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Characterizing Potential Tetrodotoxin Resistance in Domain IV of the Voltage-Gated Sodium Channel $Na_v1.4$ of Pacific Chorus Frogs, *Pseudacris regilla*

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Abstract.—Animals that frequently encounter toxins often evolve mechanisms of toxin resistance. Both predators that consume toxic prey and organisms in physical contact with a toxin or pollutant 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 hypothesized that *P. regilla* may possess resistance to TTX. We tested this prediction by comparing the amino acid sequences of the domain IV pore loop of the muscle voltage-gated sodium channel gene SCN4A ($Na_v1.4$ protein) in populations of *P. regilla* that are sympatric and allopatric with *Taricha*. We identified a single substitution in the domain IV pore loop of $Na_v1.4$ in *P. regilla* that has been hypothesized to provide TTX resistance based on computational modeling, though its role in resistance has been challenged. Nonetheless, both allopatric and sympatric *P. regilla* had this substitution, suggesting that it may be unrelated to TTX exposure from *Taricha*. Thus, there is no conclusive evidence that *P. regilla* has evolved TTX resistance encoded by amino acid substitutions in this domain. In addition, California occurrence data from the last 50 years indicate that *Taricha* activity peaks in January while the activity of *P. regilla* peaks in April. These relatively distinct activity patterns suggest that *P. regilla* may not be exposed to levels of TTX from *Taricha* that are high enough to require mutations in SCN4A. Nevertheless, other unidentified mechanisms of TTX resistance could be present in *P. regilla* and other species that are sympatric with toxic newts.

Key words: Toxin resistance; California; Environmental toxins; Target-site insensitivity; Salamanders; Taricha

Resumen.— Los animales que tienen contacto frecuente con toxinas suelen desarrollar mecanismos de resistencia a las mismas. Tanto los depredadores que consumen presas tóxicas como los organismos en contacto cercano con una toxina o contaminante en su entorno pueden experimentar una presión de selección que los lleva a evolucionar resistencia a toxinas. Basándonos en las observaciones de que las ranas coro del Pacífico (*Pseudacris regilla*) a veces comen por error y/o amplexan las salamandras del género *Taricha* que poseen tetrodotoxina (TTX), planteamos la hipótesis de que *P. regilla* podría poseer resistencia a la TTX. Probamos esta predicción comparando las secuencias de aminoácidos en el loop del poro del dominio IV en el gen del canal de sodio voltaje dependiente muscular SCN4A (proteína $Na_v1.4$) en poblaciones de *P. regilla* que son simpátricas y alopátricas con *Taricha*. Identificamos una única sustitución en el loop del poro del dominio IV de $Na_v1.4$ en *P. regilla* que se ha hipotetizado utilizando un modelo computacional que está relacionado con la resistencia a TTX. Sin embargo, tanto el *P. regilla* alopátrico como el simpátrico tienen esta sustitución, lo que sugiere que esta puede no estar relacionada con la resistencia a la TTX proveniente de *Taricha*. Por lo tanto, no hay pruebas concluyentes de que *P. regilla* haya evolucionado resistencia a la TTX causada sustituciones de aminoácidos en este dominio de la proteina. Adicionalmente, los datos de ocurrencia en California en los últimos 50 años indican una alternancia en la actividad de ambos grupos, *Taricha* alcanza su punto máximo en enero, mientras que la de *P. regilla* lo hace en abril. Estos patrones de actividad relativamente distintos sugieren que *P.*

regilla no está expuesta constantemente a niveles de TTX lo suficientemente altos como para requerir mutaciones en SCN4A. Sin embargo, otros mecanismos de resistencia a la TTX no identificados hasta ahora, podrían estar presentes en *P. regilla* y otras especies simpáticas a las salamandras tetrodotóxicas.

Palabras clave: Resistencia a las toxinas; California; Toxinas ambientales; Insensibilidad en el sitio de union; salamandras; Taricha

Many animals can synthesize or bioaccumulate toxic chemicals as an anti-predator defense. One common toxin is tetrodotoxin (TTX), which is employed as a defense by a variety of organisms including pufferfish and amphibians (e.g., dendrobatid frogs, *Atelopus, Polypedates, Brachycephalus,* salamandrid newts) (Daly et al., 1994; Tanu et al., 2001; Pires et al., 2002; Hanifin, 2010), as well as an offensive prey-capture technique by blue-ringed octopuses and flatworms (Ritson-Williams et al., 2006; Hwang et al., 2007; Williams, 2010). Bacterial symbionts of toxic newts and marine organisms produce TTX for their hosts, suggesting a bacterial origin in most if not all of these organisms (Magarlamov et al., 2017; Vaelli et al., 2020). TTX is a neurotoxin that blocks voltage-gated sodium channels by binding to highly conserved regions of the channel via hydrophobic forces and hydrogen bonds, fitting in the opening of the channel and blocking proper transport of Na^+ ions (Kao and Levinson, 1986; Tikhonov and Zhorov, 2011). TTX binding prevents action potentials from propagating, resulting in cessation of nerve signals (Kao and Levinson, 1986; Tikhonov and Zhorov, 2011). In vertebrate animals lacking TTX resistance, ingested TTX can temporarily paralyze or, in some

cases, kill the organism. The poison usually becomes fatal when respiratory muscles fail, resulting in suffocation (Brodie, 1968).

While TTX can incapacitate or kill an animal that attempts to consume it, several species are able to come into contact with TTX and survive. Resistance to TTX intoxication usually evolves via one of three common mechanisms. In the first, amino acid substitutions in voltage-gated sodium channels (VGSCs), especially in domain IV (DIV) of $Na_v1.4$, alter the shape of the pore loop (p-loop) and the surrounding area, which prevents TTX from binding (Geffeney et al., 2005; Tikhonov and Zhorov, 2005). Other regions of $Na_v1.4$, such as domain III, and other voltage-gated sodium channels, such as $Na_v1.7$, also harbor amino acid substitutions implicated in TTX resistance (Feldman et al., 2012; McGlothlin et al., 2016). Changes in the p-loops are particularly significant because they not only alter the sensitivity of the sodium channel to TTX but also to sodium ions; hence, there is often a tradeoff between TTX resistance and protein function (Chiamvimonvat et al., 1996; Geffeney et al., 2005; Lee et al., 2011; Tikhonov and Zhorov, 2011; Hague et al., 2018; but see Moniz et al., 2021). Thus, amino acid changes are usually 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.

