between amphibians and their snake predators around the world, and reveals remarkable predictability in the process of adaptation. Here we examine the repeatability of the evolution of TTX resistance in an undescribed predator–prey relationship between TTX-bearing Eastern Newts (*Notophthalmus viridescens*) and Eastern Hog-nosed Snakes (*Heterodon platirhinos*). We found that that local newts contain levels of TTX dangerous enough to dissuade most predators, and that Eastern Hog-nosed Snakes within newt range are highly resistant to TTX. In fact, these populations of Eastern Hog-nosed Snakes are so resistant to TTX that the potential for current reciprocal selection might be limited. Unlike all other cases of TTX resistance in vertebrates, *H. platirhinos* lacks the adaptive amino acid substitutions in the skeletal muscle sodium channel that reduce TTX binding, suggesting that physiological resistance in Eastern Hog-nosed Snakes is conferred by an alternate genetic mechanism. Thus, phenotypic convergence in this case is not due to parallel molecular evolution, indicating that there may be more than one way for this adaptation to arise, even among closely related species.
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INTRODUCTION

The repeated evolution of similar phenotypes in response to similar selective pressures, or convergent evolution, is a pervasive evolutionary phenomenon (Schluter, 2000; McGhee, 2011; Futuyma, 2013). Major questions remain regarding the ecological and evolutionary causes of convergence, including whether convergent evolution most often results from selection toward optimality, or from constraints on design, genetic architecture or other contingencies (Maynard Smith *et al.*, 1985; Wake, 1991; Brakefield, 2006; Christin *et al.*, 2010; Losos, 2011). Examining the genetic basis of convergence provides insight into these questions, because we can assume selection is relatively unconstrained when similar phenotypes are produced through diverse genetic routes and, conversely, that constraints may be important when phenotypes are determined by a limited subset of possible genetic mechanisms (Miller *et al.*, 2006; Weinreich *et al.*, 2006; Gompel and Prud'homme, 2009; Losos, 2011; Conte *et al.*, 2012; Feldman *et al.*, 2012). Exploration of these questions requires independent systems with similar, well-defined selection pressures. Chemically mediated interactions among species may provide especially productive systems for examining convergence, because these interactions frequently revolve around compounds with very specific biological effects (Brodie and Ridenhour, 2003).

Animals frequently employ chemical defenses to protect themselves against predation. One of the most potent chemical weapons ever discovered is tetrodotoxin (TTX), a lethal poison found across diverse animal phyla (Hanifin, 2010; Moczydlowski, 2013). TTX is highly effective because of its extremely specific mode of action. It binds selectively to the outer pore of voltage-gated sodium channels (Na_v proteins) in nerves and muscles, blocking the movement of sodium ions (Na^+) across the cell membrane and halting the initiation and propagation of action potentials (Hille, 2001; Fozzard and Lipkind, 2010). By arresting nerve impulses in muscle and nervous tissue, TTX causes immobilization, respiratory failure and often death (Brodie, 1968a; Hanifin, 2010; Moczydlowski, 2013).

Although TTX is found in a diverse array of taxa (Hanifin, 2010; Moczydlowski, 2013), only a few vertebrate groups appear to have evolved the ability to tolerate TTX, including tetraodontid fishes, newts, some frogs and a handful of snakes (for example, Soong and Venkatesh, 2006; Feldman *et al.*, 2012; Hanifin and Gilly, 2015). Of these taxa, snakes are among the only vertebrates known to regularly consume TTX-defended prey (Feldman *et al.*, 2012). Given the highly specific action of TTX, one mechanism of TTX resistance seems obvious: functional changes to the outer pore of sodium channels that reduce the affinity of TTX to the protein. Indeed, all TTX-resistant

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vertebrates examined thus far have remarkably similar mutations that alter the structure of the outer pore and reduce TTX-binding affinity to the channel (Geffeny *et al.*, 2005; Soong and Venkatesh, 2006; Jost *et al.*, 2008; Feldman *et al.*, 2012; McGlothlin *et al.*, 2014; Hanifin and Gilly, 2015). In fact, this mechanism of TTX resistance appears nearly identical across several independent, coevolutionary arms races involving predatory snakes and their TTX-defended amphibian prey (Feldman *et al.*, 2009, 2012), as well as across sodium channel paralogs within species (McGlothlin *et al.*, 2014), suggesting that adaptation to TTX is highly predictable (Jost *et al.*, 2008; Feldman *et al.*, 2012; McGlothlin *et al.*, 2014).

