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Are Pacific Chorus Frogs (*Pseudacris regilla*) Resistant to Tetrodotoxin (TTX)? Characterizing Potential TTX Exposure and Resistance in an Ecological Associate of Pacific Newts (*Taricha*)

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ABSTRACT.—**Animals that frequently encounter toxins often develop mechanisms of toxin resistance over evolutionary time. Both predators that consume toxic prey and organisms in physical contact with a toxin in their environment may experience natural selection for resistance. Based on observations that Pacific Chorus Frogs (*Pseudacris regilla*) sometimes eat and mistakenly amplect tetrodotoxin (TTX)-defended *Taricha* newts, we predicted that *P. regilla* may possess TTX resistance. We compared amino acid sequences of domain IV of the muscle voltage-gated sodium channel gene *SCN4A* ($Na_V1.4$) in populations of *P. regilla* that are sympatric and allopatric with *Taricha*. We identified a single substitution in $Na_V1.4$ of *P. regilla* at a conserved site in the pore loop where TTX binds. Although the role of this site in TTX resistance has not been functionally assessed, both allopatric and sympatric *P. regilla* had this substitution, along with several other reptiles and amphibians, suggesting that it may be unrelated to TTX exposure from *Taricha*. Thus, there is no conclusive evidence that *P. regilla* possesses TTX resistance encoded by amino acid substitutions in this domain. California occurrence data from the last 50 yr indicate that *Taricha* activity peaks in January while the activity of *P. regilla* peaks in April, with times where the species may come into contact. However, *P. regilla* may not be exposed to levels of TTX from *Taricha* high enough to select for mutations in $Na_V1.4$. Other unidentified mechanisms of TTX resistance could be present in *P. regilla* and other species sympatric with toxic newts.**

RESUMEN.—**Los animales que están expuestos frecuentemente a toxinas suelen desarrollar mecanismos de resistencia a las mismas a lo largo de su historia evolutiva. Tanto los depredadores que consumen presas tóxicas como los organismos en contacto físico con una toxina o contaminante en su entorno pueden experimentar presiones de selección natural hacia mecanismos de resistencia. Observaciones de campo han reportado a las ranas coro del Pacífico (*Pseudacris regilla*) comiendo y amplexando por error a salamandras del género *Taricha* que secretan tetrodotoxina (TTX). Por lo tanto, surge la hipótesis de que *P. regilla* podría poseer resistencia al TTX. Probamos esta hipótesis comparando las secuencias de aminoácidos del gen del canal de sodio voltaje dependiente muscular *SCN4A* (NaV1.4), que es una proteína diana de la TTX, en poblaciones de *P. regilla* que son simpátricas y alopátricas con *Taricha*. Identificamos una única sustitución en NaV1.4 de *P. regilla* en un sitio conservado en el ploop del poro donde se une la TTX. Aunque el papel de este sitio en la resistencia a la TTX no ha sido evaluada funcionalmente, tanto las *P. regilla* alopátricas como en las poblaciones simpátricas tenían esta sustitución, junto con varios otros reptiles y anfibios, lo que sugiere que esta sustitución no está relacionada con la exposición a la TTX. Por lo tanto, no hay pruebas concluyentes de que *P. regilla* posee resistencia a la TTX debida a cambios aminoacidícos en el dominio IV. Por otro lado, los datos de ocurrencia en California de los últimos 50 años indican que la actividad de *Taricha* alcanza su máximo en enero, mientras que la actividad de *P. regilla* alcanza su máximo en abril, con algunos momentos en los que las especies se superponen en actividad y pueden entrar en contacto. Sin embargo, es posible que *P. regilla* no esté expuesta a niveles de TTX de *Taricha* lo suficientemente altos como ejercer una presión de selección que fije mutaciones en el canal de sodio. No obstante, otros mecanismos no identificados para adquirir resistencia al TTX podrían estar presentes en *P. regilla* y otras especies simpáticas a las salamandras tóxicas.**

Newts of the genus *Taricha* (*Taricha torosa*, *T. granulosa*, *T. sierrae*, and *T. rivularis*, herein collectively referred to as *Taricha*) wield tetrodotoxin (TTX) on their skin (Brodie et al., 1974; Mailho-Fontana et al., 2019; Reimche et al., 2020) that likely functions as an antipredator defense (Brodie et al., 2002; Williams et al., 2010; Bucciarelli et al., 2017). TTX is a neurotoxin that blocks voltage-gated sodium channels by binding to highly conserved regions of the channel, fitting in the outer pore of the channel and blocking proper transport of Na^+ ions (Hille, 2001). TTX binding prevents action potentials from propagating, resulting in cessation of nerve signals (Hille, 2001). In vertebrate animals lacking TTX resistance, ingested TTX can temporarily paralyze or, in some cases, kill the organism—knowledge that has been held for millennia by Egyptian and Chinese cultures and more recently quantified by Western researchers (Hanifin, 2010). The poison usually becomes fatal when respiratory muscles fail, resulting in suffocation (Brodie, 1968). The San Francisco Bay Area in California is one of two known hotspots of the arms race between *Taricha* newts and their garter snake predators (Brodie et al., 2002; but see Hanifin et al., 2008). Here we explore the idea that high levels of TTX defenses in newts may select for resistance in other sympatric organisms outside the predator–prey relationship, namely Pacific Chorus Frogs, *Pseudacris regilla*.

