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The geographic mosaic in parallel: Matching patterns of newt tetrodotoxin levels and snake resistance in multiple predator–prey pairs

Jessica S. Reimche[1,2] | Edmund D. Brodie Jr.[3] | Amber N. Stokes[4] | Erica J. Ely[1,5] | Haley A. Moniz[1,2] | Vicki L. Thill[1,2] | Joshua M. Hallas[1,2] | Michael E. Pfrender[6] | Edmund D. Brodie III[7] | Chris R. Feldman[1,2]

[1]Department of Biology, University of Nevada, Reno, NV, USA; [2]Program in Ecology, Evolution, and Conservation Biology, University of Nevada, Reno, NV, USA; [3]Department of Biology, Utah State University, Logan, UT, USA; [4]Department of Biology, California State University, Bakersfield, CA, USA; [5]Department of Herpetology, California Academy of Sciences, San Francisco, CA, USA; [6]Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, USA and [7]Mountain Lake Biological Station and Department of Biology, University of Virginia, Charlottesville, VA, USA

Correspondence
Chris R. Feldman
Email: ophis@unr.edu

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Abstract

1. The Geographic Mosaic Theory of Coevolution predicts that coevolutionary arms races will vary over time and space because of the diverse ecological settings and population histories of interacting species across the landscape. Thus, understanding coevolution may require investigating broad sets of populations sampled across the range of the interaction. In addition, comparing coevolutionary dynamics between similar systems may reveal the importance of specific factors that structure coevolution.
2. Here, we examine geographic patterns of prey traits and predator traits in the relatively unstudied interaction between the Sierra garter snake (*Thamnophis couchii*) and sympatric prey, the rough-skinned newt (*Taricha granulosa*), Sierra newt (*Ta. sierrae*) and California newt (*Ta. torosa*). This system parallels, in space and phenotypes, a classic example of coevolution between predatory common garter snakes (*Th. sirtalis*) and their toxic newt prey exhibiting hotspots of newt tetrodotoxin (TTX) levels and matching snake TTX resistance.
3. We quantified prey and predator traits from hundreds of individuals across their distributions, and functional trait matching at sympatric sites.
4. We show strong regional patterns of trait covariation across the shared ranges of *Th. couchii* and newt prey. Traits differ significantly among localities, with lower newt TTX levels and snake TTX resistance at the northern latitudes, and higher TTX levels and snake resistance at southern latitudes. Newts and snakes in northern populations show the highest degree of functional trait matching despite possessing the least extreme traits. Conversely, newts and snakes in southern populations show the greatest mismatch despite possessing exaggerated traits, with some snakes so resistant to TTX they would be unaffected by any sympatric newt. Nevertheless, individual variation was substantial, and

appears to offer the opportunity for continued reciprocal selection in most populations.

5. Overall, the three species of newts appear to be engaged in a TTX-mediated arms race with *Th. couchii*. These patterns are congruent with those seen between newts and *Th. sirtalis*, including the same latitudinal gradient in trait covariation, and the potential 'escape' from the arms race by snake predators. Such concordance in broad scale patterns across two distinct systems suggests common phenomena might structure geographic mosaics in similar ways.

KEYWORDS

adaptation, arms race, coevolution, *Taricha* (Pacific newt), *Thamnophis* (garter snake), trait matching

1 | INTRODUCTION

The Geographic Mosaic Theory of Coevolution (GMTC) suggests that coevolution varies across time and space because populations are ecologically and genetically structured across the landscape (Thompson, 2005). This geographic structure can lead to diverse ecological dynamics and coevolutionary fates between interacting species (Thompson, 1994, 1999, 2005) because divergent processes (e.g. selection, gene flow, drift) can operate independently on ecologically distinct or subdivided populations, creating selection mosaics across landscapes (Brodie, Ridenhour, & Brodie, 2002; Thompson, 1994, 1997, 1999, 2005; Thompson & Cunningham, 2002). Across a range of interacting species, there may be areas of intense reciprocal selection resulting in coevolutionary hotspots. Conversely, there may also be areas of weak or no reciprocal selection, resulting in coevolutionary coldspots (Thompson, 1994, 1999, 2005).

