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Dual toxin resistance in a caddisfly sodium channel exposed to natural and anthropogenic selection

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Abstract

Populations face complex selective landscapes shaped by a combination of ecological pressures, and increasingly, novel anthropogenic challenges. Such selective forces may limit evolutionary responses and push populations to the brink of extinction, particularly when natural and anthropogenic selection target traits constrained by functional demands or pleiotropy. How ecological and human-induced pressures interact to shape molecular evolution remains poorly understood. In the western United States, the caddisfly *Limnephilus flavastellus* has been simultaneously exposed to a natural toxin, tetrodotoxin (TTX), by feeding on Rough-Skinned Newt (*Taricha granulosa*) eggs, as well as anthropogenic insecticides such as DDT and pyrethroids applied widely in the environment. These toxins have distinct effects on insect voltage-gated sodium channels (Na_v1), with TTX blocking sodium influx while pyrethroids prolonging channel activation. Using whole-animal assays to determine lethal concentrations (LC_{50}), we demonstrate that *L. flavastellus* exhibits 4.8-fold greater TTX resistance and 3.7-fold greater pyrethroid resistance compared to a closely related caddisfly species that does not experience these toxins in the wild. We used transcriptomic sequencing and comparative genomics to determine the genetic basis of these phenotypes, discovering 77 unique amino acid substitutions in Na_v1, including 4 in pore-forming regions associated with TTX resistance and 5 in regions linked to pyrethroid resistance in *L. flavastellus*. These mutations correspond to known resistance-conferring substitutions in other TTX-resistant species and insecticide-resistant species, demonstrating convergent evolution in the caddisfly sodium channel. These large-effect mutations coupled with many potentially compensatory mutations have important implications for our understanding of protein evolution and suggest that despite a complex selective landscape operating on a protein critical to cell electrophysiology, functional constraints on adaptive evolution can still be overcome.

Background

Populations experience a diverse selective landscape that drives the evolution of phenotypes [1, 2, 3]. Evolutionary responses to individual selective pressures have been well-studied. These pressures frequently arise from alterations in the ecological context including shifts in the predation regime, resource availability, or abiotic factors [4, 5]. Natural populations are increasingly challenged by anthropogenic sources of selection, such as habitat loss, introduced species, chemical exposure, and climate change that place novel pressures on morphology, physiology, and behavior [6, 7]. While these forces are often studied in isolation, organisms must contend with multidimensional selection regimes that can constrain adaptive responses and limit the effectiveness of any single adaptation [8, 9]. These complex combinations of selective pressures can lead to alternative behavioral strategies, the maintenance of polymorphisms, and trade-offs among life-history components [10, 11, 12, 13].

Determining the genetic architecture underlying organismal phenotypes is essential for understanding how populations adapt to multiple selective pressures. This architecture is generally viewed as optimized under the conditions in which populations have evolved. Yet, the process of adaptation is complicated by pleiotropic effects of genes and amino acid substitutions, epistatic interactions among

sites within genes, and the fact that mutations positively selected in one context may disrupt core functionality [14]. As the biological workhorse of the cell, proteins are critically important to organismal performance due to their diverse role in binding, catalysis, molecular transport, and structural utility [15]. As such, proteins are often the target of biological toxins, and increasingly, anthropogenic toxins.

Toxins generate a complex, often conflicting, selective landscape wherein mutations that protect protein function in response to toxin binding must also maintain the normal functional efficiency of the protein in the absence of the toxin. With the global increase in exposure to anthropogenic chemicals [16], organisms are challenged by ever-expanding cocktails of environmental toxins, many of which place multiple (potentially competing) demands on the functions of single proteins. Not surprisingly, trade-offs between resistance and function within proteins appear to constrain evolutionary responses to selection from toxins [17] and may require the evolution of compensatory mutations to ammeliorate the costs of resistant substitutions [18].

The voltage-sensitive ion channels of animals play crucial roles in cell signaling, ion balance, and other core physiological functions [19], and as a result, are common targets of natural and anthropogenic toxins [20; 21; 22]. Highly conserved across Metazoa [23; 24; 25], voltage-gated sodium channels (VGSC) are transmembrane proteins that initiate and propagate action potentials by selectively allowing the influx of Na^+ ions across the cell membrane. Because these proteins are essential for organismal homeostasis, disruption of their function is often lethal, and many toxins, both natural and synthetic, target VGSCs [19, 26]. The VGSC protein in insects (Na_V1) consist of four homologous domains (I-IV), each with six segments (S1-S6). Segments S1-S4 comprise the voltage-sensing module that detects membrane potential changes, whereas S5-S6 form the external pore of the protein that includes the selectivity filter (P-loop) that controls the passage of specific ions [27; 28]. Different toxins target different parts of the protein and thereby can affect different phases of the activation/inactivation process [29]. For instance, natural toxins like saxitoxin (STX) and tetrodotoxin (TTX) bind to the outer pore between the S5-S6 regions, blocking sodium influx and causing paralysis through the inhibition of action potentials and loss of neuronal function [28; 30; 31]. In contrast, synthetic insecticides like DDT and pyrethroids interact with intracellular linkers in domains I, II, and III, delaying channel inactivation and leading to sodium ion overload, hyperexcitability, and death [19; 20; 28].

Selection against non-resistant VGSC genotypes has driven the independent evolution of resistance in a wide variety of taxa exposed to sodium channel targeting toxins [e.g. 32; 33; 34; 35]. For example, grasshopper mice have evolved VGSC resistance to the β-toxins found in their venomous prey, the bark scorpion [34; 36]. Softshell clams (*Mya arenaria*) in red tide-prone areas have evolved sodium channels to tolerate STX accumulation, posing risks to human consumers [32; 37]. Similarly, global mosquito populations have evolved insecticide-resistant VGSCs following decades of malaria control efforts [38]. However, resistance adaptations acquired through amino acid substitutions in VGSCs often incur functional costs due to the channel's highly conserved structure-function relationship. Modifications that increase resistance to toxins can impair biophysical function and performance [33; 39]. Notably, in garter snakes capable of preying on TTX-bearing newts, mutations that confer TTX resistance in VGSCs also

destabilize the protein, causing hyperexcitability and reduced sodium conductance, resulting in reduced muscle and organismal performance compared to non-resistant relatives [17, 40, 41].