Taricha and their relatives possess amino acid changes in the muscle voltage-gated sodium channel $Na_V1.4$ that help them resist their own TTX defenses (Hanifin and Gilly, 2015; Gendreau et al., 2021b). Amino acid substitutions in voltage-gated sodium channels (VGSCs) alter the shape of the pore loop (p-loop) and prevent TTX from binding; mutations in domain IV (DIV) of $Na_V1.4$ are especially common and well studied in resistant animals (Choudhary et al., 2003; Geffeney et al., 2005; Tikhonov and Zhorov, 2005; Jost et al., 2008; Hanifin and Gilly, 2015). Amino acid substitutions in the p-loop can affect the function of VGSCs by altering sodium ion selectivity, gating properties, or ion conductance; hence, trade-offs may exist between TTX resistance and organismal function (Chiamvimonvat et al., 1996; Lee et al., 2011; Feldman et al., 2012; Hague et al., 2018; but see Moniz et al., 2021). Therefore, amino acid

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FIG. 1. Examples of *Pseudacris regilla* and *Taricha torosa* interactions: (A) Amplexus in Briones Regional Park, Martinez, California, 1 March 2019, photo credit K. Montana, user kmontana on iNaturalist (CC BY-NC 4.0; observation 100725148); (B) Another instance of amplexus in Briones Regional Park, Martinez, California, 21 February 2009, photo credit user ap2il on iNaturalist (CC BY-NC 4.0; observation 550227); (C) Attempted predation on *T. torosa* by *P. regilla* in Mark West Springs, California, 1 March 2014, photo credit user tiwane on iNaturalist (CC BY-NC 4.0; observation 2312). The frogs did not appear to be harmed in any of these scenarios, though the tongue posture in C suggests some numbing effects from newt exposure.

changes are often confined to specific conserved regions that can provide resistance while allowing the organism to maintain sodium channel functionality (Feldman et al., 2012). The presence of amino acid substitutions that alter toxin binding is a mechanism known as target-site insensitivity and is the focus of this study.

Tetrodotoxin has been shown to affect the behavior of organisms present in aquatic systems. Larval *T. torosa* use TTX as a cue to detect and avoid adult *T. torosa* that sometimes cannibalize larvae (Zimmer et al., 2006). TTX in water can also slow dragonfly nymph consumption of *T. torosa* larvae (Bucciarelli and Kats, 2015). New Zealand Mud Snails (*Potamopyrgus antipodarum*) move out of their stream environment when encountering biologically relevant levels of TTX or chemical cues from *T. torosa* containing TTX (Ota et al., 2018). Water containing TTX also increases newt trematode parasite mortality (Calhoun et al., 2017). While the concentration of TTX in natural waterways is unknown in our study, TTX produced by *T. torosa* may affect other organisms in the same aquatic environment, including *P. regilla*.

Pseudacris regilla may come into contact with TTX in pond water shared with *Taricha* or via direct contact with *Taricha*, sometimes in mistaken amplexus behavior or even attempted predation (Fig. 1). Given that *P. regilla* have permeable skin, we expected that they would be exposed to TTX through physical contact with newts or by occupying pond water that contains newts. We hypothesized that TTX exposure has caused selection for TTX resistance in *P. regilla* populations that are microsympatric with *Taricha*. We aimed to test whether sympatry of *Taricha* newts with *P. regilla* selected for target-site insensitivity in domain IV of the skeletal muscle VGSC ($Na_V1.4$) of *P. regilla*.

Our study centered on California populations, examining *Taricha* and *P. regilla* that are currently sympatric and allopatric. We conducted demographic surveys of *T. granulosa*, *T. torosa*, and *P. regilla* at three San Francisco Bay Area ponds where the species co-occur to estimate the extent to which *P. regilla* may encounter TTX. We supplemented our surveys with occurrence data available through the Global Biodiversity Information Facility (GBIF) to better estimate overlap of annual activity patterns and to determine the degree of sympatry between *Taricha* and *P. regilla*. To determine whether *P. regilla* has structural modifications in the muscle VGSC $Na_V1.4$ that may provide TTX resistance, we sequenced and reviewed amino acid residues encoded by domain IV of the *SCN4A* gene. Some amino acid substitutions in domain IV of *SCN4A* are known to confer resistance to TTX and have been found in many organisms that either have TTX defense or consume TTX-defended prey (Geffeney et al., 2005; Venkatesh et al., 2005; Jost et al., 2008; Feldman et al., 2012; Hanifin and Gilly, 2015; Gendreau et al., 2021b). We hypothesized that, if the presence of

Taricha selects for TTX resistance in *P. regilla*, populations sympatric with *Taricha* would have substitutions in the domain IV p-loop of $Na_V1.4$ while populations that are allopatric would not. Our work provides insight into the potential cascading ecological consequences of chemical defenses that evolved in the context of predator–prey dynamics.