Other mechanisms of TTX resistance include toxin-binding proteins, which have evolved in gastropods, pufferfish, horseshoe crabs, and shore crabs (Ho et al., 1994; Nagashima et al., 2002; Hwang et al., 2007; Hashiguchi et al. 2015), and diffusion

barriers, which are present in some invertebrates (Mebs et al., 2016). Some amphibians possess the toxin-binding, blood-plasma protein saxiphilin, but 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). Some species of mantids (*Tenodera sinensis*, *Sphodromantis viridis*, *Hierodula membranacea*, and *Miomantis caffra*) can survive tetrodotoxin ingestion but not toxin injection, suggesting the presence of a gut diffusion barrier (Mebs et al., 2016). Crayfish appear to have a similar mechanism (Wilson et al., 2014). Because we do not directly test for toxin-binding proteins or diffusion barriers, we cannot say whether *P. regilla* possesses these mechanisms of resistance.

Taricha newts (*Taricha torosa*, *T. granulosa*, *T. sierrae,* and *T. rivularis*, herein collectively referred to as *Taricha*) have TTX present on their skin (Brodie et al., 1974; Reimche et al., 2020) that likely functions as an anti-predator defense (Brodie et al. 2002). *Taricha* (and other salamandrids) possess amino acid changes in $Na_v1.4$ that help them resist their own TTX defenses (Hanifin and Gilly, 2015; Gendreau et al., 2021). The San Francisco Bay Area in California is one of two known hotspots of the arms race between newts and garter snake predators (Brodie et al., 2002). 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 dynamic, namely the Pacific Chorus Frog, *Pseudacris regilla*.

TTX from *Taricha* skin has been shown to enter waterways and affect the behavior of organisms present in the same 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 predatory patterns of dragonfly nymphs, which are macroinvertebrate prey of *T. torosa* (Bucciarelli and Kats, 2015). The New Zealand Mud Snail (*Potamopyrgus antipodarum*) moved out of its natural 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 negatively increases newt trematode parasite mortality (Calhoun et al., 2017). Thus, naturally occurring levels of TTX produced by *T. torosa* can affect other organisms in the same aquatic environment.

Given that *P. regilla* have permeable skin, we hypothesized that they would be exposed to TTX through physical contact with newts or by occupying pond water that contains newts. We predicted that TTX exposure has selected for TTX resistance in *P. regilla* populations that are microsympatric with *Taricha*. We recognize that our hypothesis makes 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* populations. Although both of these assumptions may be violated by changes in climate and species distributions over time, evidence from the evolutionary arms race between garter snakes and TTX-bearing newts suggest that TTX potency and the degree of TTX resistance can change over a short period of time (Brodie et al., 2002), which aligns with our study that examines approximately the past 50 years. Occasional exposure to TTX may also be sufficient to select for target-site insensitivity (Durso et al., 2021).

We aimed to test whether the sympatry of *Taricha* newts with *Pseudacris regilla* has selected for target-site insensitivity in the skeletal muscle voltage-gated sodium channel of *P. regilla*. While *P. regilla* do not regularly prey upon *Taricha* (or vice versa), they likely 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).

Our study centered on California populations, examining *Taricha* and *P. regilla* that do and do not overlap geographically in the present. We conducted demographic surveys of *Taricha 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. Because these surveys were suspended after only a few months due to the COVID-19 pandemic, we then reviewed occurrence data available through the Global Biodiversity Information Facility (GBIF) to better estimate overlap of annual activity patterns. To determine whether *P. regilla* has structural modifications in the protein muscle voltage-gated sodium channel $Na_v1.4$ that may provide TTX resistance, we sequenced and reviewed amino acid residues encoded by the SCN4A gene. Amino acid substitutions in this domain are known to confer resistance to TTX and have been found in several organisms that either have TTX defense or consume TTX-defended prey (Tikhonov and Zhorov, 2005; Venkatesh et al., 2005; Feldman et al., 2012; Hanifin and Gilly, 2015). We hypothesized that if the presence of *Taricha* selects for TTX resistance in *P. regilla,* populations sympatric with *Taricha* would have mutations in the 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, as we evaluate the unintended effects of animals wielding chemical defenses.

MATERIALS AND METHODS

Field Work.—We studied *Taricha* and *P. regilla* at four San Francisco Bay Area ponds. We conducted surveys at three of these sites where *Taricha granulosa, T. torosa,* and *P. regilla* co-occur: two in Briones Regional Park in Martinez, Contra Costa County, CA (Old Briones Road Trail 1 [OBRT 1] at 37.94571, -122.13395, and OBRT 2 at 37.94352, -122.14102, named after the trail closest to the sites) and the Japanese Pool (37.87440, -122.23760) at the University of California Botanical Garden (UCBG) in Berkeley, Alameda County, CA (Fig. 2). 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 man-made pool with a concrete floor and sides, located under tree cover within the visitor area of the UCBG (Fig. 2). We selected these three ponds because they were known to contain populations of *P. regilla* as well as *Taricha torosa* and *Taricha granulosa* (Katherine Montana, Rebecca Tarvin, pers. obs.). We also collected specimens from a fourth pond located in the Presidio of San Francisco (37.788496, -122.468605) that has historically contained *P. regilla* but no *Taricha* (records show absence of *Taricha* since at least 1966; pers. comm. J. Young, Presidio Trust). Mountain Lake is a natural lake surrounded by grass and receives run-off from the nearby Presidio Golf Course (Fig. 2). 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 three to four weeks apart from one another. We were unable to gather abundance data for more than this time period because the parks were closed from February until October 2020 during the early months of the COVID-19 pandemic.

Two researchers (usually K. Montana and V. Ramírez Castañeda) conducted surveys in the morning or early afternoon (between 8:00 AM and 1:30 PM). During each survey, surveyors split the pond perimeter into 7.62-m (25-feet) 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 and although the surface of the Japanese Pool at UCBG was glassy, the pool was filled with plants that could have obscured observations. Surveyors also counted the numbers of visible, individual adult *Pseudacris 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. Though the TTX defenses of *T. granulosa* are thought to be more potent than that of *T. torosa* (Brodie et al., 1974), for the purposes of this study, we did not differentiate between the two species when we recorded their occurrences because they both contribute to the presence of TTX in 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 *Taricha torosa* egg masses, which are conspicuous groups of eggs 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; hence 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. 3). We compared survey results to GBIF occurrence data of *P. regilla* and *Taricha* from 1 January 1971 to 23 November 2021 by plotting the GBIF data 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; Wickham, 2016; James and Hornik, 2020; Wilke, 2020; Wickham et al., 2021).

