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CellPress

Current Biology
Report

Historical Contingency in a Multigene Family Facilitates Adaptive Evolution of Toxin Resistance

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http://dx.doi.org/10.1016/j.cub.2016.04.056

SUMMARY

Novel adaptations must originate and function within an already established genome [1]. As a result, the ability of a species to adapt to new environmental challenges is predicted to be highly contingent on the evolutionary history of its lineage [2–6]. Despite a growing appreciation of the importance of historical contingency in the adaptive evolution of single proteins [7–11], we know surprisingly little about its role in shaping complex adaptations that require evolutionary change in multiple genes. One such adaptation, extreme resistance to tetrodotoxin (TTX), has arisen in several species of snakes through coevolutionary arms races with toxic amphibian prey, which select for TTX-resistant voltage-gated sodium channels (Na_v) [12–16]. Here, we show that the relatively recent origins of extreme toxin resistance, which involve the skeletal muscle channel $Na_v1.4$, were facilitated by ancient evolutionary changes in two other members of the same gene family. A substitution conferring TTX resistance to $Na_v1.7$, a channel found in small peripheral neurons, arose in lizards ~170 million years ago (mya) and was present in the common ancestor of all snakes. A second channel found in larger myelinated neurons, $Na_v1.6$, subsequently evolved resistance in four different snake lineages beginning ~38 mya. Extreme TTX resistance has evolved at least five times within the past 12 million years via changes in $Na_v1.4$, but only within lineages that previously evolved resistant $Na_v1.6$ and $Na_v1.7$. Our results show that adaptive protein evolution may be contingent upon enabling substitutions elsewhere in the genome, in this case, in paralogs of the same gene family.

RESULTS AND DISCUSSION

The role of historical contingency in adaptive evolution has been a longstanding debate in evolutionary biology [1–3]. On the one hand, past evolutionary change can be viewed as a type of negative constraint, limiting the scope of what is achievable by natural selection. On the other hand, historical quirks may open novel and previously inaccessible evolutionary pathways. Because of the pervasiveness of biological interactions, both within a protein and among genes in the genome, single amino acid replacements often change the fitness consequences of other alleles [17–19], suggesting that many or most polygenic adaptations are likely to have been facilitated by substitutions that arose in the distant past. Despite these predictions, little is known about whether adaptive evolutionary changes in natural populations tend to be contingent on previous substitutions in other genes.

Here, we use a comparative approach to assess the role of historical contingency in the evolution of tetrodotoxin (TTX) resistance in snakes. TTX is a neurotoxin used as an antipredator defense in a number of animals, including newts [20] (Caudata: Pleurodelinae). TTX binds to voltage-gated sodium channels (Na_v proteins), preventing the influx of sodium ions and impairing excitable tissue such as nerves and muscle [21]. Resistance to TTX evolves by amino acid substitutions in the channel's outer pore ("P-loops"), which are normally highly conserved across vertebrates [12, 14, 22]. Snakes possess nine Na_v channels with tissue-specific expression encoded by the nine genes of the *SCNA* family [23–25]. Because TTX can potentially bind to several of these channels, physiological resistance to high TTX

levels is a complex adaptation that requires evolutionary changes at several loci [15, 22]. Furthermore, as exemplified by the progressive stages of TTX poisoning in humans [26], tissues vary in their sensitivity to TTX. This observation suggests that in predators that consume TTX, tissues impaired by lower doses of TTX are likely to evolve resistance before those affected by higher doses.

The garter snake *Thamnophis sirtalis* preys upon highly tetrodotoxic *Taricha* newts [27, 28]. Various populations of *T. sirtalis* display physiological resistance to TTX, which can be attributed to amino acid substitutions in at least three Na_v paralogs [12, 15]: the skeletal muscle channel, $Na_v1.4$, and two peripheral nerve channels, $Na_v1.6$ and $Na_v1.7$. In mammals, $Na_v1.6$ is located in the nodes of Ranvier of myelinated axons [29], while $Na_v1.7$ is expressed in sensory fibers, sympathetic ganglia, and smooth muscle [30]. Although the precise expression patterns of these two channels are unknown in reptiles, transcriptomic data from lizards [31, 32] and snakes [33] suggest that they are expressed in peripheral nerves (see the Supplemental Experimental Procedures). The P-loop sequence of $Na_v1.4$ varies within and among *T. sirtalis* populations, with different alleles providing different levels of TTX resistance roughly matching the toxicity of local newts, suggesting a relatively recent origin of resistant skeletal muscle [12, 27, 28, 34]. In contrast, substitutions conferring resistance to $Na_v1.6$ and $Na_v1.7$ are fixed across *T. sirtalis* populations [15], suggesting that resistance in peripheral nerves has a more ancient origin. We hypothesize that the origin of resistant peripheral nerves provided baseline TTX resistance to the ancestors of garter snakes, facilitating later evolution of resistant muscle and the consequent ability to consume highly toxic prey.