We examined a previously undescribed predator–prey relationship to provide an additional test of the predictability of the evolution of TTX resistance at the molecular level, with surprising results. Eastern Hog-nosed Snakes (*H. platirhinos*) are one of a few vertebrates that regularly consume TTX-defended Eastern Newts (*N. viridescens*) (summarized in Table 1), even consuming the brightly colored efts (a terrestrial dispersal stage) that are up to 10 times more toxic than adult newts (Brodie, 1968b; Brodie *et al.*, 1974). Here we show that *H. platirhinos* are highly resistant to TTX, where they are sympatric with *N. viridescens*, indicating that these snakes have evolved resistance to their dangerous prey. However, we find no evidence of adaptive changes in the gene controlling TTX resistance in skeletal muscle of all other vertebrates (*SCN4A*) in Eastern Hog-nosed Snakes. Instead, the evolutionary path to TTX resistance in *H. platirhinos* remains enigmatic. These results highlight the unpredictable nature of genetic evolution, even for traits and ecological challenges typically characterized by a high degree of constraint.

MATERIALS AND METHODS

Prey phenotype assays

To assess prey phenotypes, we quantified TTX in the skin of 10 *N. viridescens* efts from New York and 51 from Virginia (Figure 1). We extracted TTX from 1 cm^2 skin samples (Hanifin *et al.*, 2002; Lehman, 2007) and measured TTX concentrations using high-performance liquid chromatography (New York samples) (Yasumoto and Michishita, 1985) or competitive inhibition enzymatic immunoassay (Virgania samples) (Lehman, 2007; Stokes *et al.*, 2012), using the linear range of the standard curve between 10 and 500 ng ml^{-1} to quantify TTX and scoring samples as lacking TTX with values less than the minimum level of detection (10 ng ml^{-1}; $n=1$). It is noteworthy that these two methods of quantification (high-performance liquid chromatography and competitive inhibition enzymatic immunoassay) give nearly indistinguishable measures of TTX concentrations (Lehman, 2007). We then extrapolated measures of TTX in our skin samples to the whole animal using the calculation from Hanifin *et al.* (2004, 2008).

Predator phenotype assays

To assess predator phenotypes, we assayed TTX resistance in 29 *H. platirhinos* from 9 localities (Table 2 and Figure 1) and 1 *Heterodon nasicus* (Plains Hog-nosed Snake), a species that does not consume newts. Our geographic sampling focused mainly on populations in New York, where one of us (KEB) made initial observations of *H. platirhinos* consuming efts in the wild (Barnett *et al.*, 2006). We also included two samples from more disparate locations to make a cursory examination of geographic variation of TTX resistance in *H. platirhinos*: one from northern Virginia (sympatric with newts) and one from eastern Texas (allopatric with newts) (Table 2 and Figure 1).

We measured TTX resistance using a well-established bioassay of whole-animal performance (Brodie and Brodie, 1990; Ridenhour *et al.*, 2004). Briefly, we placed snakes on a 4-m track lined with infrared sensors to record their sprint speed pre- and postinjection with TTX. After measuring the preinjection baseline speed of each snake, we injected TTX starting at 1 mass-adjusted mouse unit (MAMU) for snakes allopatric with newts (our *H. platirhinos* from TX and *H. nasicus*) and 10 MAMUs for snakes sympatric with newts (all other *H. platirhinos*). We then serially increased the doses (5, 10 and 25 MAMUs for snakes allopatric with newts; 25, 50, 100 or 250 MAMUs for sympatric snakes) until we could calculate the dose required to reduce sprint speed 50%. It is worth noting that our units of TTX are in MAMUs, where 1 MAMU is the amount of TTX needed to kill a 20-g mouse in 10 min, adjusted to the mass of each snake. Because of the prohibitive cost of TTX, in a few cases we stopped increasing the dose before a 50% reduction in speed could be estimated, resulting in a measurement that underestimates true TTX resistance. We omitted snakes that refused to crawl down the racetrack ($n=2$).

Predator functional genotype assays

To determine the genetic basis of TTX resistance in snakes, we sequenced the gene (*SCN4A*) that encodes the skeletal muscle sodium channel ($Na_v1.4$) from a subset of the hog-nosed snakes we assayed for TTX resistance. These samples included highly resistant sympatric snakes and nonresistant allopatric snakes (six *H. platirhinos* and one *H. nasicus*). The single α-subunit of $Na_v1.4$ forms a membrane-spanning channel that allows selective permeation of Na^+ ions (Goldin, 2001; Hille, 2001). The subunit consists of four domains (DI–DIV), each containing four pore forming segments (P-loops) that fold back into the cell membrane to create the outer pore, which is lined with two negatively charged rings of amino acids and terminates in a narrow selectivity filter that preferentially allows Na^+ ions to pass through the channel (Goldin, 2001; Fozzard and Lipkind, 2010). These same structures that line the outer

Table 1 Records of Eastern Newt (*N. viridescens*) predation by Eastern Hog-nosed Snakes (*H. platirhinos*)