After COVID-19 conditions permitted field work to continue in October 2020, we collected one *Pseudacris 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. We surveyed OBRT 1 and UCBG at the same time but did not find any *P. regilla* at either location; OBRT 1 was dry.

Precipitation Data.—To better interpret amphibian abundance data from field surveys, we searched for precipitation data from NOAA for the weather stations closest to our field sites in Berkeley (UCBG) and Martinez (OBRT 1 and OBRT 2): Berkeley, CA USC00040693 and Martinez 2.2 SW, CA US1CACC0011 (NOAA, https://www.ncdc.noaa.gov/cdo-web/search). We measured pond size during each

survey event by adding the number of 7.62-m transects we walked during each survey to get the circumference and calculate the area, assuming the ponds were circular.

Notes on Taxonomy.—The taxonomy of *Pseudacris regilla* has changed several times over the last decades (Recuero et al., 2006; 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.*

DNA Sequencing.—We selected 29 liver tissues of *Pseudacris regilla* from the Museum of Vertebrate Zoology at the University of California, Berkeley to broaden our geographic sampling (Table S1). We classified *P. regilla* samples as sympatric with *Taricha* 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 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 and the eight *P. regilla* from the Mountain Lake field site in the Presidio (Table S1). We predicted that $Na_v1.4$ sequences from sympatric, but not allopatric, *P. regilla* would show amino acid substitutions potentially conferring TTX resistance. We utilized the R packages ggplot2 v.3.3.5 and tidyverse v.1.3.1 to map GBIF occurrences of *Taricha* throughout California, *P. regilla* museum samples that were sympatric with *Taricha*, *P. regilla* museum samples that were allopatric with *Taricha*, and our field sites (Wickham, 2016; Wickham et al., 2019; GBIF).

Our preliminary results suggested that a single amino acid substitution is present in *P. regilla.* Thus, to estimate when this substitution evolved, 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, *Notophthalmus viridescens* (Baird, 1854; Mecham, 1967), so it may be exposed to TTX as well. Thus, 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*.

In the Evolutionary Genetics Lab (UC Berkeley), we extracted DNA from tissues according to the Miller et al. (1988) salt-extraction protocols, with modifications (see Supplementary Online Material 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: an initial denaturation for 2 min at 94 ºC; 35 cycles of: 94 ºC at 30 s for denaturation, an annealing temperature of 54 ºC for 30 s, and an elongation temperature of 72 ºC for 45 s; and a final extension time of 7 min at 72 ºC. 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 UC Berkeley DNA Sequencing Facility. For PCR and sequencing samples of *Pseudacris cadaverina* and *Acris 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'.

We also obtained previously published SCN4A sequences from species that are resistant to TTX, including *Erythrolamprus epinephelus*, which is potentially resistant to TTX from *Atelopus*, as well as *Rhabdophis tigrinus*, *Thamnophis elegans*, *Thamnophis sirtalis*, *Taricha torosa*, and *Taricha granulosa*, which are either confirmed or hypothesized to possess target-site insensitivity to TTX via mutations in $Na_V1.4$ (Feldman et al., 2009; Feldman et al., 2012; Hanifin and Gilly, 2015). We also include sequences from *Nanorana parkeri* and *Rattus norvegicus,* which have not been documented as being resistant to TTX (Table S2). We identified the location of the DIV p-loop in $Na_v1.4$ based on protein domains inferred by Tikhonov and Zhorov (2005). We

aligned sequences using Clustal Omega in Geneious (Sievers et al., 2011, Geneious Prime 2020.1.1). We reviewed the alignment by eye for amino acid differences between sequences, focusing on residues that have been shown to provide TTX resistance. We estimated a phylogeny of the species that we compared to *P. regilla* using 11 COI sequences (Table S2) and maximum likelihood on the IQTree 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., 2015; Trifinopoulos et al., 2016; Hoang et al., 2018; IQ-Tree web server http://iqtree.cibiv.univie.ac.at/). We transformed the resulting phylogeny into an ultrametric chronogram using the function *chronos* in phytools v.0.7.90 and ape v.5.5 (Revell, 2012; Paradis and Schliep, 2019) (Fig. 6). We made all figures in R and Adobe Illustrator (Adobe, Inc., 2019; R Core Team, 2021).

RESULTS

Demographic surveys indicated that *P. regilla* may encounter TTX from *Taricha* in their environment. DNA sequencing identified one amino acid substitution in domain IV of $Na_v1.4$ in *P. regilla* that could potentially be connected to TTX resistance, but the degree to which the mutation affects resistance is inconclusive.

Field Surveys.—While we never observed *P. regilla* at OBRT 2 or UCBG, their abundance at OBRT 1 peaked early in the observation period at 32 individuals on the first field trip date (20 October 2019). For OBRT field 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) (Fig. 3). California-wide GBIF observations for *P. regilla* and *Taricha* from 1 January 1971 to 23 November 2021 indicate two peaks of *P. regilla* abundance in April and August, while *Taricha* peaks in January (Fig. 4). 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 (N 38.66226, S 37.09298, E -121.12701, W -123.23579), the results are similar, indicating a peak for *Taricha* in January and a peak for *P. regilla* in March (Supplementary Fig. 1).

When we surveyed the field sites in Briones Regional Park and the UC Botanical Garden from October 2019 to March 2020, California was experiencing drought. During this period, rain began in mid-November 2019 and stopped at the end of February 2020. The most rain recorded in one day during the six-month period in Berkeley, where UCBG is located, was 30.23 mm, the average recorded rainfall during that period was 1.76 mm/day, and the total number of recorded days with rain was 40 days (NOAA field

station Berkeley, CA USC00040693, elevation 94.49 m, 37.8744° N, 122.2605° W) (NOAA, 2022). The most rain recorded in one day during the six-month period in Martinez, where Briones is located, was 89.15 mm, the average recorded rainfall during that period was 1.61 mm/day, and the total number of recorded days with rain was 33 days (NOAA field station Martinez 2.2 SW, CA US1CACC0011, elevation 210.92 m, 37.9744° N, 122.1384° W) (NOAA, 2022). 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. com.). The area of OBRT 1 ranged between 319 and 912 m^2 with a survey perimeter of about 84-122 m. The area of OBRT 2 ranged between 1550 and 1733 m^2 with a perimeter of about 130-145 m. During our study period, OBRT 1 and OBRT 2 did not merge. The area of the Japanese Pool ranged between 114 and 137 m^2 with a perimeter of about 46-53 m.