The costs and constraints on VGSCs adapting to single toxins have led to extensive convergence in sodium channel evolution across metazoans [28; 42; 43; 44; 45]. However, the response of organisms to the challenges of multiple toxins that target different aspects of VGSC function remains unclear. Some species may encounter both channel blockers and channel exciters in their habitats, demanding the simultaneous evolution of multiple protein functions. In the western United States, the caddisfly (Order: Trichoptera) *Limnephilus flavastellus* has been simultaneously exposed to just such a complex selective landscape, encountering natural TTX (a channel blocker) by feeding on Rough-Skinned Newt (*Taricha granulosa*) eggs, while also exposed to anthropogenic insecticides such as DDT and pyrethroids (channel exciters) applied widely in the environment (Fig. 1). Larvae in benthic habitats consume TTX-rich newt eggs, which provide valuable proteins and lipids for growth despite high toxin levels [Figure 1a,b; 46, 47]. Simultaneously, historical DDT applications from the 1940s to 1972—for timber protection and mosquito control—delivered over 2 million kg across forests and aquatic sites, directly impacting caddisfly habitats [Figure 1c; 48]. After the US ban on DDT use in 1972, pyrethroids surged in use, now comprising 30% of global insecticides [49], with applications targeting pests like clover seed weevils (*Protapion apricans*) in Oregon, leading to resistance in those species [50]. Pyrethroids are 10–100 times more toxic to aquatic invertebrates than terrestrial species, and caddisflies are among the most sensitive macroinvertebrates to these toxins [51, 52, 53]. Pyrethroids persist in agricultural runoff and Pacific Northwest stream sediments [54, 55, 56], causing sub-lethal effects such as reduced growth in caddisflies, even at low doses [53; 57; 58]. For decades, *L. flavastellus* has faced overlapping pressures on their sodium channels from these distinct toxin classes.

Here, we quantify TTX and pyrethroid resistance in a caddisfly species from toxin-exposed habitats, in contrast to a closely related caddisfly species (*Hesperophylax occidentalis*) from Utah with little to no exposure to these same toxins. Through whole-animal assays, we quantify resistance levels, followed by transcriptome sequencing and comparative genomics against non-resistant caddisfly species. We find numerous mutations in the voltage-gated sodium channel at amino acid sites known to confer TTX and pyrethroid resistance in other taxa. These insights illuminate how genetic architecture is shaped by multiple selective pressures, highlighting evolutionary trade-offs and constraints amid rising stress from anthropogenic toxins [16].

Methods

Sample collection

Caddisflies (of family Limnephilidae) were collected by dip net from four populations sympatric with TTX-bearing newts and from a single population allopatric with newts. *Limnephilus flavastellus*, sympatric with newts, were collected from Soap Creek Ponds, OR (44.670784 N, -123.276364 W), Stayton, OR (44.745369 N, -122.866025 W), Dupont, WA (47.097774 N, -122.623462 W), and

Sammamish, WA (47.596626 N, -122.057856 W). Allopatric *Hesperophylax occidentalis* were collected from Paradise, Utah (41.571087 N, -111.848111 W). *Hesperophylax occidentalis* does not co-occur with any known TTX-bearing organism. Historical forest pest management in Utah involved mechanical removal and targeted insecticide (non-DDT/pyrethroid) application, rather than mass spraying of DDT [59]. More recent agricultural production in Northern Utah primarily consists of cattle, alfalfa, and small grain production (e.g. barley, wheat, oats), with growers reporting no pyrethroid use across 11 counties for these crops [60] and only 2/29 counties using any pyrethroid-based insecticides [61]. Insecticide (DDT & pyrethroids) for mosquito abatement in Utah began around hospitals in urban areas (Salt Lake City, Brigham City) in 1943 and only recently (2004) expanded to the area of study [Cache County; 62]. A subset of caddisflies from Soap Creek, OR (*Limnephilus flavastellus*, N = 120) and Paradise, UT (*Hesperophylax occidentalis*, N = 66) were transported to Utah State University in ice-chests with a small amount of pond water and detritus for survivorship experiments (see below). Live animals were shipped on wet ice to the University of Notre Dame for RNA sequencing (see below).

Survivorship experiments

The relationship between caddisfly survival rate when exposed to different concentrations of TTX and Pyrethroid insecticides was assessed in LC_{50} trials. Toxin concentrations were selected based on ecologically-relevant exposure levels caddisflies experience in the wild. The TTX toxicity of individual newt eggs varies by population but can be as high as 0.004 mg TTX at Soap Creek [63; 64]; the LC_{50} toxicity of TTX in any invertebrate is unknown, although a previous study testing TTX toxicity at 3×10^{-7} mg TTX/ mL water found only 1 of 7 macroinvertebrate species (mayflies) was effected [65]. Pyrethroid concentrations in runoff or aerial application/drift can range between 1×10^{-9} to $1x10^{-12}$ ml pyrethroids / ml water [54; 56; 66]; the LC_{50} of pyrethroid insecticides is higher in aquatic invertebrates relative to terrestrial species and ranges between 2.0×10^{-8} – 3.0×10^{-10} mL Permethrin/ mL water [51; 52]. Tetrodotoxin solutions were generated by mixing purified TTX in citrate buffer (Abcam, Cambridge, MA) with dechlorinated water (henceforth: water) to produce the dosages reported below. Concentrated Permethrin E-Pro (Envincio LLC., Spartanurg, SC) was mixed with water to produce the reported pyrethroid dosages. All trials were conducted within 48 hrs of collection in 12-well non-treated tissue culture plates with 2.5 ml of the appropriate solution. We exposed caddisflies to TTX in the following concentrations: control (water), 0.1, 0.05, 0.005, 0.0005 mg TTX/ mL water; N = 6 per treatment per species) or pyrethroid ($1x10^{-4}$, $1x10^{-6}$, $1x10^{-8}$, $1x10^{-10}$ mL permethrin/ml water; N = 6 per treatment for *H. occidentalis*, N = 8–23 per treatment for *L. flavastellus*) solutions for 5 hrs. At the start of a trial, a caddisfly was haphazardly selected from the appropriate population, gently removed from its case using a blunt probe, and placed into a solution. Death was assessed at the conclusion of the experiment with an Olympus Z61 dissecting microscope. Criteria for death was the lack of movement when prodded with a blunt probe. There was no difference in mass between caddisflies from the two populations [t = 0.8, P = 0.42; *H. occidentalis* 0.0503 ± 0.006 g (mean ± SE), *L. flavastellus* 0.058 ± 0.05 g].

Survivorship data were used to create dose response curves and estimate LC_{50s}. The data were log transformed, and log-logistic models in the *drc* package [67] in R (v3.2.5) were used to estimate final LC_{50} values for each species exposed to each toxin. The LC_{50} between Utah and Oregon caddisflies for both TTX and pyrethroids were compared using the Multcomp v14-30 [68] procedure in R. In this case, non-overlapping confidence intervals indicate significant differences between LC_{50}'s. Due to the extreme TTX resistance observed in the Oregon species (see below), a total kill at 1mg TTX/ml was assumed for the analysis. The initial model fit analyses indicated that parameter estimates for the y-intercept, slope, and LC_{50} were fit in both TTX and pyrethroid models (all $P < 0.01$). Thus, the log-logistic models could produce accurate LC_{50} estimates and 95% CI's to compare species tolerance within each toxicant.

RNA extraction

Total RNA was extracted from four live larval individuals of *H. occidentalis* and *L. flavastellus* collected from Utah and Soap Creek, respectively, using a combination of Trizol (Invitrogen, Carlsbad, CA, USA) for extraction and RNeasy mini kit (Qiagen, Hilden, Germany) for purification. RNA bound to the filter column was treated with RNase-Free DNase Set (Qiagen) to reduce contaminating DNA. RNA was eluted with RNase-free water. After elution, purified RNA from each individual was quantified by NanoDrop and Qubit High Sensitivity RNA assay (ThermoFisher Scientific Inc., Waltham, MA, USA). RNA was also extracted and purified from three additional *L. flavastellus* from the Stayton, Dupont, and Sammamish populations and evaluated using Qubit High Sensitivity RNA (ThermoFisher Scientific Inc., Waltham, MA, USA) and TapeStation High Sensitivity RNA assays (Agilent Technologies, Santa Clara, CA, USA).