To test this hypothesis, we reconstructed the evolutionary history of TTX resistance in snakes by sequencing portions of the genes *SCN4A*, *SCN8A*, and *SCN9A* (encoding the proteins $Na_v1.4$, $Na_v1.6$, and $Na_v1.7$, respectively) from 78 snake species. We sequenced regions known to underlie TTX resistance in *Thamnophis* [12, 13, 15]: the P-loops in domain III and IV (DIII and DIV) of $Na_v1.4$, DIV of $Na_v1.6$, and DIII and DIV of $Na_v1.7$ (see the Supplemental Experimental Procedures). We obtained sequences from snake species known to consume TTX-bearing prey, their sister taxa, and a number of other lineages representing the breadth of snake diversity, to date the origins of resistance-conferring substitutions. We included sequences from three published snake genomes, *Boa constrictor* [35], *Python molurus* [36], and *Ophiophagus hannah* [37], and from one unpublished snake genome, *Ramphotyphlops bituberculatus*. As outgroups, we added sequences from the genomes of two lizards (*Anolis carolinensis* [38] and *Ophisaurus gracilis* [39]), a turtle (*Chrysemys picta* [40]), and a bird (*Gallus gallus* [41]). Many amino acid substitutions causing TTX resistance have been characterized experimentally [14, 22], allowing us to infer resistance from DNA sequences. Substitutions putatively conferring TTX resistance were identified from predicted translations and mapped to a time-calibrated phylogeny [42, 43] to reconstruct the origins of TTX resistance.

Stepwise Evolution of TTX Resistance in the Na_v Family

Our results show that TTX resistance of both peripheral nerve channels ($Na_v1.6$ and $Na_v1.7$) predated TTX resistance of muscle channels ($Na_v1.4$; Figure 1). Resistant $Na_v1.7$ had the most ancient origin. One substitution in DIV known to provide very high (30-fold) TTX resistance [12, 44, 45], D1684N, was present in the common ancestor of all snakes (Figures 1, 2, S1, and S2; positions refer to *T. sirtalis* sequence [15]). This substitution also occurs in the lizard *O. gracilis*, suggesting that D1684N originated in ancestral squamates at least 170 million years ago (mya), in the Middle Jurassic [42]. *A. carolinensis* has a D1684A substitution in this position instead, which provides even stronger resistance (150-fold [45]; Figure S1). In *Epicrates* sp., we found D1684H, which presumably also interferes with TTX binding, although this has not been directly tested (Figure S1).

Two additional DIV substitutions (A1681G and G1685Y) that likely contribute to TTX resistance of $Na_v1.7$ arose twice in snakes: once in *Leptotyphlops* (63 mya) and independently in the common ancestor of advanced snakes (Colubroidea, 61 mya), a group that includes garter snakes (Figures S1 and S2). The former, which occurs at the selectivity filter of the channel, is known to provide mild (1.5-fold) TTX resistance and is found naturally in several channels in TTX-bearing pufferfish [22]. We also found this substitution in the turtle *C. picta* (Figure S1). The substitution G1685Y has not been tested experimentally, but this position is thought to be associated with TTX binding, and substitutions here are often found in naturally TTX-resistant channels, either alone or together with substitutions at position 1684 [12, 14, 22]. This substitution should interfere with TTX binding, as it replaces the very small side chain of glycine with the large side chain of tyrosine. Position 1685 was quite variable across species, and many of the detected substitutions likely also interfere with TTX binding (Figure S1). An additional DIV substitution known to provide 2-fold resistance to TTX [12], I1677V, arose recently in the genus *Carphophis*.

In DIII of $Na_v1.7$, a potentially TTX-resistant substitution (D1393E) was observed in all sampled taxa except for *A. carolinensis* and *C. picta* (Figures 1, 2, S1, and S2). Here, the ancestral D refers to typical mammalian sequence (data not shown). This substitution occurs at the TTX-binding site [21], and although it has not been tested experimentally, it is found in other TTX-resistant channels [13, 14]. The lizard *A. carolinensis* has the mammalian D (aspartate) in this position, while the turtle *C. picta* has the very dissimilar proline (P). The latter has not been tested for TTX resistance but is found in $Na_v1.4a$ of the tetrodotoxic pufferfish *Arothron nigropunctatus* [22]. The adjacent position also displays two different substitutions, M1392A (*R. bituberculatus*) and M1392T (*B. constrictor* and *P. molurus*). The former has not been tested, but the latter is known to provide 15-fold resistance to TTX [22]. Although further sampling across reptiles and experimental verification of the effects of D1393E on TTX binding are necessary for confirmation, our results suggest that the common ancestor of all reptiles may have possessed $Na_v1.7$ with at least mild TTX resistance.