Amino Acid Changes.— We found one amino acid change (I1519M) within the DIV p-loop of the sodium channel protein $Na_v1.4$ in *Pseudacris regilla* compared to a sequence of *Rattus 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]) (Fig. 6) (Tables S1 and S2). Importantly, there were no observed differences in amino acid sequences between *P. regilla* specimens that were sympatric and allopatric with *Taricha*. The I1519M substitution has been previously predicted by computational modeling to provide some amount of TTX resistance (Tikhonov and Zhorov, 2005; Tikhonov and Zhorov, 2011). The I1519M

substitution is also present in the snake *Rhabdophis tigrinus,* which consumes TTX-defended treefrogs, and is inferred to be associated with TTX resistance (Feldman et al., 2012). However, it has been suggested that the characterization of the I1519M substitution as contributing to TTX resistance is erroneous because the same substitution is found in TTX-sensitive voltage-gated sodium channels of other snakes (McGlothlin et al., 2016). Additionally, we found that the amino acid change I1519M occurs in the frog *Nanorana parkeri,* which is not known for being resistant to or in contact with tetrodotoxin; though, it does occur within the range of TTX-defended salamandrids such as *Tylotritron verrucosus* (Che et al., 2010; Vitt and Caldwell, 2013; Wanninger et al., 2018). 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

Our results provide conflicting evidence regarding whether $Na_V1.4$-based TTX resistance is present in *Pseudacris regilla*. In support of resistance is the presence of a single amino acid substitution in *P. regilla* $Na_v1.4$ (Fig. 6) hypothesized to provide TTX resistance based on computational models (Tikhonov and Zhorov, 2005; Tikhonov and Zhorov, 2011), and evidence that *P. regilla* and *Taricha* overlap somewhat in annual activity patterns (Fig. 4). However, amino acid changes in important regions of the $Na_v1.4$ protein do not always translate to a resistant phenotype (e.g., see Abderemane-Ali et al., 2021). Furthermore, both allopatric and sympatric *P. regilla* possessed the

substitution, but *A. crepitans* did not, although we expected that it might possess some resistance due to its overlap in range with *N. viridescens*. The presence of an identical substitution in *Nanorana parkeri* was unexpected and could either present evidence in support of resistance (the species does live near the TTX-defended salamandrid *Tylototriton verrucosus*), or against it, potentially indicative of functionally permissive amino acid diversity at that site in anurans. Below we review in more detail these lines of evidence.

Abundance data indicate that *P. regilla* and *Taricha* annual activity patterns overlap but that peak breeding times are not synchronized (Figs. 3, 4; Supplementary Fig. 1). Observations of mistaken amplexus and predation of newts by *P. regilln* indicate that the species do come into contact (Fig. 1). Still, TTX exposure may not be frequent or potent enough for *P. regilla* to undergo strong selection for resistance to TTX. 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).

We hypothesized that we would find TTX-conferring amino acid substitutions in $Na_v1.4$ in *P. regilla* that are microsympatric 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 salamanders and snakes (Geffeney et al., 2005; Lee et al., 2011; Hanifin and Gilly, 2015). Compared to *Rattus norvegicus* DIV,

we found one amino acid change at a site hypothesized to provide TTX resistance, I1519M, which is a TTX-sensing residue located in the p-loop (Tikhonov and Zhorov, 2005; Tikhonov and Zhorov, 2011; Feldman et al. 2012). This substitution was present in *P. regilla* both sympatric and allopatric with *Taricha* and in *Nanorana parkeri*. It is also found in *Rhabdophis tigrinus*, a species of snake that has been hypothesized to be resistant to TTX (Feldman et al., 2012), although no empirical tests have been undertaken and other authors have disputed the role of I1519M in TTX resistance (McGlothlin et al., 2016; noted as I1555M in these studies). 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 years). Ultimately, our study suggests that the I1519M substitution evolved in the ancestor of *P. regilla*, which likely rules out contemporary selection from *Taricha.* The presence of the substitution in *N. parkeri* could also support that the mutation is unrelated to newt exposure. However, *N. parkeri* overlaps with other toxin-defended salamandrids like *T. verrucosus,* making it difficult to rule out resistance in *N. parkeri* or, consequently, in *P. regilla*. Similarly, *A. crepitans* co-occurs with the toxic newt *Notophthalmus viridescens,* but this species does not possess the I1519M substitution. Because there is disagreement whether the I1519M mutation provides resistance, we cannot say with certainty whether it provides TTX resistance in *P. regilla* without further experimental evidence.

While there is not strong 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, including amino acid mutations in other domains of $Na_v1.4$ or in other voltage-gated sodium channels (Feldman et al., 2012; McGlothlin et al., 2016). *Pseudacris regilla* could also possess the ability to smell TTX, as larval *Taricha* can, in order to avoid it (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* possesses a binding protein that can scavenge TTX; other frogs have saxiphillin, a protein that binds saxitoxin, which is structurally similar to TTX (Mahar et al., 1991; Llewellyn et al., 1997). Similarly, little is known about the potential interactions between *Acris crepitans* and *Notophthalmus viridescens*, which could also select for TTX resistance in the frogs. To date, none of these alternative resistance mechanisms have been studied in *Pseudacris, Acris,* or *Notophthalmus*.

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 selectivity filter of sodium channels that frequently occurs when amino acid substitutions are present (Chiamvimonvat et al., 1996; Geffeney et al., 2005; Lee et al. 2011; Tikhonov and Zhorov, 2011; Moniz et al., 2021). Evolving costly mutations may require a stronger selective pressure than is currently occurring. Given the relative quantities of TTX on newt skin [up to 4.69 mg/newt (Hanifin et al., 2008)] compared to TTX levels in newt-containing water [~$1x10^{-7}$ mol/L (Zimmer et al.,2006)], the environmental toxicity of TTX is likely to cause a 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 in animals that live in sympatry with salamandrid newts.