Location of predation by H. platirhinos	*Snake age class*	*Newt stage*	*Source*
Washington Parish, LA	Neonate/juvenile	Eft	Williams, 2011
Ozark County, MO	Subadult/adult	Adult	M. Nickerson (personal communication)
Wake County, NC	Neonate/juvenile	Eft	Palmer and Braswell, 1995
Wake County, NC	Subadult/adult	Eft	Hurst, 1963
New Hanover County, NC	Neonate/juvenile	Eft	J Hall and R Myers (personal communication)
Vinton County, OH	Subadult/adult	Eft	C. Brune and D. Sapienza (personal communication)
Saratoga County, NY	Neonate/juvenile	Eft	Barnett *et al.*, 2006; this study
Northampton County, PA	Subadult/adult	Eft	McDonald, 1987
Montgomery County, TX	Juvenile/subadult	Eft	This study
George Washington National Forest, VA	Unknown	Eft	Uhler *et al.*, 1939
Waukesha County, WI	Neonate/juvenile	Eft	Koch, 2009

It is worth noting that most predation events involve neonate or juvenile snakes and all but one record involves efts: the brightly colored sub-adult dispersal stage of Eastern Newts that are up to 10 times more toxic than the adult newts (Brodie, 1968b,Brodie *et al.*, 1974). Records are mapped in Figure 1 and suggest that hognose predation may be widespread and common. Furthermore, feeding trials in captivity suggest that Eastern Hog-nosed Snakes can take multiple efts in a single session. One of us (KEB) has fed up to three efts at a time to captive Eastern Hog-nosed Snakes, where snakes and efts were from the same location in NY; another biologist has fed up to 15 efts to a captive Eastern Hog-nosed Snakes in a single feeding session, where snakes and efts were from the same location in OH (D Sapienza, personal communication).