Because $Na_v1.7$ in *T. sirtalis* and other advanced snakes contained a large number of substitutions that had never been experimentally tested in combination, we verified the resistance of this channel by expressing it in *Xenopus* oocytes and recording sodium currents in the presence and absence of TTX (see the Supplemental Experimental Procedures). Compared to rat $Na_v1.7$ ($K_d \pm 95\%$ CI = 1.34×10^{-8} M $\pm 3.9 \times 10^{-9}$ TTX), snake $Na_v1.7$ displays 900-fold greater resistance to TTX ($K_d \pm 95\%$ CI = 1.21×10^{-5} M $\pm 5.3 \times 10^{-6}$ TTX; Figure 3), which is

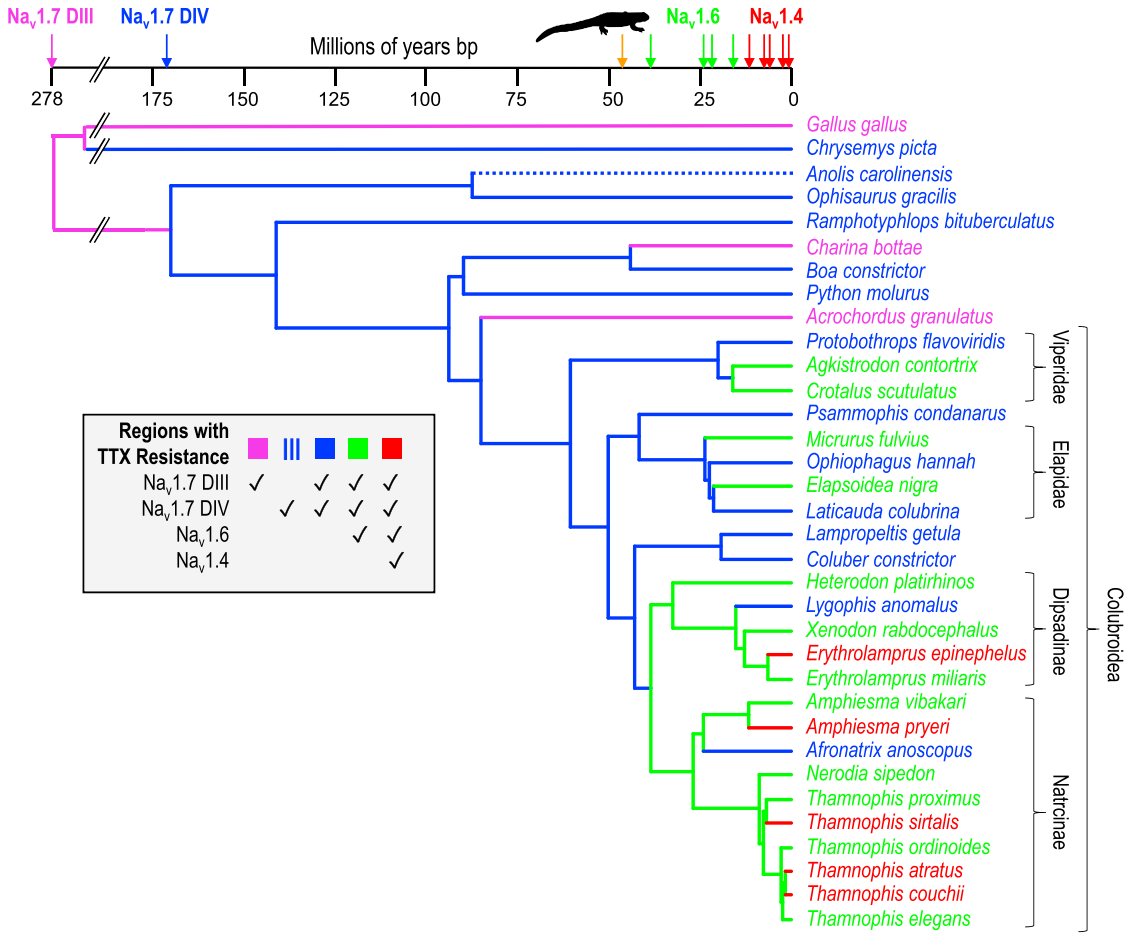

Figure 1. Extreme Resistance to TTX Evolved in a Stepwise Fashion in Snakes

A substitution in the domain III (DIII) TTX-binding region of the voltage-gated sodium Na$_v$1.7 was present in the common ancestor of all living reptiles (purple arrow on the timeline). A substitution providing extreme levels of resistance to the same channel arose in DIV at least 170 mya (blue arrow). Other substitutions conferring TTX resistance in Na$_v$1.7 DIV arose independently in turtles (*Chrysemys*) and anoles (*Anolis*), with the DIII substitution being lost in *Anolis*. Following the origin of TTX-bearing newts 44–48 mya (orange arrow), an identical I1709V substitution arose in Na$_v$1.6 in four different lineages (green arrows). Finally, Na$_v$1.4 showed five independent origins of TTX resistance in lineages with resistant Na$_v$1.7 and Na$_v$1.6 (red arrows). Branches are color-coded from fewest to most TTX-resistant regions as shown in the key above. The phylogeny [43] is pruned to a limited number of species, and the time axis (based on [42]) is truncated at the left for clarity. See also Figures S1 and S2.

comparable to the level of TTX resistance displayed by the most resistant known Na$_v$1.4 allele in *T. sirtalis* [12]. This combination of Na$_v$1.7 P-loops arose ~61 mya (Figure S2), indicating that advanced snakes inherited extremely TTX-resistant Na$_v$1.7 from their common ancestor.