Conclusions.—We do not find strong evidence for target-site insensitivity in DIV of the skeletal muscle voltage-gated sodium channel $Na_v1.4$ of *Pseudacris regilla*. Although it is possible that the I1519M substitution in the p-loop of $Na_v1.4$ may be related to TTX resistance, *P. regilla* may not encounter TTX from *Taricha* with high enough frequency or magnitude to select for TTX resistance. Thus, these animals may have behavioral or alternative physiological mechanisms to survive TTX exposure, or TTX exposure may not be severe enough to require adaptation by *P. regilla*. 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 likely absence of target-site insensitivity in *P. regilla* indicates that exposure to tetrodotoxin outside of trophic interactions may not strongly select for fixed mechanisms of toxin resistance.

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The East Bay Regional Parks District authorized the surveys at Briones under permit #19-1044, and staff at the UCBG gave written consent for surveys conducted on the premises. All animals were collected under approved collection permits and protocols: for the National Parks Service Scientific Research and 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.

Spanish translation was done using www.DeepL.com/Translator (free version), which was then edited by Valeria Ramírez Castañeda.

LITERATURE CITED

(see attached document)

SUPPLEMENTARY ONLINE MATERIAL

Supporting Methods.—Specimens sequenced in this study, SCN4A exon 24

Table S1.—DNA Extraction Procedures, Adapted from Miller et al., 1988.

Table S2.— $Na_v1.4$ (SCN4A exon 24) and COI sequences from GenBank

Supplementary Figure 1.—San Francisco Bay Area GBIF Observations of *Pseudacris regilla* and *Taricha*, 1971-2021 (N 38.66226, S 37.09298, E -121.12701, W -123.23579)

FIGURES

Fig. 1. Examples of *Pseudacris regilla* and *Taricha torosa* interactions: A. Amplexus in Briones Regional Park, Martinez, CA, 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, CA, 21 February 2009, photo credit user ap2il on iNaturalist (CC BY-NC 4.0; observation 550227); C. Attempted predation on *Taricha torosa* by *Pseudacris regilla* in Mark West Springs, CA, 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.

Fig. 2. Field sites: A. Old Briones Road Trail 1 (OBRT 1) pond, 37.94441, -122.13377; B. Old Briones Road Trail 2 (OBRT 2) pond, 37.94352, -122.14102; C. Japanese Pool at the UC Botanical Garden (UCBG), 37.87440, -122.23760; D. Presidio site, 37.788496, -122.468605; all photos taken by K. Montana.

Fig. 3. Numbers of A. *P. regilla* adults, B. *Taricha torosa* and *T. granulosa* adults and larvae, and C. *T. torosa* egg masses observed over field trips to the Old Briones Road Trail 1 (OBRT 1) (blue circle line), OBRT 2 (red triangle line), and U.C. Botanical Garden (yellow square line) ponds; 20 October 2019 to late February 2020.

Fig. 4. Percentage of total abundance of GBIF-recorded individuals of *Pseudacris regilla* (green) and *Taricha* (blue) in California by month, over the course of the last 50 years. *Taricha* includes *T. granulosa*, *T. rivularis*, *T. sierrae*, *T. torosa*, as well as those *Taricha* species no longer recognized (*T. lindoei*, *T. miocenica*, and *Palaeotaricha oligocenica*) plus individuals just identified to the genus *Taricha* (GBIF). Peak abundances for *P. regilla* occur around April and August each year, and peak abundances for *Taricha* occur in January each year. Samples included were from 1 January 1971 to 23 November 2021.

Fig. 5. Map of the state of California, USA showing location of *Pseudacris regilla* museum specimens sympatric with *Taricha* (green squares), *P. regilla* museum specimens allopatric with *Taricha* (orange triangles), and field sites (black squares) in relation to occurrences of *Taricha* (blue circles).

Fig. 6. Alignment of the p-loop of domain IV of the voltage-gated sodium channel $Na_V1.4$ in several species including *Pseudacris regilla,* mapped onto a COI phylogeny inferred using IQTREE (support values are SH-aLRT/ultrafast bootstrap values from 1000 iterations each). Amino acid positions are numbered according to *Rattus norvegicus,* UniProt accession #P15390. Species marked with an asterisk* are known to be resistant to TTX, and the AA substitutions known or hypothesized to confer TTX resistance are shown in bold (Feldman et al., 2012; Hanifin and Gilly, 2015). The amino acid substitution I1519M is found in *P. regilla* as well as *N. parkeri* and *R. tigrinus*. *R. tigrinus* consumes *Polypedates leucomystax*, which wields tetrodotoxin and bufadienolides; the I1519M substitution is thought to be linked to TTX resistance (Hutchinson et al., 2007; Tikhonov and Zhorov, 2005; Tikhonov and Zhorov, 2011). *N. parkeri* has the I1519M substitution but is not known to consume or be resistant to any toxins. Close relatives of *P. regilla*, *P. cadaverina* and *A. crepitans,* do not have the I1519M substitution or any others. *E. epinephelus* has AA substitutions that are thought

to be connected to resistance to other toxins in their diets; they eat *Atelopus* toads that have guanidine alkaloids including tetrodotoxin as well as dendrobatid poison dart frogs that wield batrachotoxin and pumiliotoxin (Myers et al., 1978; Feldman et al., 2012; Ramírez Castañeda et al., 2017).