Materials and Methods

Field Work.—We studied *Taricha* and *P. regilla* at four San Francisco Bay Area ponds and conducted surveys at three sites where *T. granulosa*, *T. torosa*, and *P. regilla* co-occur: two sites in Briones Regional Park in Martinez, Contra Costa County, California (Old Briones Road Trail 1 [OBRT 1] at 37.94571°N, 122.13395°W, and OBRT 2 at 37.94352°N, 122.14102°W, named after the trail closest to the sites) (datum WGS84 for all coordinates) and one site at the Japanese Pool (37.87440°N, 122.23760°W) of the University of California Botanical Garden (UCBG) in Berkeley, Alameda County, California (Fig. S1). OBRT 1 and OBRT 2 are in an unshaded area of grazed open grassland that lies within an oak woodland habitat, and they connect to each other when water levels are high. The UCBG Japanese Pool is a human-constructed pool with a concrete floor and sides, located under tree cover within the visitor area of UCBG (Fig. S1). We selected these three ponds because we knew them to contain populations of *P. regilla* as well as *T. torosa* and *T. granulosa*. Prior ecological work shows that *P. regilla* and *T. torosa* are sympatric in the San Francisco Bay Area (Preston and Johnson, 2012). We also collected specimens from a fourth pond (Mountain Lake) located in the Presidio of San Francisco (37.788496°N, 122.468605°W) that has historically contained *P. regilla* but no *Taricha* (records show absence of *Taricha* since at least 1966; J. Young, pers. comm.). Mountain Lake is a natural lake surrounded by grass that receives run-off from the nearby Presidio Golf Course (Fig. S1). From October 2019 to February 2020, we conducted six surveys at the two Briones ponds and five surveys at UCBG, with surveys at each site occurring 3–4 wk apart from one another. We were unable to gather abundance data after this period because the parks were closed from February until October 2020 during the early months of the COVID-19 pandemic.

Two researchers (usually KOM and VRC) conducted surveys in the morning or early afternoon (between 0800 and 1330 h). During each survey, surveyors split the pond perimeter into 7.62-m sections, and circled the pond on foot, counting how many individuals of each species were visible in the water from the shore within each transect. We note that surveyors were only able to count the number of visible newts. OBRT 1 and OBRT 2 were murky with plants and algae in which newts tended to hide. Although the surface of the Japanese Pool at UCBG was glassy, the pool was filled with plants that likely obscured observations. Surveyors also counted the numbers of visible, individual adult *P. regilla*; *P. regilla* that were calling but not visible were not counted because surveyors could not ascertain if multiple calls were coming from a single or a few individuals. For the purposes of this study, we did not differentiate between *T. torosa* and *T. granulosa* when we recorded their occurrences because they both wield TTX that may affect organisms in the pond water, and we could not easily identify individuals to species from the pond shores. We counted large (~5 cm or longer) *Taricha* larvae, adult *Taricha*, and *T. torosa* egg masses, which are conspicuous groups of eggs; in the sister taxon *T. granulosa*, eggs are known to possess TTX via vertical transfer from the parent (Gall et al., 2012). The much smaller *T. granulosa* eggs are typically hidden in the substrate and are more difficult to see, so we did not include them in our survey. To simplify our survey protocol, we counted larval *Taricha*, which also have some amount of TTX (Gall et al., 2011), together with adults. We plotted survey results using R version 4.1.1 and the packages ggplot2 v.3.3.5 and ggpubr v.0.4.0 (Wickham, 2016; Kassambara, 2020; R Core Team, 2021) (Fig. S2).

After COVID-19 conditions permitted field work to continue in October 2020, we collected one *P. regilla* from the dry bed of OBRT 2 and eight *P. regilla* from the east arm of Mountain Lake in the Presidio of San Francisco for sequencing. We surveyed OBRT 1 and UCBG at the same time but did not find any *P. regilla* at either location; OBRT 1 was dry.

GBIF Occurrences and Sympatry.—We estimated seasonal patterns of *P. regilla* and *Taricha* activity across the state of California and within the San Francisco Bay Area (north boundary: 38.66226°N, south boundary 37.09298°N, east boundary: 121.12701°W, west boundary 123.23579°W) by reviewing annual patterns of GBIF occurrence data of *P. regilla* and *Taricha* from 1 January 1971 to 23 November 2021 (GBIF) using the following R packages: RColorBrewer v.1.1.2, ggplot2 v.3.3.5, chron v.2.3.56, cowplot v.1.1.1, and dplyr v.1.0.7 (Neuwirth, 2014; James and Hornik, 2020; Wilke, 2020; Wickham et al., 2021). In our search terms for GBIF, we took into account that the taxonomy of *Pseudacris regilla* has changed several times over the last few decades (Recuero et al., 2006a,b; Fouquette and Dubois, 2014; Duellman et al., 2016). iNaturalist categorizes some *Pseudacris regilla* (e.g., in the San Francisco Bay Area) as *Pseudacris sierra*. However, these distinctions are not universally accepted by the herpetology community (Barrow et al., 2014; Crother et al., 2017). Here we consider *P. sierra*, *P. hypochondriaca*, and *Hyla regilla* synonyms of *Pseudacris regilla*. We downloaded occurrence data from GBIF for the *P. regilla* synonyms listed here and for all currently recognized species of *Taricha* (*T. granulosa*, *T. rivularis*, *T. sierrae*, *T. torosa*), as well as those no longer recognized (*T. lindoei*, *T. miocenica*, and *Palaeotaricha oligocenica*) and individuals identified only to the genus *Taricha*. GBIF data include Research Grade iNaturalist observations in addition to museum and specimen records.

We aimed to sequence $Na_V1.4$ from *P. regilla* that were sympatric and allopatric with *Taricha*. We originally classified *P. regilla* tissue samples as sympatric if they were collected within 35 km or 500 m of elevation from the nearest *Taricha* occurrence documented on GBIF as of 23 November 2021; this included 11 individuals from collections and the single *P. regilla* collected at OBRT 2. We classified allopatric *P. regilla* samples as those individuals collected at least 35 km or 500 m in elevation away from the nearest *Taricha* observation; this included 18 individuals from collections and the eight *P. regilla* from the Mountain Lake field site in the Presidio (Table S1).