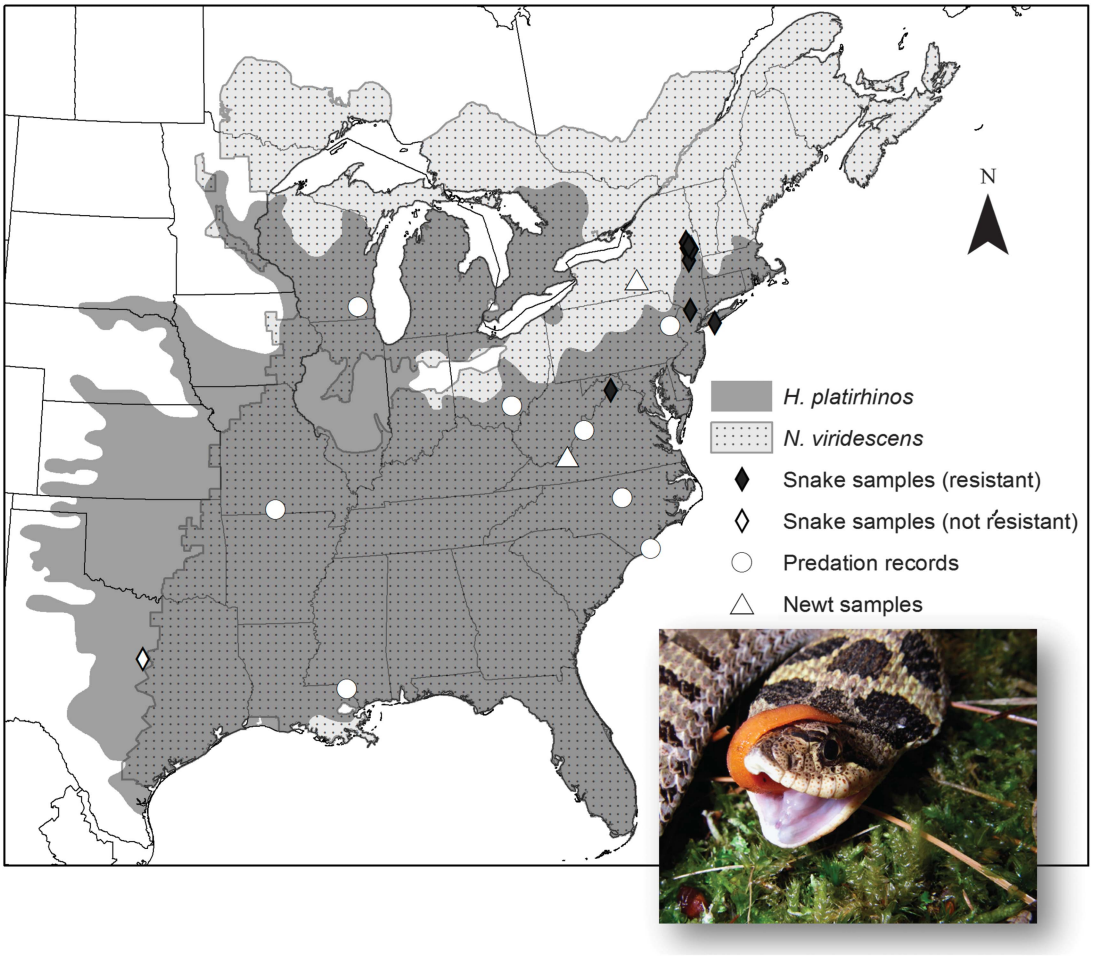

Figure 1 Distribution of Eastern Newts *N. viridescens* (stippled) and Eastern Hog-nosed Snakes *H. platirhinos* (gray), showing documented records of newt predation by snakes (circles; Table 1), as well as sample localities of newts (triangles; Figure 2) and snakes (diamonds) used in this study (Table 2). Inset shows an *N. viridescens* eft being ingested (only the tail can be seen) by a *H. platirhinos* from eastern NY (photo by KEB).

Table 2 Locality information, phenotypes, and sample sizes of hog-nosed snakes *(Heterodon)*

Species locality	*TTX resistance (50% MAMU)*	n *TTX bioassay*	n *SCN4A sequence*
H. platirhinos			
Saratoga Springs, Saratoga County, NY	>250	5	1[a]
Greenfield Center, Saratoga County, NY	>250	8	3
Albany, Albany County, NY	100–>250	6	—
Town of Oyster Bay, Nassau County, NY	75–>250	5	—
Sterling Forest, Orange County, NY	>100	1	—
Luzerne, Warren County, NY	>100	1	—
Wilton, Saratoga County, NY	>100	1	—
no specific locality, Frederick County, VA	>100	1	1
Lake Whitney, Bosque County, TX	5	1	1
H. nasicus			
no locality data (captive bred)	5	1	1

Abbreviations: MAMU, mass-adjusted mouse unit; TTX, tetrodotoxin.
Locality information of Eastern Hog-nosed Snakes (*H. platirhinos*) and Plains Hog-nosed Snakes (*H. nasicus*) sampled for phenotypic (TTX resistance) and genotypic (*SCN4A*) variation, along with corresponding sample sizes (*n*).
[a]Sample also used to obtain full transcript (cDNA) of *SCN4A*.

pore and permit selectivity and permeability of Na^+ through the channel also bind strongly to TTX, which fits into the outer pore through a combination of chemical bonds and steric attraction, essentially docking in the outer pore and blocking Na^+ movement (reviewed in Fozzard and Lipkind, 2010). Thus, we focused on variation in the portions of the four domains (DI–DIV) of *SCN4A* that code for the P-loops that interact with TTX; changes at specific P-loop sites