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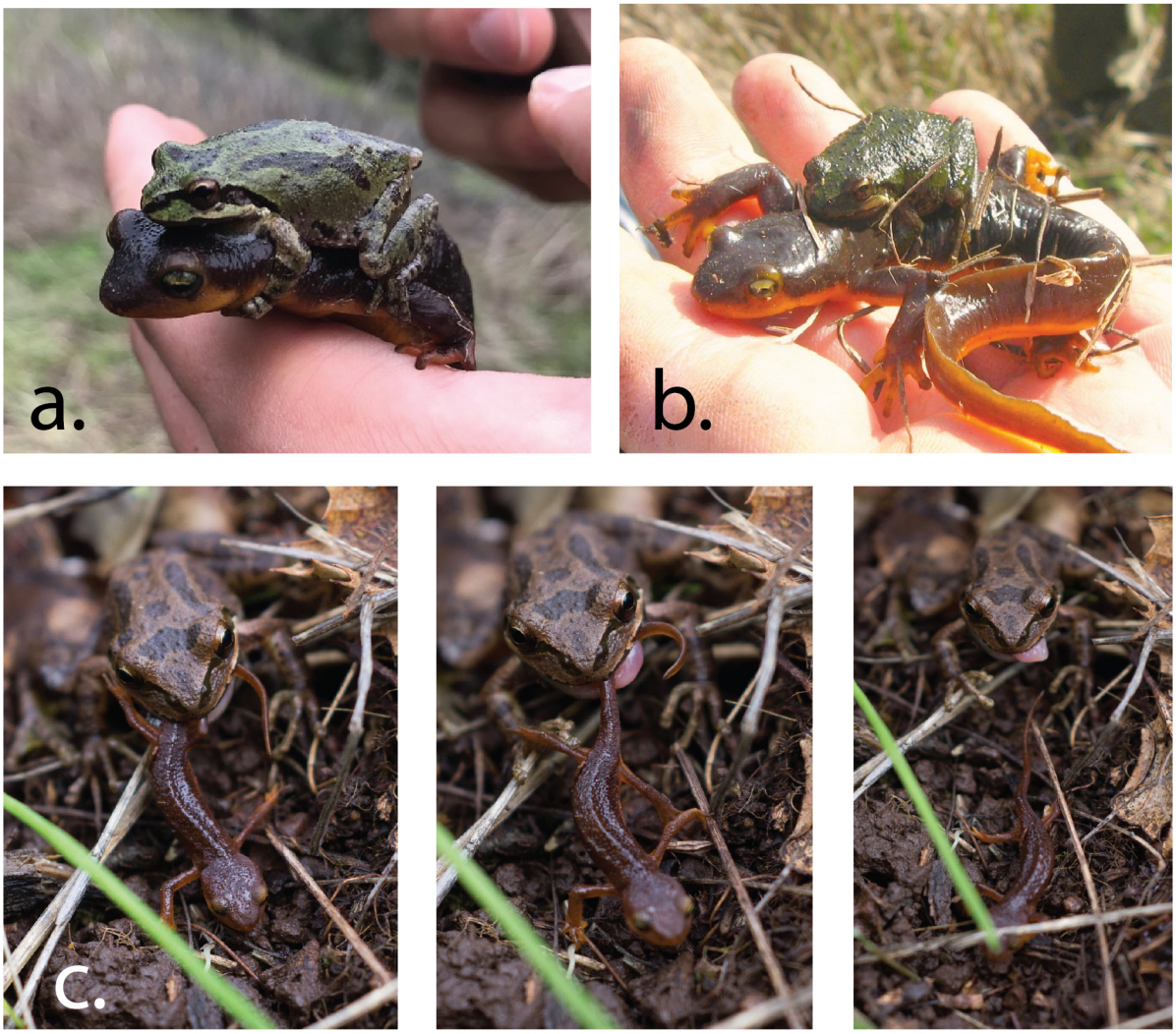
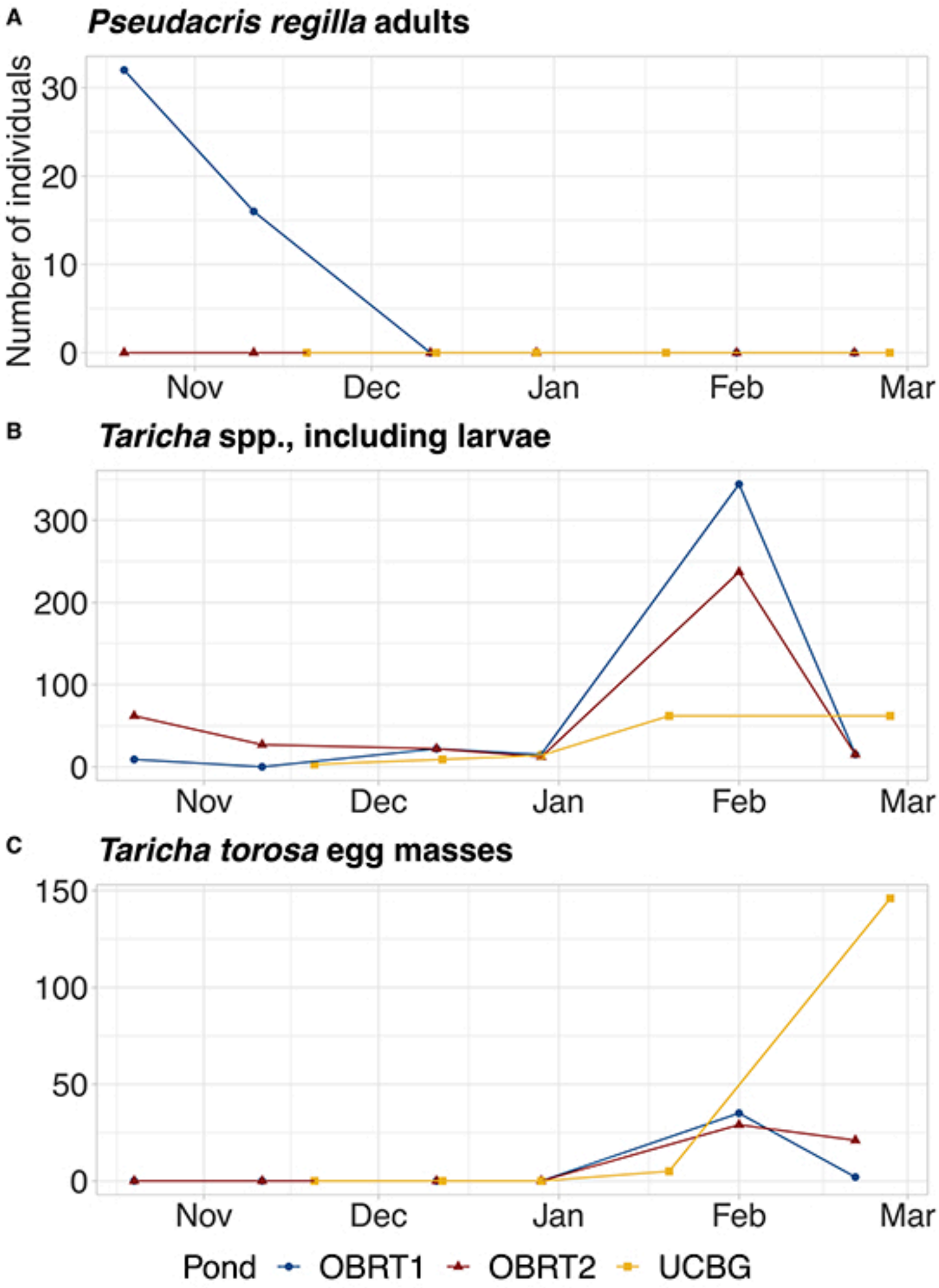

a.
b.
c.