A second nerve channel, Na$_v$1.6, evolved TTX resistance independently at least four times within snakes: once in the common ancestor of two large subfamilies (Natricinae, which includes garter snakes, and Dipsadinae, 38 mya), once in the New World Viperidae (16 mya), and twice within the family Elapidae ($\leq$24 mya; Figures 1, S1, and S2) [42]. In each case, TTX resistance arose by an identical substitution in DIV (I1709V) (Figures S1 and S2). Although this substitution has not been tested in Na$_v$1.6, it is known to confer a 2-fold increase in TTX resistance when expressed in Na$_v$1.4 [12], which has a nearly identical TTX-binding region [15]. Each of the origins of I1709V occurred in lineages that had possessed TTX-resistant Na$_v$1.7 for over 100 million years. A second substitution likely to confer TTX resistance, G1717M, is found within the species *Erythrolamprus* (= *Liophis*) *epinephelus* ($\leq$6.4 mya). This substitution has not been experimentally tested, but it is found naturally in the channel Na$_v$1.1Lb in *A. nigropunctatus* [22]. TTX resistance was lost from domain IV on at least two occasions: in the natricine clade containing *Afronatrix*, *Rhabdophis*, and *Xenocrophis* (23 mya) and in the dipsadine *Lygophis anomalus* ($\leq$15 mya).

TTX-resistant skeletal muscle channels (Na$_v$1.4) arose only in lineages that historically expressed resistance in both Na$_v$1.7 and Na$_v$1.6, suggesting that the presence of two resistant channels in peripheral nerves facilitated the evolution of resistant muscle (Figures 1 and 2). Indeed, the origin of resistance in Na$_v$1.4 was significantly contingent on the presence of resistance in Na$_v$1.6 (χ^2_1 = 5.28, p = 0.02, Pagel's Discrete [46]). TTX resistance in Na$_v$1.4 evolved independently in five snake species that consume toxic amphibians via substitutions in DIII and/or DIV [14] (Figure S1). One species, *E. epinephelus*, is found in the subfamily Dipsadinae, and four are found in the related

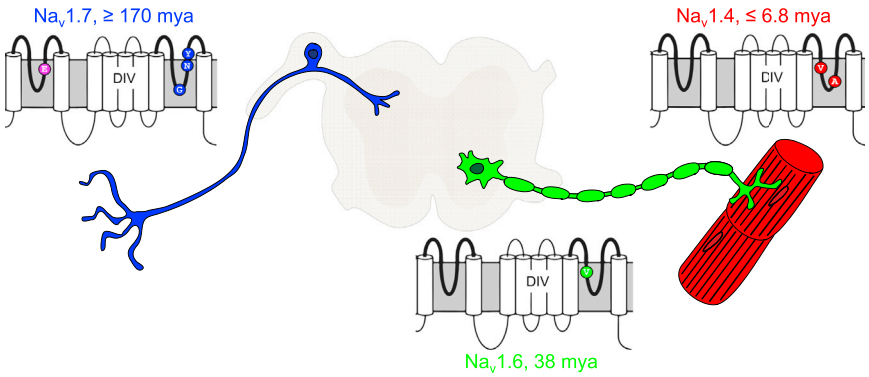

Figure 2. History and Physiological Context of TTX-Resistance Substitutions in Garter Snakes

Evolutionary changes in DIII and DIV of Na$_v$1.7 provided the common ancestor of all snakes with TTX resistance of small sensory neurons (blue). TTX resistance of larger myelinated axons subsequently evolved four times via a substitution in DIV of Na$_v$1.6 (green). These changes provided certain snake lineages with baseline resistance to low TTX levels, facilitating predator-prey arms races and recent evolution of resistant skeletal muscle via substitutions in Na$_v$1.4 (red). The substitutions illustrated above derive from *T. sirtalis* in Benton County, Oregon [15]. Although nerve channels do not vary across the species range, Na$_v$1.4 is polymorphic and varies according to the toxicity of local newts [12, 15, 28, 34]. See also Figures S1 and S2.

subfamily Natricinae: *T. sirtalis*, *T. atratus*, *T. couchii*, and *Amphiesma pryeri*. In a sixth natricine, *Rhabdophis tigrinus*, Na$_v$1.4 was previously interpreted as TTX-resistant via the substitution I1555M [14]; however, because this methionine is present in nearly every TTX-sensitive Na$_v$ channel, this characterization is most likely erroneous, and we do not consider this species to possess resistant Na$_v$1.4 here. All origins of TTX-resistant Na$_v$1.4 are independent, and none are shared with extant sister species. Based on the dated phylogeny we present, all origins have occurred relatively recently, with *E. epinephelus* evolving resistance $\leq$6.4 mya, *T. sirtalis* $\leq$6.8 mya, *T. atratus* and *T. couchii* $\leq$1.5 mya, and *A. pryeri* $\leq$11.7 mya. However, most of these origins likely occurred much more recently. In particular, in at least two species, *T. sirtalis* and *T. atratus*, Na$_v$1.4 is highly polymorphic within and among populations and covaries with prey toxicity, which both indicates ongoing coevolutionary arms races and suggests a very recent origin [12, 27, 28, 34].