Hotspots and coldspots can be detected by quantifying geographic variation in the traits that mediate the coevolutionary interaction between species (i.e. the phenotypic interface of coevolution; Brodie & Ridenhour, 2003; summarized in Thompson, 1994, 2005). Areas where coevolved traits are well matched indicate hotspots of reciprocal selection, while areas where traits are not well matched (coldspots) are expected in regions where reciprocal selection is weak or absent, though directional selection may be intense for one member of the interaction (Brodie et al., 2002; Thompson, 1994, 1997, 1999, 2005; but also see Gomulkiewicz et al., 2007; Nuismer, Gomulkiewicz, & Ridenhour, 2010). Although much theoretical work has been done to establish a framework for understanding coevolution, relatively few coevolutionary systems have been investigated at a landscape level to test the GMTC (Anderson & Johnson, 2008; Brodie et al., 2002; Hanifin, Brodie, & Brodie, 2008; Mezquida & Benkman, 2005; Thompson & Cunningham, 2002; Zangerl & Berenbaum, 2003). In addition, historical contingency and other ecological or evolutionary forces might conspire to produce hotspots despite weak reciprocal selection or coldspots in the face of strong reciprocal selection (Gomulkiewicz, Thompson, Holt, Nuismer, & Hochberg, 2000; Gomulkiewicz et al., 2007; Nuismer et al., 2010). For example, the traits of ecological partners could match because of diffuse coevolution or selection from other members of a community, because of similar abiotic or other ecological conditions, or from historical selection pressures and genetic constraints (e.g. pleiotropy) that prevent trait change (Gomulkiewicz et al., 2000, 2007; Nuismer et al., 2010). Likewise, mismatches are predicted under the GMTC, depending on patterns of gene flow and drift, selection intensity, the genetic architecture of coevolved traits, ecological constraints on adaptations and even the temporal stage of the species interaction (Brodie et al., 2002; Feldman, Brodie, Brodie, & Pfrender, 2010; Gomulkiewicz et al., 2000, 2007; Hanifin et al., 2008; Kopp & Gavrilets, 2006; Nuismer et al., 2010; Nuismer, Ridenhour, & Oswald, 2007; Nuismer, Thompson, & Gomulkiewicz, 1999; Thompson, 1994, 1999, 2005). Thus, it may be difficult to infer the specific forces behind individual patterns in any one system. Multiple independent coevolutionary systems provide an opportunity to examine whether geographic patterns occur in repeatable and predictable ways, thereby allowing greater inferences about the role of ecological mechanisms responsible for coevolutionary patterns. We address this problem by investigating phenotypic matching between prey and predator traits in a largely unstudied predator–prey system involving poisonous newts and their resistant snake predators. We then compare the geographic patterns to those from a parallel predator–prey system. By evaluating geographic patterns of covariation in two distinct systems, we can produce rare insights into the generality of landscape scale phenomena that might structure geographic mosaics.

The interaction between toxic newts (*Taricha*) and resistant garter snakes (*Thamnophis*) provides a model system to investigate patterns of predator–prey coevolution across the landscape (Brodie & Brodie, 1999). Pacific newts possess a lethal neurotoxin, tetrodotoxin (TTX), which provides a nearly impenetrable chemical defence (Brodie, 1968). TTX binds to the outer pore of voltage-gated sodium channels in nerves and muscles (Na_v proteins), blocking the movement of sodium ions across the cell membrane and halting action potentials (Fozzard & Lipkind, 2010; Hille, 2001). By arresting electrical impulses in muscles and nerves, TTX causes immobilization, respiratory

failure and often death (Abal et al., 2017; Brodie, 1968; Isbister & Kiernan, 2005). Despite the fact that TTX is one of the most potent natural toxins ever discovered, some species of *Thamnophis* prey on sympatric newts (Brodie et al., 2002, 2005; Greene & Feldman, 2009; Wiseman & Pool, 2007), and these different species have independently evolved high tolerance of TTX (Feldman, Brodie, Brodie, & Pfrender, 2009).