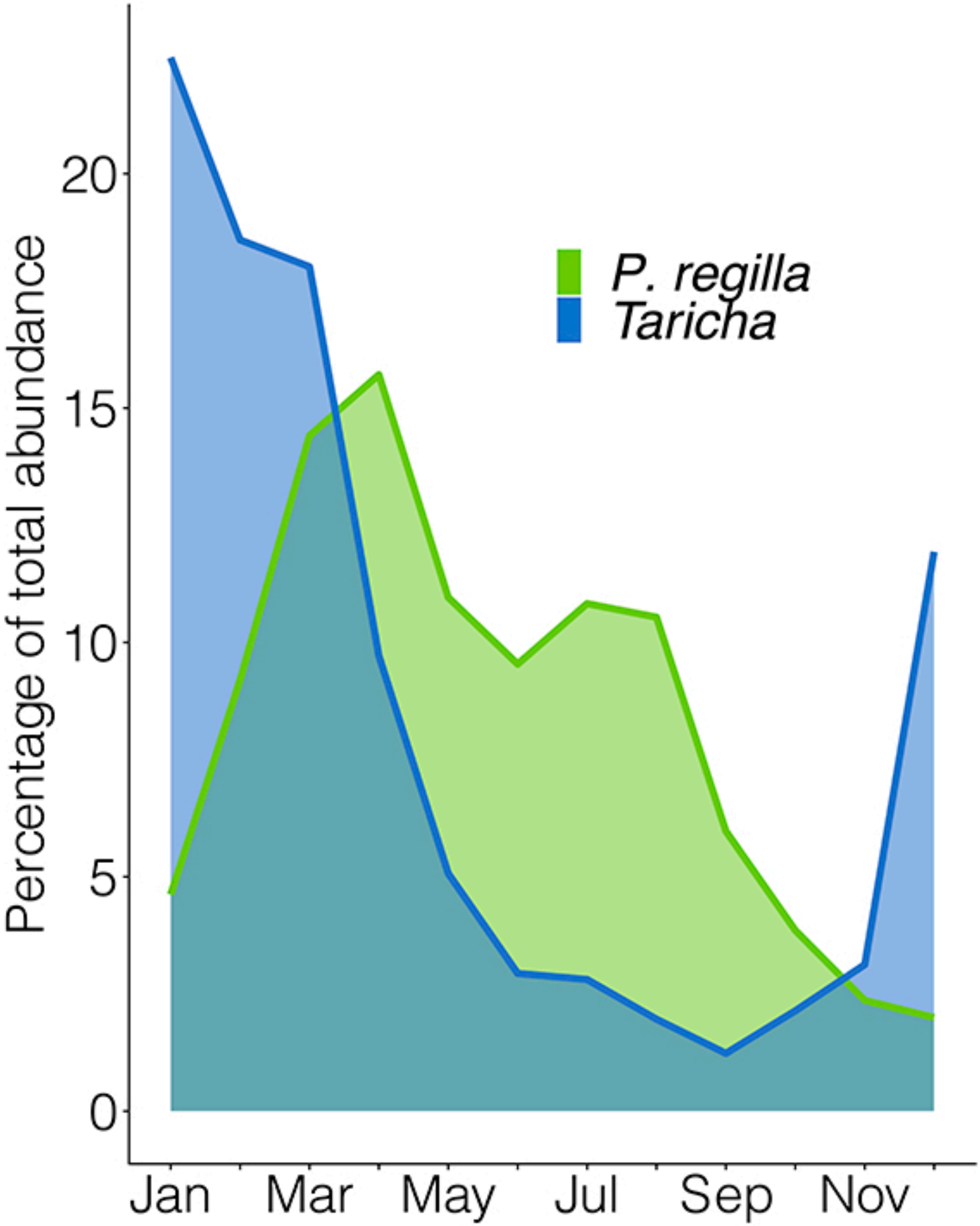
a.

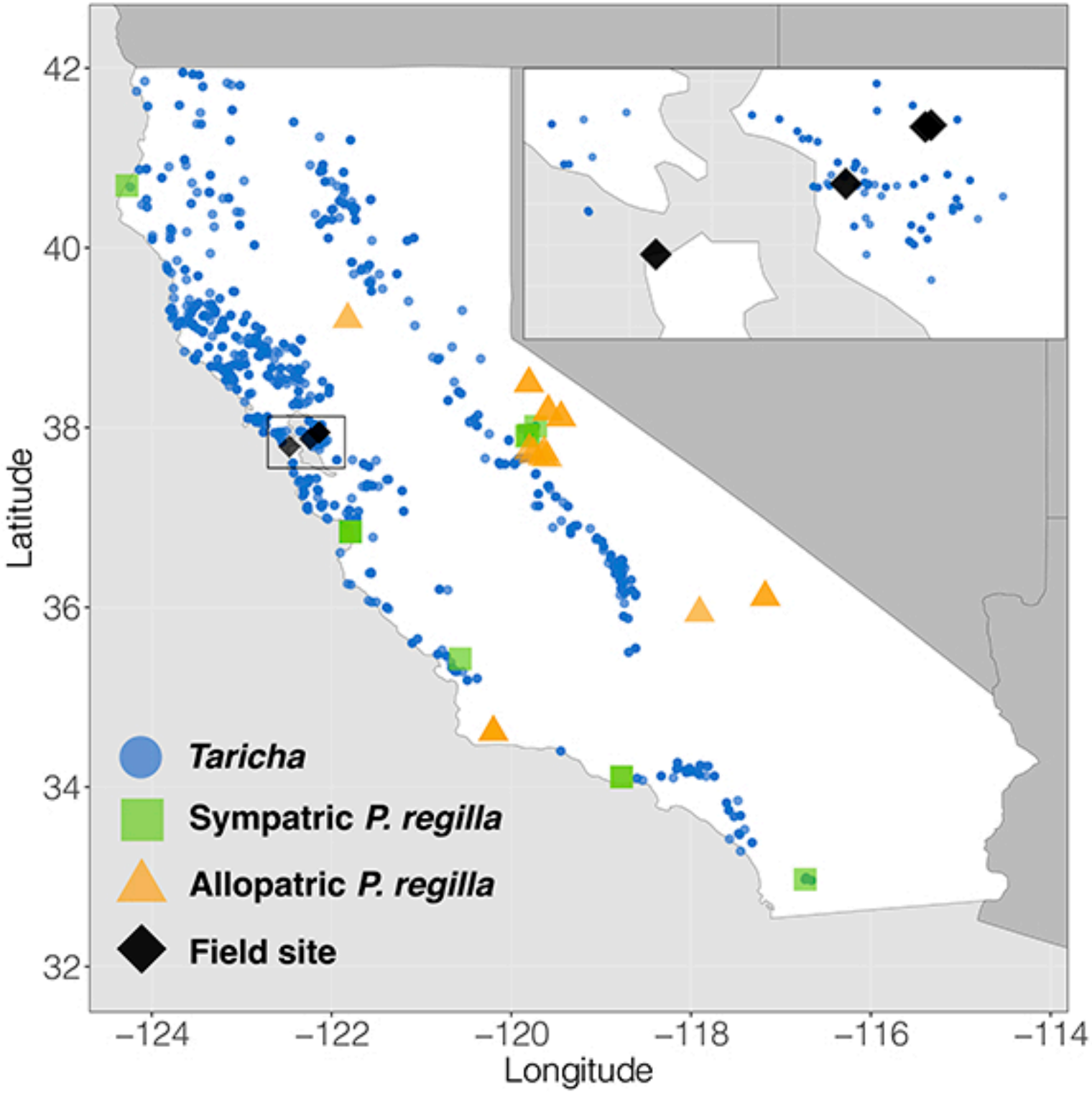
b.