Library preparation and sequencing

Two sequence library protocols were used. In the first round, 15 ug of RNA from four individuals was pooled to create a single mixed sample for each species (*H. occidentalis* and *L. flavastellus).* From these pools, 500ng of RNA was used for library preparation using the NEBNext® Ultra™ II Directional RNA Library Prep with Sample Purification Beads (NEB #E7765), in conjunction with the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) (New England Biolabs, Ipswich, MA). Library preparation followed the manufacturer's protocol with fragmentation for 15 minutes at 94°C. Completed libraries were QCed by Qubit ds DNA High-Sensitivity Assay (ThermoFisher Scientific Inc., Waltham, MA, USA) and Bioanalyzer DNA 7500 kit (Agilent, Santa Clara, California, USA) and then pooled in equimolar amounts for final QC and sequencing. The library pool was assessed using Qubit High Sensitivity DNA, Bioanalyzer DNA 7500 assay kit, and KapaHiFi qPCR (Kapa Biosystems, Wilmington, MA, USA) assays. Libraries were sequenced on an Illumina MiSeq platform using 250bp paired-end reads in the Notre Dame Genomics and bioinformatics Core Facility. In the second round, 3 individual libraries were prepared with input of 200ng of total RNA using the NEBNext® Ultra™ II Directional RNA Library Prep with Sample Purification Beads (NEB #E7765), in conjunction with the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) (New England Biolabs, Ipswich, MA). The following modifications were

made to the protocol to target a larger (350bp) insert size to support genome guided transcript assembly. Fragmentation times were reduced to 7 minutes at 94°C, and during first strand cDNA synthesis, the incubation at 42°C was increased to 50 minutes. Adaptor ligated DNA was size selected with 30ul for the 1st bead selection and 10ul for the 2nd bead selection. Completed libraries were pooled in equimolar amounts for final QC and sequencing. The library pool was assessed using Qubit High Sensitivity DNA, Tapestation High Sensitivity D1000, and KapaHiFi qPCR (Kapa Biosystems, Wilmington, MA, USA) assays. Libraries were sequenced on a NovaSeq X Series 10B (300 cycle) flowcell (Illumina Inc., San Diego, CA, USA) using paired 150bp reads at the Indiana University School of Medicine Center for Medical Genomics.

Data filtering and transcriptome assembly

Trimmomatic v0.39 [69] was used to remove adapter contaminations and low-quality reads. The Trimmomatic parameter “ILLUMINACLIP” was used, which means that Trimmomatic first searched for seed matches (16-bp) in sequencing reads allowing maximally 2 mismatches to the adapter sequences [70]. These seeds were extended and clipped if in the case of paired-end reads a score of 30 was reached (about 50-bp), or in the case of single-end reads a score of 10 was reached (about 17-bp). For palindromic reads, the ends of reads with more than 8-bp match to Illumina adapters were clipped. The ends of reads were clipped if the quality of the base pairs at the start or the end of the read was below three, and interior portions of the reads were clipped if the average quality was below 15 using a sliding window of 4-bp. After filtering, any reads shorter than 36-bp were removed. If the paired-end reads were palindromic, both reads were kept for assembly. All the single-end reads were combined with the forward reads of the paired-end reads for assembly. Reads were assembled using Trinity v2.15.2 [71] using default settings with maximum memory of 200 G and 45 CPUs. Commands related to read filtering and assembly are available on GitHub (https://github.com/lyy005/VGSC, last accessed August 30, 2025).

Species identification based on COX1 genes

To identify caddisfly samples collected in this study, MitoZ [72] v3.6 was used to assemble the cytochrome c oxidase I (COX1) genes from the transcriptome data. In particular, COX1 genes were assembled using metaSPAdes [73] based on 2G of transcriptome sequencing data for HO and LiFl samples. For the *L. flavastellus* samples, all transcriptome sequencing data were used to retrieve the COX1 gene. Assembled COX1 gene sequences were identified using the animal species-level library (public + private) and rapid species search option on the Barcode of Life Data Systems (BOLD) (https://boldsystems.org/)

Outgroup sodium channels

Multiple data sets were used for comparative analysis of potential amino acid replacements related to TTX and insecticide resistance. These data sources included the transcriptome sequences of five other

caddisfly species from the 1KITE (1K Insect Transcriptome Evolution) project [74], the draft genome sequence of the caddisfly, *Limnephilus lunatus* [75; 76), and the sodium channel genes of *Drosophila melanogaster* [77], *Musca domestica* [78], *Bombyx mori* [79], and *Tribolium castaneum* [80] (Table S8).

Completeness of transcriptome assemblies

Benchmarking Universal Single-Copy Orthologs (BUSCO) v5.4.2 [81] was used to assess the completeness of the transcriptomes. The OrthoDB v10 Insecta BUSCOs were used for the evaluation. Three metrics were used to assess the level of transcriptome completeness: the percentage of complete BUSCOs, fragmented BUSCOs and missing BUSCOs found in the transcriptome assembly. Complete BUSCOs represented the genes that match the BUSCO profile and are within the 95% expectation range of alignment length. Fragmented BUSCO matches indicate the genes match to the BUSCO profile but not within the 95% expectation range of length. Missing BUSCOs indicated no significant matches to the BUSCO profile. The commands relating to BUSCO assessment are available on GitHub (https://github.com/lyy005/VGSC, last accessed August 30, 2025).

Sodium channel gene annotation

Voltage-gated sodium channel (VGSC) genes in vertebrates comprise a diverse gene family with as many as 10 paralogs with tissue specific patterns of expression [25]. In contrast, insect genomes lack this complex array and often harbor a single voltage-gated sodium channel [28]. Therefore, sodium channel genes were annotated based on a homolog searching algorithm. The reference database for homologous search included the sodium channel genes from *Drosophila melanogaster* [77], *Musca domestica* [78], *Bombyx mori* [79] and *Tribolium castaneum* [80] (Table S8).

To annotate sodium channels in transcriptomes, protein-coding sequences were searched using TransDecoder v5.7.1. As multiple protein-coding regions could be found on a Trinity scaffold, all proteins were used for annotation.

To further explore amino acid replacements in the sodium channel among caddisflies, five caddisfly genomes from the Darwin Tree of Life project were included (https://wellcomeopenresearch.org/treeoflife) (Table S8). The corresponding genome annotations were retrieved from Ensembl (https://projects.ensembl.org/darwin-tree-of-life/). The longest isoform of each gene in the genome assemblies was used for annotation.

For homologous annotation, protein sequences from both transcriptomes and genomes were aligned to the sodium channel gene amino acid sequences of the four reference insects, *B. mori, D. melanogaster* (the paralytic gene), *M. domestica*, and *T. castaneum* using BLASTP v2.13.0 [82] with e-value = 1e-10. BLASTP hits with sequence identity ≥ 50% were kept. The longest BLAST hit of each species was annotated as sodium channel genes and used for downstream analysis.