Historically Contingent Origins of Extreme TTX Resistance

The ancient emergence of resistance in Na$_v$1.7 indicates that it did not evolve as a direct response to selection from TTX-bearing newts, which did not appear until ~44 mya [20, 47, 48], or from other amphibians, which likely also did not possess TTX in the Jurassic [20]. Because TTX-resistant substitutions are located in the Na$_v$ outer pore, they typically influence other biophysical properties [14], suggesting that the evolution of TTX-resistant Na$_v$1.7 may have occurred as a side effect of selection for other functions. For example, the D1684N substitution observed in snakes is known to decrease channel conductance [45] and may also affect ion selectivity [49]. In contrast, the substitution A1681G is known to increase channel conductance [22]. In mammals, Na$_v$1.7 is involved in setting the threshold for action potentials in a number of neuron types including nociceptors and olfactory receptors [30, 50], suggesting that such changes in channel function may have been selected for via effects on neuron excitability. In snakes, Na$_v$1.7 appears to be the primary sodium channel expressed in the snake vomeronasal organ [33] (see the Supplemental Experimental Procedures), which implicates the channel's role in chemosensation as a potential driver of the observed evolutionary changes.

In contrast, resistant Na$_v$1.6 may have arisen as a direct response to selection by toxic prey. All origins of TTX-resistant Na$_v$1.6 postdate the origin of modern newts (Figure 1) and either postdate or roughly coincide with the origin of the more highly toxic North American newts (~36 mya [20, 48]). Further, the groups with resistant Na$_v$1.6 contain most of the snake species that commonly feed on amphibians; in particular, many dipsadine and natricine snakes are amphibian specialists [51]. All of these snake groups also overlap geographically with newts, suggesting that Na$_v$1.6 resistance may have evolved via past interactions between snakes and newts.

The historical sequence of evolution of TTX-resistance across the Na$_v$ family in snakes suggests that modern predator-prey arms races were possible only after the sequential accumulation of toxin resistance in more sensitive tissues (Figure 2). The localization of Na$_v$1.7 on small-diameter neurons suggests that its function would be impaired by relatively small concentrations of TTX [52]. Accordingly, mild cases of TTX poisoning in humans involve solely sensory symptoms [26], likely mediated by Na$_v$1.7. Reduced affinity of Na$_v$1.7 to TTX likely would have rendered early snakes less sensitive to the numbing sensation caused by small doses of TTX, which if present, would have resulted in the avoidance of tetrodotoxic prey. The slightly higher concentrations necessary to block Na$_v$1.6 in larger neurons [52] could be delivered by ingesting tetrodotoxic prey. The consequent motor impairment [26] caused by blockade of action potentials in peripheral motor neurons would then provide a source of selection for Na$_v$1.6 resistance in lineages that frequently consumed such prey. In this genetic background, the appropriate ecological interactions between predators and prey should occasionally trigger escalating arms races such as those seen in *Thamnophis* and *Taricha*, where TTX resistance of Na$_v$1.4 in garter snake skeletal muscle coevolves with the magnitude of newt TTX [14, 27, 28].

Conclusions

Neurotoxins are an effective defense mechanism against many predators because the evolution of physiological resistance requires changes in multiple sensitive proteins. Such adaptations could conceivably arise in a predator species in one of two ways: either all of the proteins in question evolve resistance effectively simultaneously, or they acquire resistance

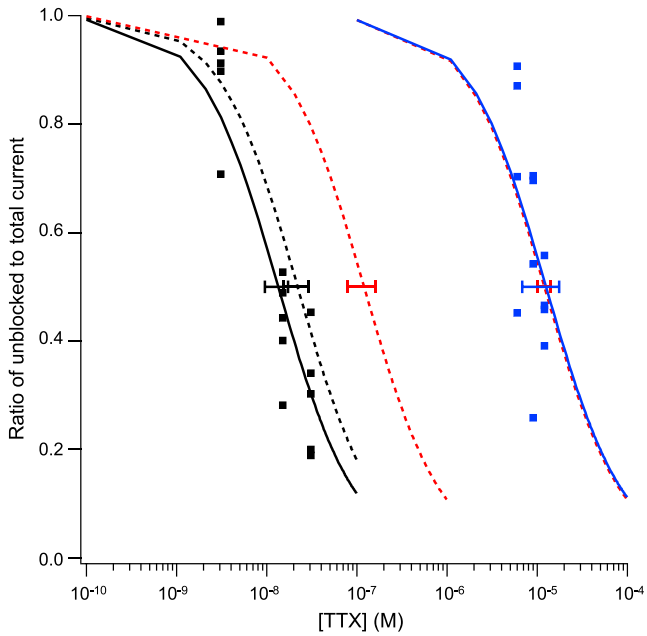

Figure 3. Garter Snake $Na_v1.7$ Shows Strong Resistance to TTX

Garter snake $Na_v1.7$ is blocked at TTX concentrations 900-fold higher than rat $Na_v1.7$, a level of resistance comparable to the most resistant garter snake $Na_v1.4$. From left to right, the traces are rat $Na_v1.7$ (black, solid), garter snake or garter snake-human chimeric $Na_v1.4$ from three different populations (dashed lines; Bear Lake chimera [black, non-resistant], Benton garter snake [red, moderately resistant], Willow Creek chimera [red, strongly resistant]; data from [12]), and garter snake $Na_v1.7$ (blue). Each symbol corresponds to the ratio of unblocked to total current for an oocyte expressing the indicated channel and exposed to TTX. The TTX concentration that blocked 50% of the channels (K_d) for each channel type was calculated from pooled channel data (see the Supplemental Experimental Procedures). K_d values (±95% confidence limits) are shown for each channel type with a bar. The lines represent the equation fitted to the data with the estimated K_d for each channel type.