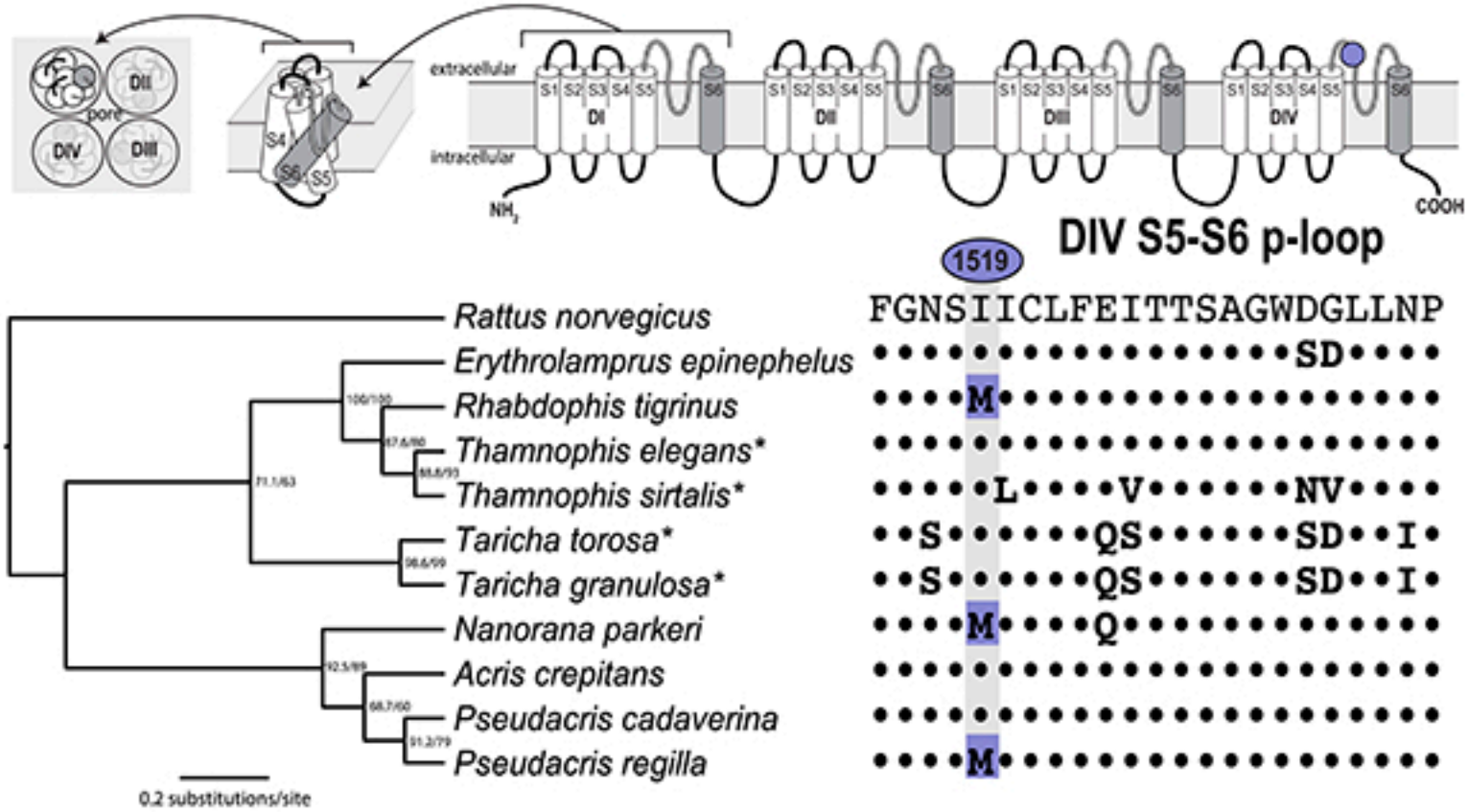
c.

d.


A
Pseudacris regilla adults
Number of individuals
30
20
10
0
Nov
Dec
Jan
Feb
Mar
B
Taricha spp., including larvae
300
200
100
0
Nov
Dec
Jan
Feb
Mar
C
Taricha torosa egg masses
150
100
50
0
Nov
Dec
Jan
Feb
Mar
Pond
OBRT1
OBRT2
UCBG


Percentage of total abundance
20
15
10
5
0
P. regilla
Taricha
Jan
Mar
May
Jul
Sep
Nov


42
40
38
36
34
32
Latitude
−124
−122
−120
−118
−116
−114
Longitude
Taricha
Sympatric P. regilla
Allopatric P. regilla
Field site


DII
pore
DIV
DIII
S4
S6
S5
extracellular
intracellular
S1
S2
S3
S4
S5
S6
DI
DII
DIII
DIV
NH2
COOH
DIV S5-S6 p-loop
1519
FGNSIICLFEITTSAGWDGLLNP
Rattus norvegicus
Erythrolamprus epinephelus
Rhabdophis tigrinus
Thamnophis elegans*
Thamnophis sirtalis*
Taricha torosa*
Taricha granulosa*
Nanorana parkeri
Acris crepitans
Pseudacris cadaverina
Pseudacris regilla
0.2 substitutions/site