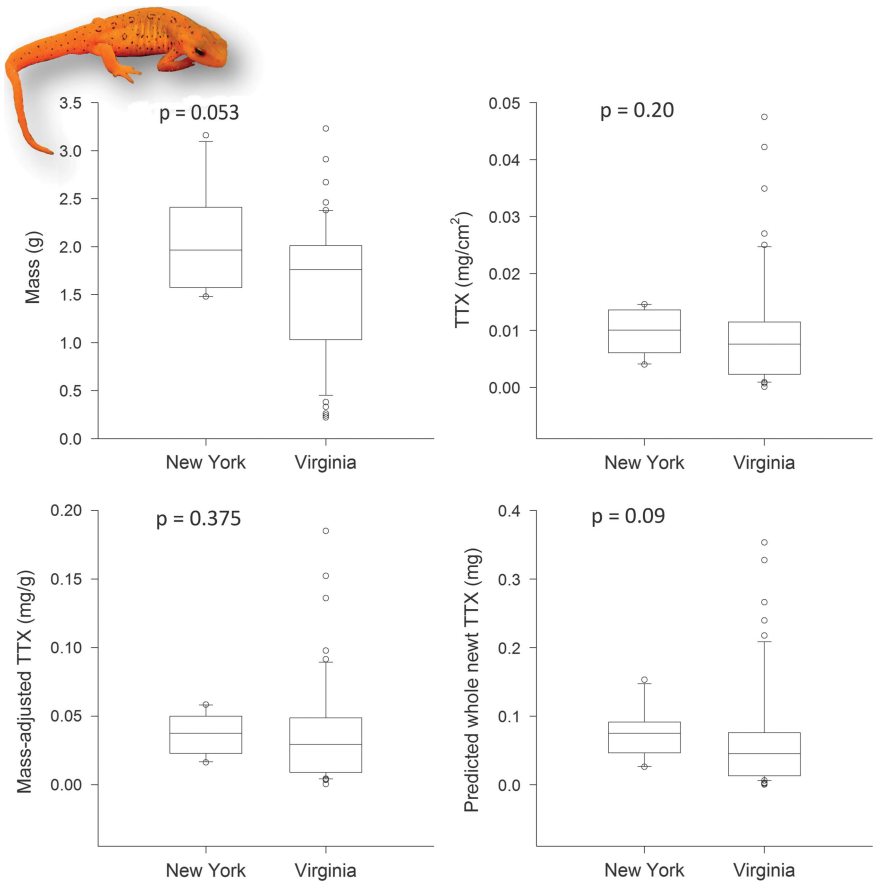

Figure 2 Box plots of body size and TTX levels in terrestrial stage (eft) Eastern Newts (*N. viridescens*) from Cortland County, NY ($n=10$), and Giles County, VA ($n=51$). These two populations did not differ in comparisons of size or measures of TTX (see Results). Left top: newt body mass (g); Right top: concentration of TTX per skin sample (mg of TTX per cm^2). Left bottom: mass-adjusted concentration of TTX per individual (mg of TTX per gram of newt). Right bottom: estimated total amount of TTX per individual (mg). Inset shows the brightly colored eft stage of *N. viridescens* (photo by AMD).

are known to contribute to TTX resistance in vertebrates (for example, Geffeny *et al.*, 2005; Soong and Venkatesh, 2006; Jost *et al.*, 2008; Hanifin and Gilly, 2015).

We isolated and purified genomic DNA from muscle or liver tissue with the DNeasy Tissue Kit (Qiagen, Inc., Germantown, MD, USA). We amplified and sequenced the four P-loops of *SCN4A* using primers we designed specifically for snakes (Feldman *et al.*, 2009).

We also attempted to sequence the entire coding region (coding DNA sequence) of a resistant *H. platirhinos* to potentially identify other novel genetic changes (for example, splice variation) that might provide TTX resistance. We isolated and purified mRNA from fresh skeletal muscle with the RNeasy Mini Plus Kit (Qiagen, Inc.). We reverse transcribed total mRNA to cDNA with the iScript Select cDNA Synthesis Kit (BioRad, Hercules, CA, USA) and oligo(dT) primer. We then amplified and sequenced a series of overlapping pieces of *SCN4A* to construct a complete contig of the locus using primers we designed for snakes *SCN4A* (Feldman *et al.*, 2009). We edited sequences by eye in Sequencher 4.9 (Gene Codes Corp., Ann Arbor, MI, USA), aligned sequences with Clustal W 1.83 (Thompson *et al.*, 1994) and translated coding regions into amino acid sequences using MacClade 4.08 (Maddison and Maddison, 2005). We deposited all sequences in GenBank (accession numbers: KT277675–KT277703).

RESULTS

Newt and snake phenotypes

Eastern Newts collected from central New York, just outside the distribution of Eastern Hog-nosed Snakes, contained a median concentration of 0.0374 mg of TTX per gram of newt, which can be converted to a total quantity of 0.075 mg of TTX in an eft (mean = 0.075 mg, max = 0.153 mg). Newts from Virginia, where Eastern Hog-nosed Snakes occur, contained a median concentration of 0.0294 mg of TTX per gram, for total estimate of 0.045 mg of TTX in an eft (mean = 0.067 mg, max = 0.353 mg). Although the population of newts sympatric with *Heterodon* displayed a greater range of toxicity, levels of TTX between the two sites were not statistically different (t-value = 356 000, DF = 39, $P=0.375$; Figure 2).

All *H. platirhinos* sympatric with *N. viridescens* that we examined possessed highly elevated levels of TTX resistance (Table 2). Resistance in these populations ranged from 75 MAMUs to well over 250 MAMUs, with some snakes crawling at over 90% of their baseline speed even after injections of 250 MAMUs; doses of TTX that would kill almost any other terrestrial vertebrate (Hanifin, 2010). In contrast, the allopatric *H. platirhinos* and *H. nasicus* showed only base levels of resistance, on par with other nonresistant snakes (Table 2) (Feldman *et al.*, 2012).

Snake *SCN4A* genotypes

To our surprise, all *Heterodon* possessed the same TTX-sensitive *SCN4A* allele ('wild type') seen in all nonresistant snakes (Figure 3) (Geffeny *et al.*, 2005; Feldman *et al.*, 2010, 2012; McGlothlin *et al.*, 2014).