To ensure that potential paralogs of sodium channel genes were not missed, all BLAST hits were extracted and aligned to check whether they shared the same substitutions in the key TTX- and insecticide-resistant sites.

In addition to the BLASTP approach, all genes were annotated through KEGG using GhostKOALA [83]. Genes annotated as voltage-gated sodium channel (KEGG Orthology ID: K04834) were extracted to confirm that the same set of genes was annotated by BLAST and KEGG approaches.

Lastly, domains of the caddisfly sodium channels were annotated based on the gene structure of *D. melanogaster* (UniProt ID: P35500) from UniProt [84] and visualized in Geneious v2025.2.1 (https://www.geneious.com). Sequences of the newly assembled sodium channel genes, other caddisfly sodium channel genes and the four insect reference sequences were aligned based on codon sequences [85]. The script for codon alignment is available on GitHub (https://github.com/lyy005/codon_alignment, last accessed August 30, 2025).

Amino acid replacements relating to TTX and insecticide resistance

To estimate the overall amino acid divergence, the pairwise similarities of the sodium channel genes among the 14 samples (from 11 insect species) were calculated using Geneious v2025.2.1 (https://www.geneious.com). In addition, a phylogenetic tree was constructed using the protein sequences of the sodium channel genes. Sodium channel sequences were first aligned using MAFFT L-INS-i v7.526 [86]. The IQ-Tree v3.0.1 [87] was used with the best-fit model (JTT + I+R2), 1,000 ultrafast bootstrap approximation. *B. mori, D. melanogaster*, *M. domestica*, and *T. castaneum* were used as outgroups.

A Perl script was used to filter amino acid replacements if the replacement is consistent within the four resistant caddisflies and different from other ten species. Amino acid replacements among the 11 insect species were then matched to known amino acid replacements relating to TTX and insecticide resistance. Known amino acid replacements associated with TTX resistance were collected from previous studies [30; 44; 88], including 23 TTX-resistant substitutions from 12 invertebrates and 13 substitutions from 9 vertebrates. All amino acid sites were annotated and indexed following the *M. domestica* gene model [28]. Known replacements in voltage-gated sodium channel genes related to resistance to DDT, Pyrethrins, and Pyrethroid were collected from Dong et al. [28], including 50 insecticide-resistance related substitutions from 55 invertebrate species. The TTX- and pyrethroid-resistance information was combined and incorporated into the sequence alignment.

Calling single nucleotide variants (SNV) in samples

It is possible that Trinity only assembled one allele of sodium channel genes of the genome. SNV calling based on the sequencing reads to the sodium channel assembly was used to avoid missing TTX- or pyrethroid-resistance alleles. The GATK best practices (https://github.com/trinityrnaseq/trinityrnaseq/wiki/SuperTranscripts) for variant calling on

transcriptome data were applied. In particular, assembled transcriptomes were first combined into SuperTranscripts [89] using the Trinity_gene_splice_modeler.py script in Trinity v2.15.2. Then, based on the tools implemented in the Trinity v2.15.2 singularity image, STAR v2.7.8a [90] was used to align reads to the SuperTranscripts. Picard ve2.25.7 [91] was used to add read group, sort, mark duplicates and create an index. GATK v4.2.1.0 [92] was used to split reads into exon segments, remove “N” base pairs and reassign mapping qualities. GATK HaplotypeCaller [93] was then used to call single nucleotide variants (SNV) with default parameters. The result of variant calling for each nucleotide locus was also added into the combined file in Table S6.

Molecular test for the signature of selection

PAML version 4.10.9 [94] and HyPhy version 2.5.73 [95] were used to test whether substitutions were under positive selection. The PAML branch model [96] and HyPhy aBSREL [97] were used to test for positive selection in the branch leading to *L. flavastellus*. The PAML branch-site model [96] and HyPhy BUSTED were used to test for codons under positive selection in the test clade [98].

The sodium channel gene tree and the codon alignment was used as the input for all PAML analyses. The test branches were labeled in two different ways. First, as the TTX-resistance phenotype was gained in the ancestral stage and many amino acid substitutions were shared among the *L. flavastellus* clade, the ancestral node leading to the TTX-resistant *L. flavastellus* was labeled as the test branch for all four analyses. The signature of positive selection was assessed by labeling the clade of *L. flavastellus* and testing for positive selection in the whole TTX-resistant clade.

For the PAML branch model, the Null model was used first, which estimated one *dN/dS* ratio across the tree. Then the Alternative model was used to estimate two different *dN/dS* ratios for the test branch and the background branch. Lastly, another Alternative model that estimates two different *dN/dS* ratios, with the test branches fixed with *dN/dS* = 1 was used to test whether the test branches have a *dN/dS* ratio close to 1.

To test for codons under positive selection, the setups of PAML branch-site model A were followed [96]. The codon alignment and the node-labeled and clade-labeled trees were the same as the branch model. The Null hypothesis was that the codons in the test branches had *dN/dS* ratios < 1, while the Alternative hypothesis was that the codons in the test branches had *dN/dS* ratios > 1.

Similar to the PAML branch model, the HyPhy aBSREL model was used to test for branches under positive selection. The input codon alignment was the same as the PAML analyses. The online version of HyPhy version 2.5.73 on the datamonkey website (datamonkey.org)was used for this analysis. A gene tree was generated on the datamonkey website and the gene tree was rerooted using the four outgroup species, i.e. *Bombyx mori*, *Drosophila melanogaster*, *Musca domestica*, and *Tribolium castaneum*. The gene tree was labeled in two ways, on the ancestral node and the whole clade leading to *L. flavastellus*. To test for codons under positive selection, the BUSTED model was used with HyPhy version 2.5.73 on the datamonkey website. The inputs codon alignment was the same as used in aBSREL.

Results

Survivorship experiments

Caddisflies (*L. flavastellus*) from Oregon were significantly less sensitive to TTX and pyrethroids compared to those from Utah (*H. occidentalis*; Fig. 2a,c). For Oregon caddisflies exposed to TTX, the LC_{50} was 0.10 mg/mL (95% CI: 0.07–0.13 mg/mL) compared to 0.021 mg/mL (95% CI: 0.004–0.04 mg/mL) for Utah caddisflies, a 4.8-fold difference (Fig. 2b). The same pattern held for pyrethroids with Oregon caddisflies (*L. flavastellus*) having an LC_{50} of 2.02×10^{-8} mL/mL (95% CI: 1.9–2.1 $\times 10^{-8}$ mL/mL), approximately 3.7 times higher than Utah caddisflies (*H. occidentalis*; 5.4×10^{-9} mL/mL; 95% CI: 4.9–5.8 $\times 10^{-9}$ mL/mL; Fig. 2d).

Sequencing data filtering, assembly and annotation

After data filtering, 14.6 to 182.9 million reads passed the quality filtering for the four samples of *L. flavastellus* and one sample of *H. occidentalis* (Table S1). These filtered reads were used for subsequent analysis (Table S1). After Trinity assembly, 78.2% to 98.40% of the Insecta BUSCOs were complete in the five samples. Only 0.90% to 10.80% BUSCOs were missing (Table S2), indicating a high completeness of the transcriptome assemblies.