To review general patterns in geographic range overlap between species, we plotted *P. regilla* and *Taricha* GBIF occurrence data from the western United States, as well as localities of museum samples we sequenced using ggplot2 v.3.3.5 and tidyverse v.1.3.1 (Wickham et al., 2019; GBIF, 2022). We plotted occurrence data of these species to confirm that museum tissues were from locations where *P. regilla* and *Taricha* are allopatric or sympatric. Specimens that were collected from localities within overlapping hexagons were considered to be sympatric; none of our categorizations changed. The binwidth of each hexagon was 0.05 in the vertical and horizontal directions. We note that this visualization, though qualitatively useful, is limited in several ways. For example, our designations of sympatry versus allopatry are not indicative of species

distributions prior to 1971. Additionally, iNaturalist data included in the GBIF data can be biased; for instance, individuals are unlikely to post multiple images of a single species at a site and observations can be more highly concentrated in regions where iNaturalist is well known (Hochmair et al., 2020). Future studies may consider estimating more accurate species distribution models.

DNA Sequencing and Analysis.—We selected 29 liver tissues of *P. regilla* from the Museum of Vertebrate Zoology at the University of California, Berkeley to broaden our geographic sampling beyond the San Francisco Bay Area (Table S1). In total, we sequenced $Na_V1.4$ from 12 *P. regilla* that were sympatric with *Taricha* and 26 that were allopatric, including museum samples and samples that we collected from our field sites in the Bay Area. We predicted that $Na_V1.4$ sequences from sympatric, but not allopatric, *P. regilla* would show amino acid substitutions potentially conferring TTX resistance. We also sequenced $Na_V1.4$ from two close relatives of *P. regilla*, *Pseudacris cadaverina*, and *Acris crepitans* (Table S1). The individuals of *P. cadaverina* and *A. crepitans* are from locations far from *Taricha* and, thus, were likely not exposed to TTX from *Taricha*. However, the range of *A. crepitans* overlaps with another TTX-bearing newt species, *Notophthalmus viridescens* (Powell et al., 2016), so they may be exposed to TTX as well. Additionally, while *P. cadaverina* can be sympatric with *Taricha* (Stebbins and McGinnis, 2018), we chose a specimen from the midwestern United States that was far from any known *Taricha* localities.

If TTX from newts selects for mutations in $Na_V1.4$ in sympatric frog species, we would expect to find amino acid substitutions in $Na_V1.4$ of *A. crepitans* as well as in $Na_V1.4$ of *P. regilla* sympatric with *Taricha*. In contrast, we expected to find no resistance-conferring amino acid substitutions in allopatric *P. regilla* or in *P. cadaverina*.

We extracted DNA from tissues according to the Miller et al. (1988) salt-extraction protocols, with modifications (see Supplementary Data for details). The primers used for *P. regilla* samples targeted exon 24 (DIV segment 6 and p-loop) of the gene *SCN4A*: 2268F_SCN4A 5′-TCTCCCGGCCCTCTTCAATA-3′ and 2681R_SCN4A 5′-TCGTCCTCGCATAAAGGCTC-3′ (Tarvin et al., 2016). We added 0.5 μL of $MgCl_2$ to each PCR sample. PCR protocols were as follows: an initial denaturation for 2 min at 94°C; 35 cycles of 94°C at 30 sec for denaturation, an annealing temperature of 54°C for 30 sec, and an elongation temperature of 72°C for 45 sec; and a final extension time of 7 min at 72°C. PCR products were run on an agarose gel, purified with ExoSAP-IT PCR Product Cleanup Reagent (ThermoFisher Scientific, 78201.1.ML) and sequenced using the reverse primer 2681R_SCN4A at the University of California, Berkeley DNA Sequencing Facility. For PCR and sequencing samples of *P. cadaverina* and *A. crepitans*, we used the same protocol and forward primer, but the reverse primer was redesigned based on the DIV *SCN4A P. regilla* sequences we produced: P.regilla_SCN4AD4_R 5′-ACTGCTTTCTTCTGTGGCCAC-3′.

Using BLAST (Altschul et al., 1990) with the *P. regilla* $Na_V1.4$ sequence as a query, we obtained 180 previously published representative sequences of *SCN4A* from other herpetofauna including salamandrids, dendrobatids, snakes, and lizards with available data on GenBank and DataDryad (Table S2; Gendreau et al., 2021a). We compare these *SCN4A* sequences, as well as those of *P. cadaverina* and *A. crepitans*, with *SCN4A* of our *P. regilla* samples. Of the additional 180 sequences added, *Erythrolamprus epinephelus* (=*Liophis epinephelus*), *Rhabdophis tigrinus*, *Thamnophis sirtalis*, *Thamnophis atratus*, *T. torosa*, *T. granulosa*, *S. salamandra*, *Tylototriton shanjing*, and other salamandrids are either confirmed or hypothesized to possess target-site insensitivity to TTX via mutations in DIV of $Na_V1.4$ (Feldman et al., 2009, 2012; Hanifin and Gilly, 2015; Ramírez-Castañeda, 2017; Gendreau et al., 2021b; Vaelli et al., 2020). We used *Rattus norvegicus* as a reference (UniProt number P15390) species that has not been documented to be resistant to TTX.