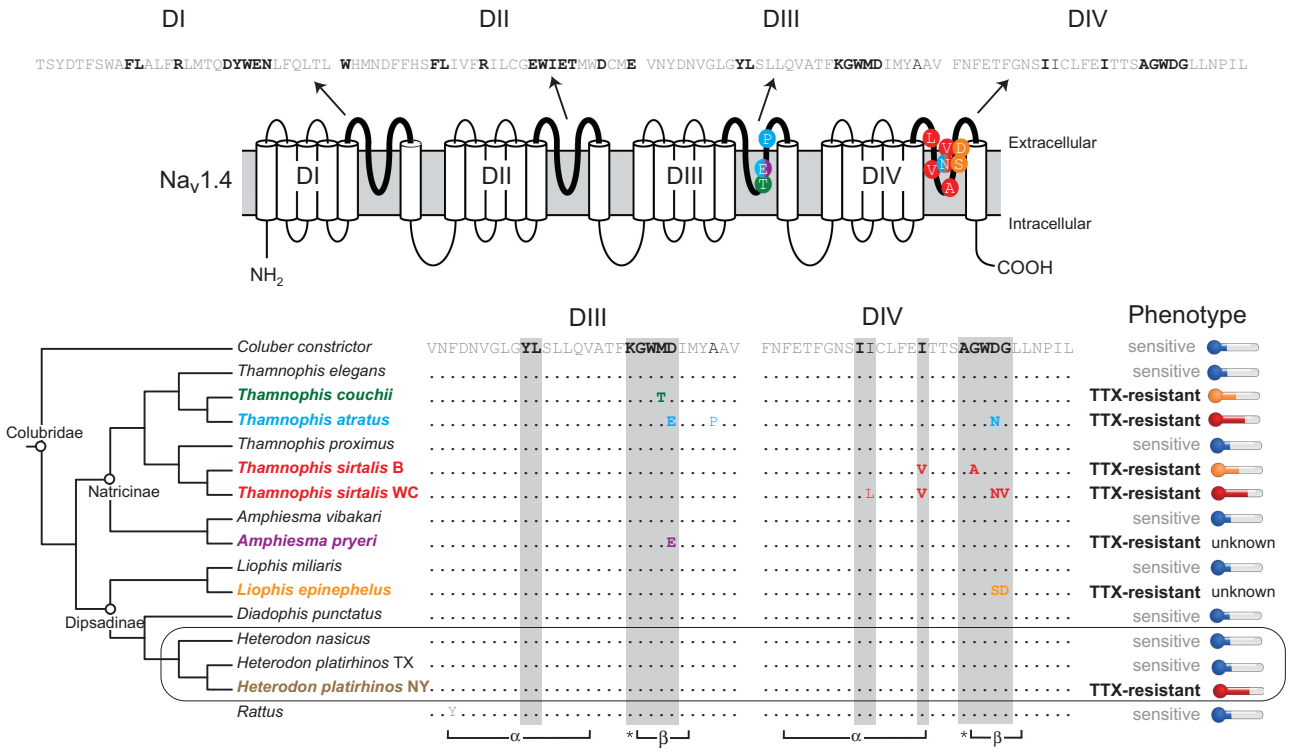

Figure 3 Amino acid replacements are found at sites critical in TTX ligation in the pore-forming loops (P-loops) of Na$_v$1.4 in all snakes known to prey on TTX-bearing amphibians, except for Eastern Hog-nosed Snakes (*H. platirhinos*). Top: amino acid sequence of the 4P-loops (DI-DIV) of Na$_v$1.4; sites in dark black have been demonstrated to reduce TTX-binding affinity at least twofold (see Feldman *et al.*, 2012). Middle: two-dimensional model of Na$_v$1.4 showing the placement of resistance-conferring substitutions in snakes. Bottom: genotypes and phenotypes of resistant snakes and close relatives (and *Rattus*) arranged phylogenetically (after Pyron *et al.*, 2013). Taxa that prey on TTX-bearing amphibians (bold colors) also possess mutations (colors) at sites known to alter TTX ligation to the pore (bold, outlined in grey), with the exception of *H. platirhinos*. Pore features indicated below sequence: α-helix (α); β-strand (β); selectivity filter (*). Phenotypic data are categorized as follows: blue thermometers indicate TTX-sensitive animals (1–5 MAMUs), orange thermometers indicate moderate to high levels of TTX resistance (40–80 MAMUs), whereas red thermometers indicate extreme levels of TTX resistance (100–>250 MAMUs). It is noteworthy that most *H. platirhinos* display extreme levels of TTX resistance (Table 2), similar to the levels seen in *Thamnophis* that possess functionally important replacements in Na$_v$1.4. Phenotypic data are from this study, Brodie *et al.* (2002, 2005), Feldman *et al.* (2009, 2012); two snake species (*A. pryeri* and *L. epinephalus*) are known to be resistant to their TTX-bearing prey and possess convergent TTX-resistant substitutions (Feldman *et al.*, 2012), but actual levels of whole-animal TTX resistance have yet to be quantified (unknown).

No *H. platirhinos* possessed derived *SCN4A* alleles found in the other TTX-resistant taxa or paralogs that have been examined thus far (Figure 3) (Geffeny *et al.*, 2005; Soong and Venkatesh, 2006; Jost *et al.*, 2008; Feldman *et al.*, 2012; McGlothlin *et al.*, 2014; Hanifin and Gilly, 2015). Thus, the resistance-conferring mutations in *SCN4A* known to reduce the binding affinity of TTX to the channel appear to be entirely lacking in TTX-resistant *H. platirhinos*.