Based on BLASTP search to the sodium channel genes of the four reference insect species, one complete sodium channel gene with a start codon and a stop codon was annotated for each transcriptome and genome. The sodium channel genes ranged from 2,065 to 2,146 amino acids (aa) for the published caddisfly genomes and 2,050 aa for the transcriptome of *H. occidentalis*. Interestingly, the sodium channel genes in *L. flavastellus* samples were relatively short, ranging from 1,625 to 1,773 aa.

Inferred toxin-resistance amino acid replacements in L. flavastellus

The average pairwise amino acid similarity among the sodium channels of the 11 species was used to estimate the divergence among the sodium channel genes. The average pairwise amino acid similarity among all species was 77.97% (Table S3). Among caddisfly sodium channels, the average amino acid similarity was 82.97%. The sodium channels of *L. flavastellus* were more similar to each other (average similarity = 93.33%) than to other caddisfly species (average similarity = 75.79%). The phylogeny of sodium channel sequences (Figure S1) shows that *L. flavastellus* forms a monophyletic group with a relatively long branch length separating this clade from other caddisfly species.

The annotations from the well-investigated sodium channel of *D. melanogaster*, were used to investigated replacements specific to the TTX-resistant caddisflies. To investigate amino acid replacements unique to the TTX-resistant caddisfly species, *L. flavastellus*, the amino acid locus was defined as a unique replacement if: (1) the locus was fixed for a novel amino acid in our sample of *L. flavastellus* (N = 4) and not shared with any of the non-resistant species; and (2) the locus had no gaps

across all species. All amino acid sites were annotated and indexed following the *M. domestica* gene model [28]. In total, 77 unique amino acid replacements were found in *L. flavastellus* compared to the other 10 non-resistant insect species (Table S4). These replacements were found in both the transmembrane domains and the cytoplasmic regions of sodium channel genes. Of these unique replacements, five replacements were at sites known to confer pyrethroid resistance in other insect species (Fig. 3, Table S4). Four were found within the outer pore, which is critical to TTX resistance (Fig. 3, Table S4). None of the amino acid replacement locations showed single-nucleotide variants (SNVs) among the *L. flavastellus* individuals (Table S5). In addition, all *L. flavastellus* samples showed deletions in the cytoplasmic regions at the beginning of Domain II and the last cytoplasmic region at the end Domain IV, even when considering all possible alleles. These deletions explained the relatively short sodium channel gene compared to other species.

SNVs related to TTX- and insecticide-resistance

The distribution of SNVs in the VGSC varied among different resistant samples (Table S5). LiFl_CF_27 had the lowest number of SNVs (3 SNVs) while LiFl_CF_36 showed the highest number of SNVs (45 SNVs). Samples showed different levels of polymorphisms along the sodium channel gene. Among the known TTX- and insecticide-resistant replacements (Fig. 3), three sites (E435K, V1010A, and A1410T) did not show any polymorphism among the four resistant populations (notation follows that for *D. melanogaster*).

Molecular signature of selection

To test whether the substitutions in the sodium channels were due to positive selection, four different approaches were used: the branch and branch-site models [96] in PAML [94], and the aBSREL [97] and BUSTED [98] models in HyPhy [95]. The gene tree was labeled in two different ways. The ancestral node leading to *L. flavastellus* was labeled to test for positive selection at the ancestral stage of *L. flavastellus*. The whole clade of *L. flavastellus* was labeled to test for positive selection in the TTX-resistant clade. Based on the branch model, both the ancestral node and the whole clade were under positive selection (Table S6). The ancestral node had a significantly higher *dN/dS* ratio (*dN/dS* = 0.484) than the rest of the tree (*dN/dS* = 0.0485) (c^2 test statistics = 167.955, degrees of freedom = 1, c^2 test p-value < 0.00001). Positive selection was also detected in the whole clade with dN/dS = 0.459 and a background *dN/dS* = 0.0463 (c^2 test statistics = 234.398, degrees of freedom = 1, c^2 test p-value < 0.0000). Consistent with the PAML branch model, aBSREL also found evidence of episodic diversifying selection in the ancestral branch leading to *L. flavastellus*, based on both the node- and clade-labeled trees.

The PAML branch-site model was used to examine for codons under positive selection in the test branches. Labeling only the whole TTX-resistant clade gave a significantly better model (c^2 tests p-value = 0.00298). In this case, twelve codons were under positive selection (Table S7). Three of these sites overlap with *L. flavastellus*-specific amino acid replacements (Table S4). For BUSTED, labeling only the ancestral node gives a significantly better model (c^2 tests p-value = 0.00000160). Five sites showed

signals of episodic diversifying selection. All five sites were also detected under positive selection by the PAML branch-site model (Table S7). Two of the five codons were *L. flavastellus*-specific amino acid replacements.

Conclusions

When environmental toxins exert distinct functional demands on a single target protein, they generate multiple selection pressures that pose a major challenge for adaptation via molecular evolution. Here we show that caddisflies (*L. flavastellus*) from regions exposed to both TTX and pyrethroid insecticides display high levels of physiological resistance to both toxins, as compared to allopatric populations. Transcriptome data reveal that individuals from a single population in Oregon exhibiting TTX and pyrethroid resistance, and individuals from 3 other populations of the same species from the western US, have 77 unique amino acid substitutions in coding regions of the sodium channel gene, 9 of which are known to confer resistance to these toxins in various organisms [e.g., 28, 30, 88]. The remaining substitutions have unknown effects but may be involved in compensatory changes that help retain core function of the channel in light of these dramatic structural changes. These results show that even in a highly conserved protein, adaptive evolution can occur in response to multiple environmental toxins with distinct functional effects.

The physiological effects of TTX and pyrethroid insecticides on caddisflies were assessed by immersing caddisfly larvae in solutions of these toxins. Caddisflies (*L. flavastellus*) from Oregon had 4.8 times greater resistance to TTX and 3.7 times greater resistance to insecticides relative to a caddisfly lacking contemporaneous exposure to these toxins (*H. occidentalis*). Caddisflies exposed to the highest dose of TTX received 10 – 20% of the human oral lethal dose (1–2 mg) and 400% the intravenous lethal dose, yet mortality was only 50%. These caddisflies showed similar levels of resistance to pyrethroid insecticides, with survival at 10–100 ug/L or 100 – 10,000 times higher concentrations than required to kill naïve caddisflies [99], and comparable to various “super-resistant” pest insects [100, 101]. Singular resistance to TTX or pyrethroids has been documented in numerous species. For example, multiple species of garter snakes from the western US display resistance to TTX found in their newt prey [102, 103, 104, 105]. More broadly, a taxonomically diverse snake fauna have independently evolved TTX resistance to toxic amphibian prey at a global scale [33]. In parallel, anthropogenic pressures have driven similar adaptations in insects, where resistance has evolved to virtually every class of insecticide [106, 107, 108, 109, 110]. While simultaneous resistance to multiple classes of insecticide has been reported [111, 112], in these cases either the target sites of the compounds operate on disparate parts of cell physiology or involve enzymes with cross specificity to multiple compounds. In contrast, TTX and pyrethroid insecticides target different regions of the same protein, with distinct effects on channel function. Whereas TTX targets the outer pore and blocks sodium influx [28, 30, 31], DDT and pyrethroids hold the channel open preventing inactivation leading to hyperexcitability [19, 20, 28]. The presence of resistance to two compounds with distinct effects on the voltage-gated sodium channel raises important questions about the basis of resistance and the molecular adaptations enabling such dual tolerance.