We identified the location of the DIV p-loop in $Na_V1.4$ based on protein domains inferred by Tikhonov and Zhorov (2005), and we numbered the residues according to *SCN4A* of *R. norvegicus* (UniProt number P15390). We aligned sequences using Clustal Omega in Geneious Prime 2020.1.1 (Sievers et al., 2011) and reviewed the alignment by eye for amino acid differences between sequences, focusing on residues that have been shown to provide TTX resistance. To visualize the evolutionary history and variety of substitutions in the DIV p-loop in $Na_V1.4$ in our samples and other amphibians and reptiles, we estimated a phylogeny of the $Na_V1.4$ DIV sequences using maximum likelihood on the IQ-Tree web server, with two partitions (one for codon positions 1 and 2, another for codon position 3), the GTR + G substitution model, and support assessed using 1000 ultrafast bootstraps and the SH-aLRT test (Nguyen et al., 2015; Chernomor et al., 2016; Trifinopoulos et al., 2016; Hoang et al., 2018; IQ-Tree web server http://iqtree.cibiv.univie.ac.at/). We edited and visualized the phylogeny using the Interactive Tree of Life (Letunic and Bork, 2021).

Results

Field Surveys.—While we did not observe *P. regilla* at OBRT 2 or UCBG, their abundance at OBRT 1 peaked early in the observation period at 32 individuals on the first survey date (20 October 2019). For OBRT sites, *Taricha* abundance peaked at the beginning of February; for the UCBG, abundance was low, and we may not have observed the seasonal peak (surveys were suspended at the end of February 2020 because of the COVID-19 pandemic). *Taricha torosa* egg masses were most abundant at the beginning of February 2020 at OBRT 1 and OBRT 2, while they were most abundant at UCBG at the end of February (though we cannot be certain these are true peaks because our surveys ended before March 2020) (Fig. S2).

GBIF.—California-wide GBIF observations for *P. regilla* and *Taricha* from 1 January 1971 to 23 November 2021 indicate two seasonal peaks of *P. regilla* abundance in April and August, while *Taricha* peaks in January (Fig. S3). Though the abundance peaks do not occur at precisely the same time, *P. regilla* and *Taricha* do overlap with one another and, thus, the species have opportunities to interact such that *Taricha* could expose *P. regilla* to TTX. These data are summarized across California and thus represent average activity across a range of habitat types. Nevertheless, when the data are constrained to a square roughly representing the San Francisco Bay Area (north boundary 38.66226°N, south boundary 37.09298°N, east boundary 121.12701°W, west boundary 123.23579°W), the results are similar, indicating a peak for *Taricha* in January and a peak for *P. regilla* in March (Fig. S4). Along the west coast of the United States, patterns of geographic sympatry estimated from GBIF occurrence data vary substantially (Fig. 2). Hot spots of sympatry were found in Humboldt County, the San Francisco Bay Area, the Los Angeles area, and northern Oregon, although much of each taxon's range does not overlap with that of the other.

Amino Acid Changes.—We found one amino acid change (I1519M, numbered according to *R. norvegicus*, UniProt number

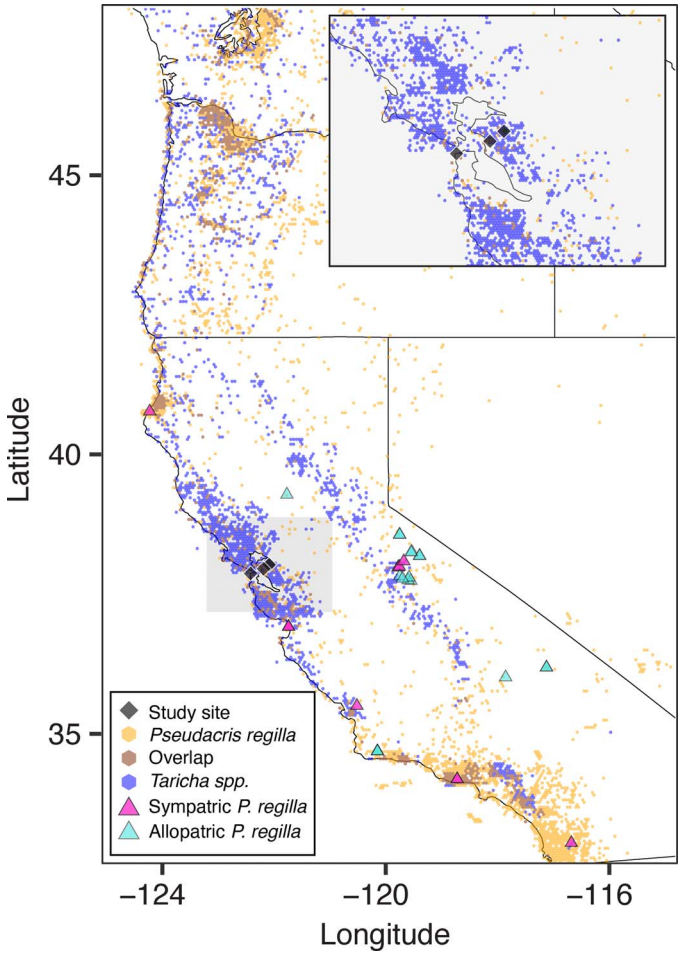

FIG. 2. Map showing overlap of *Pseudacris regilla* and *Taricha* occurrences on the west coast of the United States. Data are from GBIF, collected through 23 May 2022 for *Taricha* and through 6 June 2022 for *P. regilla*. *P. regilla* museum specimens that are sympatric with *Taricha* are indicated by pink triangles; *P. regilla* museum specimens allopatric with *Taricha* are indicated by blue triangles; field sites are indicated by black diamonds. Geographic regions with only *Taricha* occurrences are indicated by blue hexagons; those with only *P. regilla* are indicated by orange hexagons; regions where *Taricha* and *P. regilla* overlap are indicated by brown hexagons.