sequentially over longer periods of evolutionary time. We have shown that the arms races that drive exaggerated evolution of TTX toxicity and resistance arise only after a stepwise pattern of accumulated changes in paralogous proteins expressed in diverse tissues. Our results emphasize both the predictable and capricious aspects of adaptive evolution. The convergent origins of extreme TTX resistance, which occurred multiple times in snakes through predator-prey coevolution, were facilitated by earlier changes in the lineage's distant evolutionary past.

ACCESSION NUMBERS

The accession numbers for new sequences are GenBank: KX063539–KX063606 and KX079340–KX079444. The accession numbers for new annotations from *Ophiophagus* are GenBank: BK009415–BK009419. The accession numbers for previously published sequences are given in Table S1. New annotations of genes from *Ophisaurus* and *Boa*, along with a time-calibrated phylogeny, have been deposited in Dryad: http://dx.doi.org/10.5061/dryad.tm65d.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.04.056.

AUTHOR CONTRIBUTIONS

J.W.M. provided project leadership, designed the project, collected and analyzed sequence data, and performed bioinformatic analyses. M.E.K. and C.R.F. collected and analyzed sequence data. T.A.C. performed bioinformatic analyses. S.L.G., C.T.H., and G.T. tested TTX resistance of $Na_v1.7$. F.J.V. and M.K.R. sequenced the *Ramphotyphlops* genome. E.D.B., Jr., M.E.P., and E.D.B. III provided project leadership and designed the project. All authors prepared the manuscript.

ACKNOWLEDGMENTS

Funding for this work was provided by NSF grants DEB 0922216 (E.D.B. III), DEB 1034686 (M.E.P.), and DEB 1457463 (J.W.M.) and a USU New Faculty Success in Scholarship Grant (C.T.H.). We thank C. Alencar for assistance in the laboratory; C. Cox, J. Vindum (California Academy of Sciences), J. McGuire and C. Spencer (Museum of Vertebrate Zoology), and F. Sheldon and D. Dittmann (Louisiana Museum of Natural Science) for tissue grants; D. Melton, G. Mandel, and T. Olivera for providing plasmids and the rPN1 construct; M. Augat, R. Costello, S. Davies, A. de Queiroz, T. Fetters, M. Hague, A. Hornsby, J. Jahner, and B. Sanderson for comments on the manuscript; and J. Abramyan, R. Cox, and H. Greene for helpful discussions.

Received: February 17, 2016
Revised: March 15, 2016
Accepted: April 20, 2016
Published: June 9, 2016

REFERENCES

1. Jacob, F. (1977). Evolution and tinkering. Science *196*, 1161–1166.
2. Gould, S.J. (1989). Wonderful Life: The Burgess Shale and the Nature of History (New York: W.W. Norton).
3. Gould, S.J. (2002). The Structure of Evolutionary Theory (Belknap Press).
4. Stern, D.L., and Orgogozo, V. (2009). Is genetic evolution predictable? Science *323*, 746–751.
5. Blount, Z.D., Borland, C.Z., and Lenski, R.E. (2008). Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*. Proc. Natl. Acad. Sci. USA *105*, 7899–7906.
6. Meyer, J.R., Dobias, D.T., Weitz, J.S., Barrick, J.E., Quick, R.T., and Lenski, R.E. (2012). Repeatability and contingency in the evolution of a key innovation in phage lambda. Science *335*, 428–432.
7. Weinreich, D.M., Delaney, N.F., Depristo, M.A., and Hartl, D.L. (2006). Darwinian evolution can follow only very few mutational paths to fitter proteins. Science *312*, 111–114.
8. Bridgham, J.T., Ortlund, E.A., and Thornton, J.W. (2009). An epistatic ratchet constrains the direction of glucocorticoid receptor evolution. Nature *461*, 515–519.
9. Bloom, J.D., Gong, L.I., and Baltimore, D. (2010). Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. Science *328*, 1272–1275.
10. Harms, M.J., and Thornton, J.W. (2014). Historical contingency and its biophysical basis in glucocorticoid receptor evolution. Nature *512*, 203–207.
11. Shah, P., McCandlish, D.M., and Plotkin, J.B. (2015). Contingency and entrenchment in protein evolution under purifying selection. Proc. Natl. Acad. Sci. USA *112*, E3226–E3235.
12. Geffeney, S.L., Fujimoto, E., Brodie, E.D., 3rd, Brodie, E.D., Jr., and Ruben, P.C. (2005). Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. Nature *434*, 759–763.
13. Feldman, C.R., Brodie, E.D., Jr., Brodie, E.D., 3rd, and Pfrender, M.E. (2009). The evolutionary origins of beneficial alleles during the repeated adaptation of garter snakes to deadly prey. Proc. Natl. Acad. Sci. USA *106*, 13415–13420.