To understand the molecular underpinnings of this dual toxin resistance, we used transcriptome sequencing to compare variation in sodium channel genes from four populations of the resistant caddisfly (*L. flavastellus*) to toxin-sensitive caddisflies and other insects. We identified 77 unique amino acid replacements in the sodium channel gene of the resistant caddisfly species, which were present in all four populations from the western US. Comparisons with published sequences revealed four amino acid replacements related to increased TTX resistance, located in the pore-forming regions of Domains I and III. Notably, the Q376L substitution in Domain I [mutations numbered after *M. domestica* sodium channel gene (GenBank AAB47604.1), following Dong et al. [28] mirror those in TTX-resistant sea anemones (*Aiptasia pallida*) and jellyfish (*Cyanea capillata* and *Polyorchis penicillatus*) [30]. The pore-forming region of Domain III is a hotspot for replacements in other invertebrates [30, 113], as well as vertebrates such as pufferfish [42, 114], salamanders [35, 115], and snakes [33, 115]. Two replacements discovered in caddisflies are in the pore α-helix region of Domain III, while a third is next to the β-strand. These are adjacent to regions critical for TTX binding [117, 118, 119], where a key substitution seen in garter snakes, newts, and pufferfish reduces TTX affinity 15-fold [104, 114, 115]. While functional validation via *ex vivo* expression is needed, these replacements likely underpin TTX-resistance in *L. flavastellus*. Beyond TTX resistance, five additional replacements in the same sodium channel confer resistance to pyrethroids and DDT, highlighting the protein's versatility under dual selective pressures. These include replacements in the S4-S5 linker of Domain II associated with knockdown resistance in thrips and *Anopheles* [120, 121], two others that confer resistance in *Anopheles sinensis* and the German cockroach (*Blattella germanica*) [122, 123], and an A1410T substitution linked to 18-fold increase in DDT resistance in *Drosophila melanogaster* [124]. These nine sodium channel replacements reveal the convergent nature of sodium channel evolution in *L. flavastellus* adapting to dual neurotoxins. Despite the seperate effects of TTX and pyrethroid toxins, the sodium channel of *L. flavastellus* shows remarkable adaptability having evolved resistance to both toxins. The number of amino acid substitutions detected in caddisflies suggest that sodium channels may be more evolutionarily labile than previously assumed, perhaps because the distinct structures of the channel can evolve independently of one another in a modular fashion.

Beyond these nine toxin-resistant replacements, we identified 68 additional unique substitutions in *L. flavastellus*, 25 of which are fixed across populations. Given the physiological importance of this protein and the predictable molecular patterns that arise in association with resistance [33, 44], this extreme number of replacements in a single lineage is noteworthy. These findings seem at odds with the remarkable level of conservation in VGSC amino acid sequences across diverse vertebrae lineages, and the observation of striking parallelism in the amino acid substitutions that contribute to the evolution of TTX resistance. Taken together, these observations suggest that VGSC proteins are under strong purifying selection driven by functional constraints. Previous work on mutations associated with either TTX or pyrethroid resistance has found a small number of large-effect replacements that dramatically modify channel function. For instance, TTX-resistant snakes typically show five or fewer amino acid replacements [33, 104, 116, 125, 126], and pyrethroid resistance in cockroaches or mosquitoes requires just three or four mutations [127, 128]. Furthermore, the handful of channel mutations that alter toxin

resistance also tend to impair channel function, imposing biophysical tradeoffs that have negative fitness consequences [17, 41, 129]. In the case of TTX-resistant replacements, mutations affect the electrophysiological performance of the channel by reducing ion conductance thereby altering channel excitability [17]. In insects, pyrethroid-resistant replacements also similarly lead to reduced fitness [130, 131]. Thus, the nine amino acid substitutions that presumably alter TTX and pyrethroid resistance in *L. flavastellus* likely impair native sodium channel gating or conductance, rendering them adaptive only in toxin-exposed environments [14]. We hypothesize that the remaining 68 substitutions found only in *L. flavastellus* serve a compensatory role, mitigating the costs of large-effect resistance alleles and enabling dual tolerance despite the opposing actions of TTX and pyrethroids on the sodium channel. Questions over the specific functional roles of these substitutions remain (e.g., permissive or rescue function). The specific effects of individual, and combinations of, amino acid substitutions may also be moderated by the posttranscriptional mechanisms of alternative splicing and RNA editing [132]. It has been suggested that these posttranscriptional modifications allow for modulation of protein quantity and may increase functional diversity in voltage-gated ion channels, which are known to occur in insect voltage-gated sodium channels [28]. These mechanisms may play a significant role in insects as they typically have a single (or few) copies of the voltage-gated sodium channel gene, in contrast to vertebrates that have a large array of paralogs with tissue and developmental stage specific patterns of expression. Examination of available caddisfly genomes and transcriptomes suggest that there is a single copy of the voltage-gated sodium channel in the toxin resistant populations. Neither mechanism is likely to explain the larger number of fixed substitutions we observe in the toxin resistant caddisflies, however, either could play a significant role in the functional attributes of the mature protein. Further biophysical work will be required to understand the effects of single and combined substitutions and metabolomic assays will be required to assess the role of posttranscriptional modifications. While it seems unlikely that the long branch of channel substitutions found in *L. flavastellus* could arise through neutral processes like drift, there may be additional sources of selection on the sodium channels of these caddisflies.

The order in which these mutations may have accumulated during an adaptive walk to their current configuration is unknown. Yet this order and the timescale of selection carry critical implications for channel evolution, with the selective response in one context potentially allowing resistance to be more easily conferred in another. If TTX resistance evolved first, pre-existing substitutions could have lowered the fitness costs or expanded the mutational landscape for subsequent insecticide resistance. In newts, TTX resistance (and thus its likely use as a defensive chemical) traces to substitutions in ancestral sodium channels ~ 145 MYA, followed by a series of mutations in the common ancestor of modern newts ~ 69 MYA [115]. Aside from neutral processes or an unknown source of selection, caddisflies exposure to any insecticide (and the associated mutations) is contemporary (~ 75 years). Thus, if TTX-resistant substitutions were already fixed or standing in caddisfly populations long before selection from insecticides, they could reduce pleiotropic costs or open mutational pathways once exposed to this new selective source [especially given insects have only one Na_V gene; 25]. In TTX-resistance evolution, functional constraints and epistatic interactions shape the order of Na_V substitutions with early

substitutions often enabling later ones without catastrophic trade-offs [33, 125]. Similarly, adaptations to natural xenobiotics (e.g. plant toxins) are known to pre-adapt insects to synthetic pesticides that act on the same channel via standing variation or reduced pleiotropy [133]. Thus, any historical TTX-driven modifications to the caddisfly Na_v channel may have facilitated subsequent changes conferring insecticide resistance. Alternatively, the opposite scenario is also possible, that possession of TTX-resistant residues in Na_v rendered *L. flavastellus* more susceptible to synthetic pyrethroids. Thus, modern exposure to these insecticides imposed especially intense selection, resulting in rapid fixation of pyrethroid-resistant changes in *L. flavastellus* Na_v. Additional research, ideally with museum specimens that predate the application of pyrethroids, might elucidate the pattern of mutations during the adaptive walk to the current phenotype.