P15390) within the DIV p-loop of the sodium channel protein $Na_V1.4$ in *P. regilla* compared with a sequence of *R. norvegicus* (a common species used in comparative TTX studies [e.g., Tikhonov and Zhorov, 2005; Tarvin et al., 2016] as it represents the mammalian TTX-sensitive $Na_V1.4$ [Chahine et al., 1994]) (Figs. 3, S5). Importantly, there were no observed differences in amino acid sequences between *P. regilla* specimens that were sympatric and allopatric with *Taricha*. Close relatives of *P. regilla* (*P. cadaverina* or *A. crepitans*) did not possess the I1519M substitution, suggesting that the mutation arose within *P. regilla* or its ancestor.

DISCUSSION

The *Taricha–Thamnophis* system is a well-studied example of predator–prey interactions involving a toxin (Brodie, 1968; Brodie et al., 2002; Feldman et al., 2009, 2016). Here, we extend the system to include an ecological associate, *P. regilla*. We document ecologically relevant interactions between *Taricha* and *P. regilla* with new field observations (Fig. 1) and an assessment of GBIF data (Figs. 2, S2–S4). We show the absence of any obvious TTX-resistance-conferring amino acid mutations in the $Na_V1.4$ DIV p-loop of *P. regilla* (Figs. 3, S5). It remains unknown how *P. regilla* survive living in close proximity with toxic *Taricha* newts and whether they encounter high enough levels of TTX to necessitate some mechanism of resistance. Our data have added to the complex story involving newt toxicity and its effects on members of the surrounding community.

Abundance data indicate that *P. regilla* and *Taricha* annual activity patterns overlap but that peak breeding times are not synchronized (Figs. S2–S4). When we surveyed the field sites in Briones Regional Park and the UCBG from October 2019 to March 2020, California was experiencing drought. The area of the ponds fluctuated due to changes in precipitation and, in the case of the Japanese Pool, some drainage for maintenance (UCBG personnel, pers. comm.). Thus, weather and pond conditions may have influenced our survey observations.

Observations of mistaken amplexus and predation of newts by *P. regilla* indicate that the species do come into direct contact (Fig. 1), though the frequency of these events is unclear. Predation of *P. regilla* on *Taricha* in their various life stages has not been reported previously but it could contribute to *P. regilla* exposure to TTX. Months of overlapping activity patterns of frog tadpoles and newt larvae (Figs. S2–S4) could further expose tadpoles to TTX, potentially while tadpoles scavenge for food (i.e., they may eat newt eggs). Still, TTX exposure may not be frequent or potent enough for *P. regilla* to experience strong selection for resistance to TTX, especially when TTX levels can vary at different life stages in *T. granulosa* (Gall et al., 2022). In cases where environmental selective regimes are not predictable, more plastic responses like upregulation of detoxifying enzymes may be the primary way animals deal with toxin exposure (Zanger and Schwab, 2013). Nevertheless, even occasional exposure to TTX can result in maintenance of target-site insensitivity (Durso et al., 2021). In the case of the *Thamnophis–Taricha* arms race, *Thamnophis* activity peaks in April (according to iNaturalist records), not matching that of *Taricha* (Figs. S2–S4), yet TTX resistance has evolved in *Thamnophis*.

We hypothesized that we would find TTX-resistance-conferring amino acid substitutions in $Na_V1.4$ in *P. regilla* that are sympatric with *Taricha* and no substitutions in *P. regilla* that are allopatric with *Taricha*, with a greater number of substitutions indicating a higher level of resistance as has been shown in various Na_V channels in salamanders and snakes (Geffeney et al., 2005; Feldman et al., 2010; Hanifin and Gilly, 2015; McGlothlin et al., 2016). Compared with the TTX-sensitive *R. norvegicus* $Na_V1.4$ DIV, we found one amino acid change, I1519M, at a conserved hydrophobic residue located in proximity to TTX-binding residues in the p-loop (Geffeney et al., 2005; Tikhonov and Zhorov, 2005, 2011; Feldman et al., 2012; Gendreau et al., 2021b). This substitution was present in *P. regilla* that were sympatric and allopatric with *Taricha*. Amino acid changes in important regions of the $Na_V1.4$ protein do not always translate to a resistant phenotype or vice versa (e.g., see Abderemane-Ali et al., 2021; Reimche et al., 2022). Furthermore, the presence of this substitution in all *P. regilla* samples does not support our hypothesis that substitutions would only be found where *P. regilla* is exposed to TTX from sympatric *Taricha* (at least based on occurrence data from the last 50 yr). We recognize that our hypothesis made a simplifying assumption that the designation of *P. regilla* populations as sympatric or allopatric with *Taricha* is relevant and valid during the time in which selection may have occurred for TTX resistance and that gene flow does not occur between sympatric and allopatric *P. regilla*