14. Feldman, C.R., Brodie, E.D., Jr., Brodie, E.D., 3rd, and Pfrender, M.E. (2012). Constraint shapes convergence in tetrodotoxin-resistant sodium channels of snakes. Proc. Natl. Acad. Sci. USA *109*, 4556–4561.
15. McGlothlin, J.W., Chuckalovcak, J.P., Janes, D.E., Edwards, S.V., Feldman, C.R., Brodie, E.D., Jr., Pfrender, M.E., and Brodie, E.D., 3rd. (2014). Parallel evolution of tetrodotoxin resistance in three voltage-gated sodium channel genes in the garter snake Thamnophis sirtalis. Mol. Biol. Evol. *31*, 2836–2846.
16. Brodie, E.D., 3rd, and Brodie, E.D., Jr. (2015). Predictably convergent evolution of sodium channels in the arms race between predators and prey. Brain Behav. Evol. *86*, 48–57.
17. Phillips, P.C. (2008). Epistasis–the essential role of gene interactions in the structure and evolution of genetic systems. Nat. Rev. Genet. *9*, 855–867.
18. Hansen, T.F. (2013). Why epistasis is important for selection and adaptation. Evolution *67*, 3501–3511.
19. Natarajan, C., Inoguchi, N., Weber, R.E., Fago, A., Moriyama, H., and Storz, J.F. (2013). Epistasis among adaptive mutations in deer mouse hemoglobin. Science *340*, 1324–1327.
20. Hanifin, C.T. (2010). The chemical and evolutionary ecology of tetrodotoxin (TTX) toxicity in terrestrial vertebrates. Mar. Drugs *8*, 577–593.
21. Lipkind, G.M., and Fozzard, H.A. (1994). A structural model of the tetrodotoxin and saxitoxin binding site of the Na^+ channel. Biophys. J. *66*, 1–13.
22. Jost, M.C., Hillis, D.M., Lu, Y., Kyle, J.W., Fozzard, H.A., and Zakon, H.H. (2008). Toxin-resistant sodium channels: parallel adaptive evolution across a complete gene family. Mol. Biol. Evol. *25*, 1016–1024.
23. Catterall, W.A., Goldin, A.L., and Waxman, S.G. (2005). International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol. Rev. *57*, 397–409.
24. Widmark, J., Sundström, G., Ocampo Daza, D., and Larhammar, D. (2011). Differential evolution of voltage-gated sodium channels in tetrapods and teleost fishes. Mol. Biol. Evol. *28*, 859–871.
25. Zakon, H.H., Jost, M.C., and Lu, Y. (2011). Expansion of voltage-dependent Na^+ channel gene family in early tetrapods coincided with the emergence of terrestriality and increased brain complexity. Mol. Biol. Evol. *28*, 1415–1424.
26. Isbister, G.K., and Kiernan, M.C. (2005). Neurotoxic marine poisoning. Lancet Neurol. *4*, 219–228.
27. Brodie, E.D., Jr., Ridenhour, B.J., and Brodie, E.D., 3rd. (2002). The evolutionary response of predators to dangerous prey: hotspots and coldspots in the geographic mosaic of coevolution between garter snakes and newts. Evolution *56*, 2067–2082.
28. Hanifin, C.T., Brodie, E.D., Jr., and Brodie, E.D., III. (2008). Phenotypic mismatches reveal escape from arms-race coevolution. PLoS Biol. *6*, e60.
29. Caldwell, J.H., Schaller, K.L., Lasher, R.S., Peles, E., and Levinson, S.R. (2000). Sodium channel $Na_{(v)}1.6$ is localized at nodes of ranvier, dendrites, and synapses. Proc. Natl. Acad. Sci. USA *97*, 5616–5620.
30. Dib-Hajj, S.D., Yang, Y., Black, J.A., and Waxman, S.G. (2013). The $Na_{(V)}1.7$ sodium channel: from molecule to man. Nat. Rev. Neurosci. *14*, 49–62.
31. Eckalbar, W.L., Hutchins, E.D., Markov, G.J., Allen, A.N., Corneveaux, J.J., Lindblad-Toh, K., Di Palma, F., Alföldi, J., Huentelman, M.J., and Kusumi, K. (2013). Genome reannotation of the lizard *Anolis carolinensis* based on 14 adult and embryonic deep transcriptomes. BMC Genomics *14*, 49.
32. Hutchins, E.D., Markov, G.J., Eckalbar, W.L., George, R.M., King, J.M., Tokuyama, M.A., Geiger, L.A., Emmert, N., Ammar, M.J., Allen, A.N., et al. (2014). Transcriptomic analysis of tail regeneration in the lizard *Anolis carolinensis* reveals activation of conserved vertebrate developmental and repair mechanisms. PLoS ONE *9*, e105004.
33. Brykczynska, U., Tzika, A.C., Rodriguez, I., and Milinkovitch, M.C. (2013). Contrasted evolution of the vomeronasal receptor repertoires in mammals and squamate reptiles. Genome Biol. Evol. *5*, 389–401.
34. Feldman, C.R., Brodie, E.D., Jr., Brodie, E.D., 3rd, and Pfrender, M.E. (2010). Genetic architecture of a feeding adaptation: garter snake (*Thamnophis*) resistance to tetrodotoxin bearing prey. Proc. Biol. Sci. *277*, 3317–3325.