We also obtained a nearly complete transcript of the *SCN4A* locus from a highly resistant Eastern Hog-nosed Snake (we were unable to obtain the first ~640 bp and ~430 bp encoding most of exon 9 and all of exon 10, respectively). The coding DNA sequence from this snake appears consistent with that of other snakes (and mammals), as does gene structure (intron/exon arrangement), providing no indication that other changes to the protein or exon shuffling explain resistance in *H. platirhinos*. Furthermore, changes in the regions we were unable to sequence are not likely to contribute to resistance, because they do not interact with TTX (Fozzard and Lipkind, 2010). Specifically, the N-terminus of Na$_v$1.4 we could not sequence is not part of the outer pore, and although the missing portion of exons 9 and 10 are just C-terminal to the DI P-loop, these regions actually encode a portion of the inner pore, which is an inner membrane and cytoplasmic structure (thus unexposed to TTX) involved in channel inactivation (Catterall, 2000; Hille, 2001).

DISCUSSION

Across much of the eastern United States, it is now apparent that Eastern Hog-nosed Snakes (*H. platirhinos*) prey on Eastern Newts (*N. viridescens*) (Table 1; Figure 1) and may be engaged in an arms race mediated by TTX, similar to the well-characterized garter snake (*Thamnophis*) and Pacific newt (*Taricha*) system (Brodie and Brodie, 1999). Eastern Newts in sympatry and allopatry with Eastern Hog-nosed Snakes possess similar levels of TTX (Figure 2). Although the most toxic newt contained only 0.353 mg TTX, roughly 80 times less than the most potent *Taricha* (Stokes *et al.*, 2015), the amount of toxin present in Eastern Newts is still sufficient to provide protection against almost any predator (Brodie and Brodie, 1990). However, Eastern Hog-nosed Snakes, one of only a few vertebrates known to consume the terrestrial efts (summarized in Table 1), show remarkable levels of toxin resistance (Table 2), on par with the most resistant populations of *Thamnophis* (Brodie *et al.*, 2002; Feldman *et al.*, 2009, 2010) (Figure 3). In fact, all but the smallest *H. platirhinos* in these populations could safely consume the most toxic *N. viridescens* known

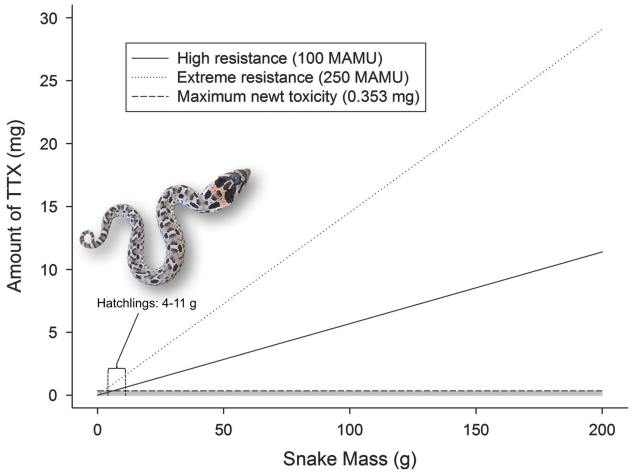

Figure 4 Projected levels of TTX required to slow *H. platirhinos* (NY samples) with high resistance (100 MAMUs; solid line) and extreme levels of resistance (250 MAMUs; dotted line) to 50% of their normal crawling ability, relative to the maximum amount of total TTX (0.353 mg) in an individual *N. viridescens* (horizontal dashed line). Inset shows that *H. platirhinos* range in size from 4 to 11 g on hatching (Ernst and Ernst, 2003) and the curve for 100-MAMU snakes intersects the maximum newt toxicity threshold at 6.2 g, whereas that of 250-MAMU snakes intersects at 2.4 g, meaning that all but the smallest *H. platirhinos* from these populations could safely consume the most toxic *N. viridescens* known and would suffer $<50\%$ reduction in crawling speed. It is worth noting that as the effects of TTX are mass dependent, projections can be made for any level of resistance and are useful in estimating the amount of TTX needed to slow a snake of a specific size compared with the amount of TTX in newt prey (after Brodie *et al.*, 2005) (photo by AMD).

(this study) and suffer only mild reductions in crawling ability (Figure 4). Whether or not predator and prey traits are well matched across the distribution of both Eastern Hog-nosed Snakes and Eastern Newts remains an open question. Although our TTX-resistant Eastern Hog-nosed Snakes were sampled from within Eastern Newt range and our nonresistant *H. platirhinos* was from a locale outside newt range, we were unable to sample predator and prey from syntopic locations. Thus, there may be regions where Eastern Hog-nosed Snakes are less resistant to TTX and syntopic efts are more toxic than the populations we sampled, such that predator and prey can impose reciprocal selection on one another (for example, Hanifin *et al.*, 2008). Much more extensive sampling across the distribution of these two interacting species is needed to sketch a picture of the geographic mosaic of coevolution in this system. Regardless of any geographic variation predator and prey traits, it is clear that some *H. platirhinos* populations have evolved phenotypic resistance in a parallel manner to the *Thamnophis—Taricha* system, but through a novel mechanism.