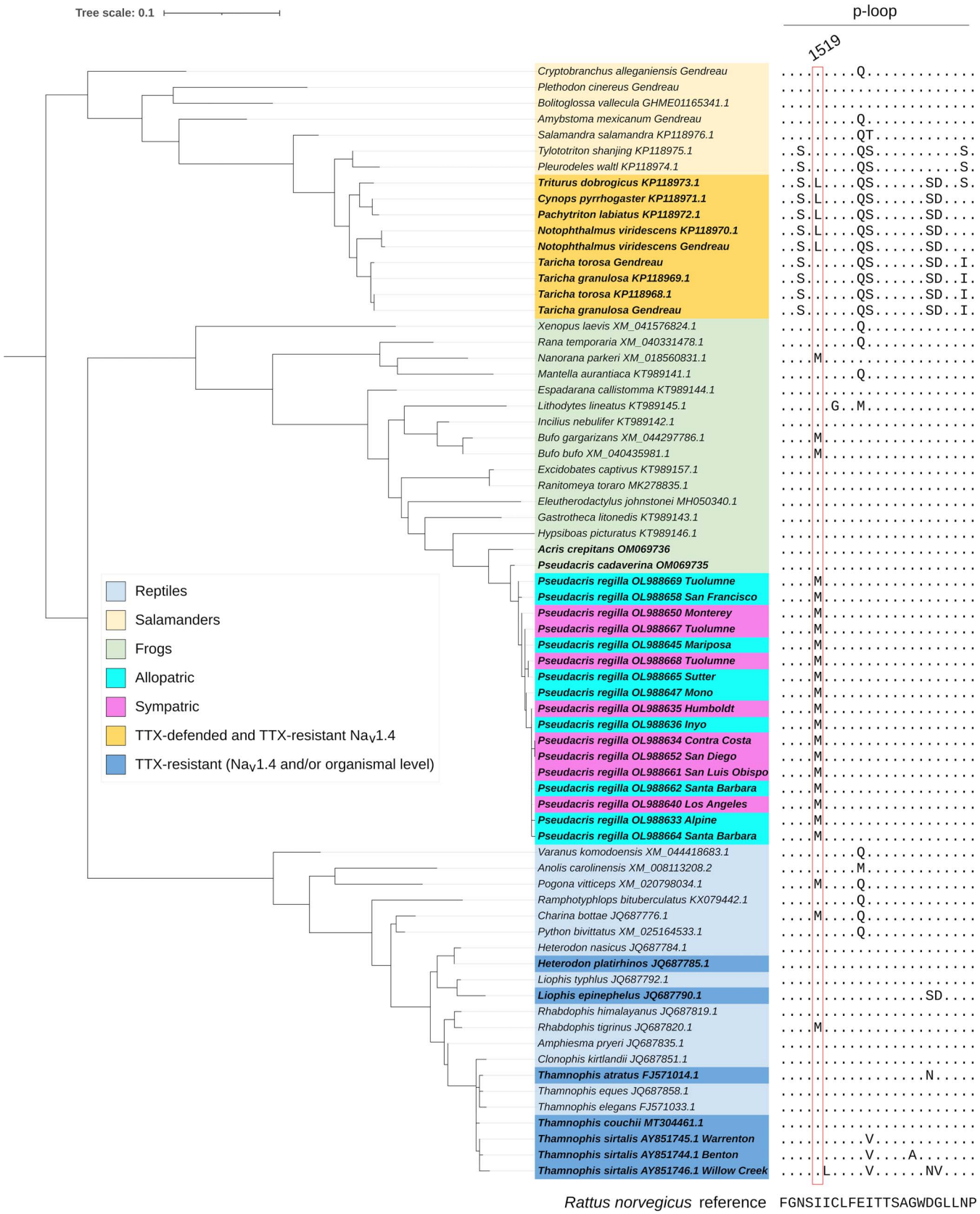

FIG. 3. Amino acid sequence variation in the domain IV p-loop of the voltage-gated sodium channel $Na_V1.4$ in *Pseudacris*, *Acris*, and outgroup sequences retrieved from GenBank, mapped onto a gene tree inferred from 381 bp of $Na_V1.4$ domain IV. Amino acid positions are numbered according to *Rattus norvegicus*, UniProt number P15390, and the p-loop position is drawn according to Tikhonov and Zhorov (2005). The amino acid substitution I1519M is found in *Pseudacris regilla* as well as *Nanorana parkeri*, *Bufo gargarizans*, *Bufo bufo*, *Pogona vitticeps*, *Charina bottae*, and *Rhabdophis tigrinus*. Taxa that are TTX-defended and have a TTX-resistant $Na_V1.4$ are shown in yellow (Hanifin et al., 2015; Gendreau et al., 2021b). Groups that are not TTX-defended but have TTX resistance via target-site insensitivity in $Na_V1.4$ or another mechanism are shown in dark blue (Feldman et al., 2012; Feldman et al., 2016). See Fig. S5 for branch support values and additional sequence data.

populations. Nonetheless, our study suggests that the I1519M substitution evolved in the ancestor of *P. regilla*, which likely rules out contemporary selection from *Taricha*. Our survey of *SCN4A* sequences indicates that this site is somewhat variable in reptiles and amphibians (Figs. 3, S5), further suggesting that the substitution may not be directly related to TTX resistance.

In addition to *P. regilla*, the I1519M substitution is present in *Nanorana parkeri*, *Bufo bufo*, *Bufo gargarizans*, *Pogona vitticeps*, *Charina bottae*, and *R. tigrinus* (Figs. 3, S5). None of these species are known for having a strong association with TTX; though, *R. tigrinus* has been observed consuming TTX-defended treefrogs (Feldman et al., 2012), and *N. parkeri* occurs within the range of the toxic and relatively TTX-resistant newt species *Tylototriton verrucosus* (though its toxin is yet to be identified; Brodie et al., 1984; Hanifin and Gilly, 2015). In contrast, *A. crepitans* co-occurs with the TTX-defended newt *N. viridescens* (Mebs et al., 2010), but this species does not possess the I1519M substitution. Thus, the presence or absence of I1915M does not appear to closely track history of TTX exposure.