35. Bradnam, K.R., Fass, J.N., Alexandrov, A., Baranay, P., Bechner, M., Birol, I., Boisvert, S., Chapman, J.A., Chapuis, G., Chikhi, R., et al. (2013). Assemblathon 2: evaluating de novo methods of genome assembly in three vertebrate species. Gigascience *2*, 10.
36. Castoe, T.A., de Koning, A.P.J., Hall, K.T., Card, D.C., Schield, D.R., Fujita, M.K., Ruggiero, R.P., Degner, J.F., Daza, J.M., Gu, W., et al. (2013). The Burmese python genome reveals the molecular basis for extreme adaptation in snakes. Proc. Natl. Acad. Sci. USA *110*, 20645–20650.
37. Vonk, F.J., Casewell, N.R., Henkel, C.V., Heimberg, A.M., Jansen, H.J., McCleary, R.J.R., Kerkkamp, H.M.E., Vos, R.A., Guerreiro, I., Calvete, J.J., et al. (2013). The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system. Proc. Natl. Acad. Sci. USA *110*, 20651–20656.
38. Alföldi, J., Di Palma, F., Grabherr, M., Williams, C., Kong, L., Mauceli, E., Russell, P., Lowe, C.B., Glor, R.E., Jaffe, J.D., et al. (2011). The genome of the green anole lizard and a comparative analysis with birds and mammals. Nature *477*, 587–591.
39. Song, B., Cheng, S., Sun, Y., Zhong, X., Jin, J., Guan, R., Murphy, R.W., Che, J., Zhang, Y., and Liu, X. (2015). A genome draft of the legless anguid lizard, *Ophisaurus gracilis*. Gigascience *4*, 17.
40. Shaffer, H.B., Minx, P., Warren, D.E., Shedlock, A.M., Thomson, R.C., Valenzuela, N., Abramyan, J., Amemiya, C.T., Badenhorst, D., Biggar, K.K., et al. (2013). The western painted turtle genome, a model for the evolution of extreme physiological adaptations in a slowly evolving lineage. Genome Biol. *14*, R28.
41. Hillier, L.W., Miller, W., Birney, E., Warren, W., Hardison, R.C., Ponting, C.P., Bork, P., Burt, D.W., Groenen, M.A.M., Delany, M.E., et al.; International Chicken Genome Sequencing Consortium (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature *432*, 695–716.
42. Pyron, R.A., and Burbrink, F.T. (2012). Extinction, ecological opportunity, and the origins of global snake diversity. Evolution *66*, 163–178.
43. Pyron, R.A., Burbrink, F.T., and Wiens, J.J. (2013). A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC Evol. Biol. *13*, 93.
44. Penzotti, J.L., Fozzard, H.A., Lipkind, G.M., and Dudley, S.C., Jr. (1998). Differences in saxitoxin and tetrodotoxin binding revealed by mutagenesis of the Na^+ channel outer vestibule. Biophys. J. *75*, 2647–2657.
45. Choudhary, G., Yotsu-Yamashita, M., Shang, L., Yasumoto, T., and Dudley, S.C., Jr. (2003). Interactions of the C-11 hydroxyl of tetrodotoxin with the sodium channel outer vestibule. Biophys. J. *84*, 287–294.
46. Pagel, M. (1994). Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. Proc. R. Soc. Lond. *255*, 37–45.
47. Roelants, K., Gower, D.J., Wilkinson, M., Loader, S.P., Biju, S.D., Guillaume, K., Moriau, L., and Bossuyt, F. (2007). Global patterns of diversification in the history of modern amphibians. Proc. Natl. Acad. Sci. USA *104*, 887–892.
48. Zhang, P., Papenfuss, T.J., Wake, M.H., Qu, L., and Wake, D.B. (2008). Phylogeny and biogeography of the family Salamandridae (Amphibia: Caudata) inferred from complete mitochondrial genomes. Mol. Phylogenet. Evol. *49*, 586–597.
49. Lee, C.H., Jones, D.K., Ahern, C., Sarhan, M.F., and Ruben, P.C. (2011). Biophysical costs associated with tetrodotoxin resistance in the sodium channel pore of the garter snake, *Thamnophis sirtalis*. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. *197*, 33–43.
50. Ahn, H.S., Black, J.A., Zhao, P., Tyrrell, L., Waxman, S.G., and Dib-Hajj, S.D. (2011). $Na_v1.7$ is the predominant sodium channel in rodent olfactory sensory neurons. Mol. Pain *7*, 32.
51. Wells, K.D. (2007). The Ecology and Behavior of Amphibians (Chicago: University of Chicago Press).
52. Staiman, A., and Seeman, P. (1977). Conduction-blocking concentrations of anesthetics increase with nerve axon diameter: studies with alcohol, lidocaine and tetrodotoxin on single myelinated fibers. J. Pharmacol. Exp. Ther. *201*, 340–349.