Functional trade-offs are hypothesized to drive the molecular evolution of resistance in organisms evolving in response to environmental toxins [17, 44, 134]. These trade-offs occur between toxin resistance and ion channel performance and enforce limitations on the adaptive evolution of the sodium channel protein [17, 33, 41]. When coupled with purifying selection over evolutionary time, adaptive evolution in a functionally constrained protein may be restricted to relatively few large-effect mutations [14]. Because *L. flavastellus* has been exposed to multiple toxins that target the same protein, we expected an even narrower set of mutations would preserve ion channel function and provide resistance. We instead found a large number of mutations in the sodium channel of *L. flavastellus*. These findings reveal unexpected variation in a conserved ion channel, with implications for models of protein evolution under multi-constraint regimes, including the role of compensatory networks in stabilizing large-effect alleles with potentially competing effects. Ultimately, understanding the order in which these substitutions arose, as well as their independent and epistatic effects, will be critical to reveal how organisms evolve resistance in such a conflicting adaptive landscape.

Declarations

Ethics Approval and Consent to Participate

Not applicable. This study did not involve human or vertebrate participants, data, or tissue requiring ethical approval.

Consent for Publication

Not applicable

Data Availability

Transcriptome sequence data are archived in the open access NCBI SRA (BioProject PRJNA1474585, BioSample IDs SAMN60600636-40). LC_{50} data are available from the corresponding author on request.

Competing Interests

The authors declare no competing interests.

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Figures

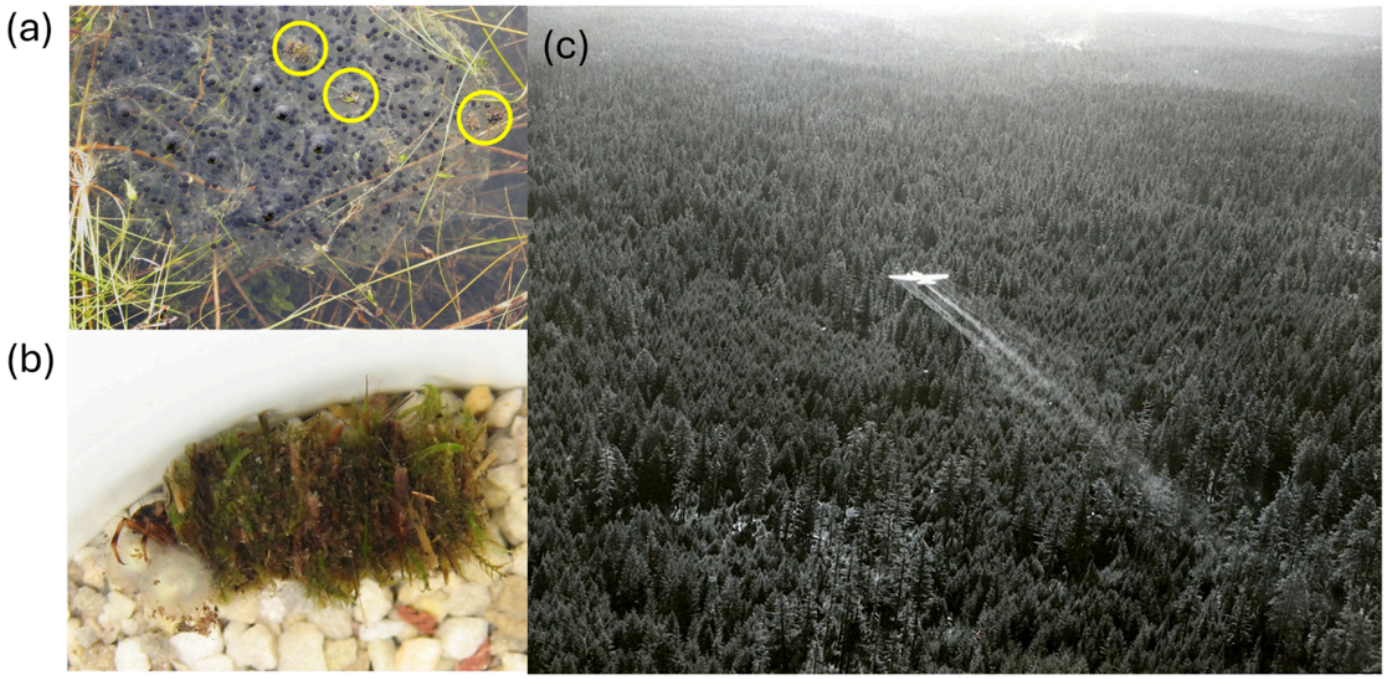


Figure 1

Photo of caddisfly larvae (*Limnephilus flavastellus*) consuming amphibian eggs at the Soap Creek study site (a) and an individual *L. flavastellus* consuming a toxic newt egg in the laboratory (b). (c) Photo of aerial application of DDT in the Mt. Hood area (Oregon) as part of Western spruce budworm control project in 1949. Photos (a) and (b) by BGG. (c) by Robert L. Furniss and John F. Wear with credit to USDA Forest Service, Pacific Northwest Region, State and Private Forestry, Forest Health Protection. Collection: Portland Station Collection; La Grande, Oregon. Image: PS-796.