Although the I1519M substitution has previously been hypothesized to provide TTX resistance in *R. tigrinus* (Feldman et al., 2012), this conjecture has been challenged because the same substitution is found in other VGSC paralogs known to be TTX-sensitive (McGlothlin et al., 2016; noted as I1555M in their study). Another hydrophobic change in the same position (I1519L) is found in several TTX-resistant newts (Figs. 3, S5: *Triturus dobrogicus*, *Cynops pyrrhogaster*, *Pachytriton labiatus*, *N. viridescens*); however, it is hypothesized that this change is not directly related to TTX resistance either (Hanifin and Gilly, 2015). Mutagenesis studies suggest that substituting methionine for isoleucine has limited effects on a protein (Ohmura et al., 2001), but in some cases minor changes can have substantial effects on toxin binding (e.g., see the isoleucine to valine substitution in Geffeney et al. [2005]). In any case, because there is no direct evidence that the I1519M substitution (or the site itself) is involved in resistance, we cannot say with certainty whether it provides TTX resistance in *P. regilla* without further experimental evidence.

While there is no direct evidence that *P. regilla* possesses target-site insensitivity in the p-loop of DIV in $Na_V1.4$, it is plausible that *P. regilla* may have other ways of avoiding the effects of TTX. Other regions of $Na_V1.4$, such as domain III, and other VGSCs, such as $Na_V1.7$, also harbor amino acid substitutions implicated in TTX resistance (Feldman et al., 2012; McGlothlin et al., 2016). *Pseudacris regilla* could possess the ability to smell TTX, such as larval in *Taricha*, enabling avoidance (Zimmer et al., 2006). Alternatively, they could possess a diffusion barrier in the skin or the gut that would prevent high levels of TTX exposure, as occurs in mantids (Mebs et al., 2016). It is also possible that *P. regilla* possess a binding protein that can scavenge TTX. Other frogs have saxiphilin, a protein that binds saxitoxin, which is structurally similar to TTX. However, saxiphilin is incapable of binding TTX, at least in the animals that have been tested (Mahar et al., 1991; Llewellyn et al., 1997; Abderemane-Ali et al., 2021). Similarly, little is known about the potential interactions between *A. crepitans* and *N. viridescens*, or between *N. parkeri* and *T. verrucosus*, which could also select for toxin resistance in the frogs. To date, none of these alternative resistance mechanisms have been studied in *Pseudacris*, *Acris*, *Nanorana*, or salamandrids.

A number of reasons could explain why *P. regilla* would lack target-site insensitivity in $Na_V1.4$. Evolving TTX resistance could be costly to *P. regilla* due to possible alterations in the outer pore of sodium channels that frequently occur when amino acid substitutions are present (Chiamvimonvat et al., 1996; Lee et al., 2011; Feldman et al., 2012; Hague et al., 2018; but see Moniz et al., 2021). Evolving costly substitutions may require a stronger selective pressure than is currently occurring. Given the relative quantities of TTX on newt skin (up to 28 mg/newt [Stokes, 2015]) compared with TTX levels in newt-containing water ($\sim 1 \times 10^{-7}$ mol/L [Zimmer et al., 2006]), the environmental toxicity of TTX is likely to cause weaker selection for TTX resistance than exposure via chemical defenses from prey. The cost of exhibiting TTX resistance combined with the low potential for *P. regilla* to interact with high concentrations of TTX in its environment may explain why we did not unequivocally identify target-site insensitivity in *P. regilla* DIV $Na_V1.4$. Future work exploring the effects of TTX as an environmental toxin could include measuring TTX concentrations in newt-containing ponds, performing whole organism or in vitro assays of *P. regilla* to determine TTX sensitivity, and testing for other modes of TTX resistance.

Conclusions.—We did not find direct evidence for target-site insensitivity in the DIV p-loop of the skeletal muscle voltage-gated sodium channel $Na_V1.4$ of *P. regilla*. Although it is possible that the I1519M substitution in the DIV p-loop of $Na_V1.4$ may be related to TTX resistance, experimental evidence is necessary to determine the impact of this substitution on TTX binding. Abundance data indicate that *P. regilla* may not encounter *Taricha* with high enough frequency or magnitude to cause selection for TTX resistance. Future seasonal measurements of pond-water concentrations will help evaluate this hypothesis. If *P. regilla* are indeed exposed to significant levels of TTX, they may have mechanisms to survive TTX exposure other than target-site insensitivity in $Na_V1.4$ DIV, such as resistance-conferring mutations in other domains or sodium channels, diffusion barriers, avoidance mechanisms, or scavenging proteins. Investigating the environmental toxicity of TTX in the relationship between *P. regilla* and *Taricha* is an opportunity to explore the nexus of ecological interactions, neurotoxins, and resistance mechanisms. The hypothesized absence of target-site insensitivity in *P. regilla* suggests that exposure to tetrodotoxin outside of trophic interactions may not strongly select for fixed mechanisms of toxin resistance.

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Collecting Permit 2 number PRSF-2020-SCI-0002 within study PRSF-00035, with a written extension after expiration of the original permit; University of California IACUC AUP-2019-08-12457; and California Department of Fish and Wildlife Scientific Collection Permit S-190980001-19111-001.

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Supplementary Data

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