We then examined the genetic basis of the predator adaptation to determine whether a common genetic mechanism underlies TTX-resistant phenotypes in these disparate newt–snake systems (*Heterodon* and *Thamnophis* are members of distinct subfamilies and only distantly related; Figure 3). To date, the evolution of TTX resistance in all animal lineages examined appears to involve a predictable subset of replacements in the outer pore of the sodium channel proteins (Na_v) that interact directly and strongly with TTX (Hille, 2001; Fozzard and Lipkind, 2010). Remarkably, in *H. platirhinos*, the *SCN4A* gene (encoding $Na_v1.4$), a locus that appears to underlie a major portion of resistance in other vertebrates (Geffeny *et al.*, 2005; Soong and Venkatesh, 2006; Jost *et al.*, 2008; Feldman *et al.*, 2012; Hanifin and Gilly, 2015), contains no allelic variation that would alter TTX ligation to the pore. *Thamnophis* with comparable levels of TTX resistance typically have two to four amino acid substitutions in this protein (Feldman *et al.*, 2009, 2010) (Figure 3). Thus, *Heterodon* appears to have evolved a novel mechanism of TTX resistance.

Multiple, non-mutually exclusive hypotheses might explain resistance to TTX in *Heterodon* in the absence of allelic variation in *SCN4A*. The first involves simple posttranscriptional changes to the locus, such as RNA editing or alternative splicing (Liu *et al.*, 2004; Onkal *et al.*, 2008). However, our sequence of a nearly complete transcript reveals no such modifications. The second possibility involves changes in the expression patterns of sodium channels (for example, Lopez-Santiago *et al.*, 2006). Simple upregulation of *SCN4A* might produce more copies of $Na_v1.4$ than can be blocked by TTX. However, this mechanism seems unlikely, because major changes in sodium channel densities negatively impact electrophysiology and even impair normal cell and organ function (Chen *et al.*, 2002). However, of the nine functional sodium channel paralogs in amniotes ($Na_v1.1$–1.9), each of which is expressed in a particular tissue type (Goldin, 2001), three are natively resistant to TTX ($Na_v1.5$ in cardiac muscle; $Na_v1.8$ and 1.9 in peripheral nerves), owing to a major amino acid replacement in the outer pore (Backx *et al.*, 1992). It is possible that one of these insensitive channels is expressed in the muscle tissue of *H. platirhinos*, thereby rendering the muscles impervious to TTX. An alteration in tissue-specific expression of this nature would likely compromise muscle performance to some degree, because the natively expressed $Na_v1.4$ allows rapid, large amplitude changes in membrane potential compared with other channels (Geffeney and Rubin, 2006), allowing quick and intense muscular contractions. However, hog-nosed snakes are stout-bodied predators that rely on defensive displays rather than speed to avoid enemies (Durso and Mullin, 2014) and thus might tolerate some compromise in muscle function. Finally, an intriguing possibility is that *H. platirhinos* circumvent TTX not by possessing resistant sodium channels (targets of TTX) but by 'grabbing' TTX before the poison reaches sensitive tissues. In marine systems, some arthropods, gastropods and even pufferfish, possess TTX-binding proteins in specific tissues and blood (Matsui *et al.*, 2000; Nagashima *et al.*, 2002; Hwang *et al.*, 2007). These proteins essentially wrap TTX and prevent it from binding to sodium channels, thereby disabling the toxin. Although TTX-binding proteins are unknown from snakes (or any other tetrapods), analogous proteins are employed by some snakes to defeat the effects of prey toxins or provide innate immunity to their own venoms (Weinstein *et al.*, 1992; Mackessy, 2009). The fact that North American hog-nosed snakes are major predators of toxic toads (Ernst and Ernst, 2003) and these hog-nosed snakes are themselves venomous (Young, 1992) lends some credence to the hypothesis that binding agents already in place for dealing with prey toxins or self-immunity may have been co-opted for TTX resistance.

Determining whether the genetic pathways to adaptation are predictable or unpredictable remains a central question in evolutionary biology (Stern and Orgogozo, 2009; Martin and Orgogozo, 2013) and one with real ramifications for human health, agriculture and other practical applications. Here, even in chemically mediated systems thought to be characterized by a high-degree of predictability, it is apparent that phenotypic convergence may still result from diverse genetic mechanisms. This example stands out as a reminder that even when common pathways and mechanisms exist, evolution may not be as constrained as we often assume.

DATA ARCHIVING

All sequence data are available from GenBank (accession numbers: KT277675–KT277703).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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