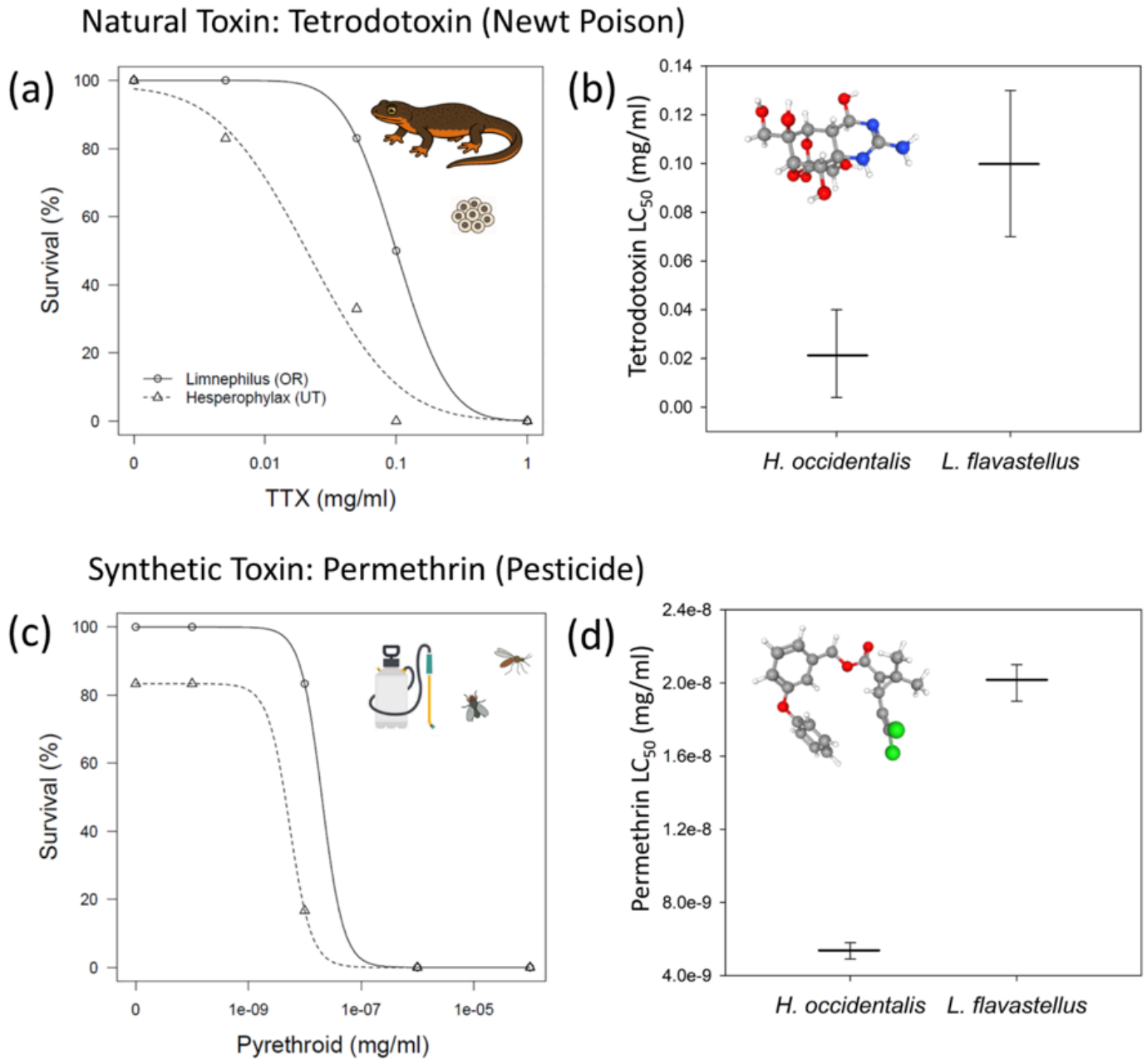


Figure 2

Dose response curves and corresponding LC_{50} (± 95% CI) for two species of caddisfly exposed to purified Tetrodotoxin (a, b) and Pyrethroid insecticide (c, d). *L. flavastellus* (open circles) were collected from a population that co-occurs with toxic newts and has historical exposure to DDT and pyrethroid insecticides. *Hesperophylax occidentalis* (open triangles) were collected in Paradise, UT and do not co-occur with newts. *Limnephilus* exhibits a significantly higher TTX LC_{50} (0.1 mg TTX/ml) and significantly higher Pyrethroid LC_{50} (2.02×10^{-8} ml permethrin/ml) compared to allopatric *Hesperophylax* (0.02 mg TTX/ml, 5.4×10^{-9} ml permethrin/ml).

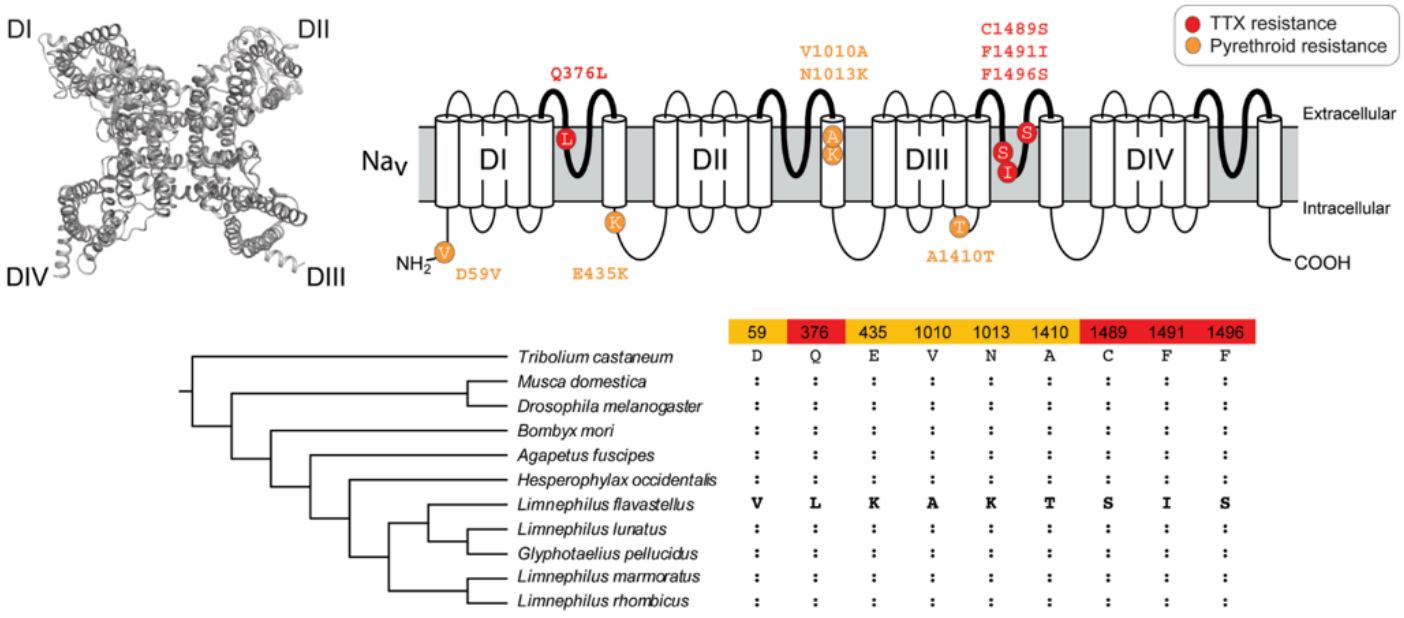


Figure 3

Voltage-gated sodium channels (Nav) are the targets of naturally occurring neurotoxins and synthetic pesticides. Ribbon diagram of Nav protein (view from top down), alongside 2D cartoon of the channel. The sodium channel contains four homologous domains (Domains I to IV). Each domain contains six transmembrane segments (S1-S6). Segments S1-S4 form the voltage sensing module and S5-S6 forms the pore module (P-loop, dark black). Amino acid substitutions only found in *L. flavastellus*related to insecticide and TTX resistance. Mutations are numbered after Dong et al. [28] based on the *M. domestica* sodium channel gene deposited in GenBank (Accession number: AAB47604.1). Numbers in yellow indicate that the replacements are known to affect insecticide resistance. Numbers in red indicate the replacements are within the pore region of the sodium channel gene and lead to TTX resistance. Phylogeny based on our analyses of sodium channel genes (see Fig S1).

Supplementary Files

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