Work on the coevolution between newts and snakes, largely centred around the interaction between the rough-skinned newt (*Ta. granulosa*) and the common garter snake (*Th. sirtalis*), demonstrates extensive geographic variation in both prey and predator traits in Western North America (Brodie & Brodie, 1990, 1991; Brodie et al., 2002; Hague, Feldman, Brodie, & Brodie, 2017; Hague et al., 2016; Hanifin, Yotsu-Yamashita, Yasumoto, Brodie, & Brodie, 1999; Hanifin et al., 2008). Newt TTX levels and snake resistance appear well matched across much of the sympatric range of these two species. In localities where *Ta. granulosa* have low levels of TTX (or TTX is absent altogether), *Th. sirtalis* populations have low levels of resistance to TTX. In contrast, in areas where populations of *Ta. granulosa* possess higher levels of TTX, sympatric populations of *Th. sirtalis* display correspondingly elevated levels of TTX resistance (Brodie & Brodie, 1991; Brodie et al., 2002; Hanifin et al., 1999, 2008). While the general pattern of phenotypic variation supports reciprocal selection in predator–prey interactions, a few regions (e.g. San Francisco Bay Area and middle Sierra Nevada of California) show high levels of phenotypic mismatch, with extreme levels of TTX resistance in some snake populations. Snakes at these sites possess elevated TTX resistance that far surpasses the levels required to withstand the amounts of TTX in sympatric newts, suggesting snake predators have 'escaped' the arms race in these populations (Hanifin et al., 2008).

An independent predator–prey system was recently discovered in the Sierra Nevada Mountains of California, involving the Sierra garter snake *Th. couchii* and sympatric species of Pacific newts *Taricha* (Brodie et al., 2005; Feldman et al., 2009; Wiseman & Pool, 2007). The Sierra garter snake is a highly aquatic predator that occupies a wide range of communities in the Lower Cascade and Sierra Nevada Mountains (Rossman, Ford, & Seigel, 1996; Stebbins, 2003). This snake cohabits creeks, springs, ponds and lakes with three newt species: the rough-skinned newt (*Ta. granulosa*) in the Lower Cascades; the Sierra newt (*Ta. sierrae*) along almost the entire Sierra Nevada Range; and the California newt (*Ta. torosa*) in the Southern Sierra. Though this snake is a voracious predator of aquatic and semi-aquatic vertebrates (Fitch, 1949; Rossman et al., 1996; Stebbins, 2003), it was only recently discovered preying on Sierra and California newts (Brodie et al., 2005; Wiseman & Pool, 2007). This interaction provides an opportunity to investigate the extent to which independent predator and prey phenotypes covary across the landscape.

Here, we examine geographic variation in both prey and predator traits across the geographic range of this interaction, characterizing the phenotypic variation and correlation between prey and predator traits at sympatric locations. Our goals are to (a) understand the spatial scale at which coevolution may be occurring; (b) evaluate the presence and extent of trait mismatches across populations and (c) determine whether phenotypes in *Th. couchii* and sympatric newts vary in a geographically parallel fashion to the well-established *Th. sirtalis* and *Ta. granulosa* system.

2 | MATERIALS AND METHODS

2.1 | Prey phenotype assays

To assess prey phenotypes, we quantified TTX in the skin of 108 newts (*Ta. granulosa*, *Ta. sierrae* and *Ta. torosa*) from 10 localities across the Lower Cascade and Sierra Nevada mountain ranges, representing nine distinct watersheds that we used as our population-level samples (Table S1). We briefly housed newts (2–6 weeks) prior to TTX sampling by keeping animals from the same location together in 10 gallon tanks with 5 cm of chloride-free water, rocks and other features. We kept newts on a 12L:12D cycle with temperatures ranging from 15 to 20°C and fed newts blood worms every other day.

From each newt, we took 3-mm diameter skin biopsies from the dorsal surface (the mid back) between the pectoral and pelvic girdle (Hanifin, Brodie, & Brodie, 2002; Lehman, 2007) and extracted TTX following Hanifin et al. (2002). We measured TTX concentrations with a competitive inhibition enzymatic immunoassay (Lehman, 2007; Stokes, Williams, & French, 2012). We used standards from the linear range of the curve in concentrations of 10–500 ng/ml, and diluted all samples 1:1 in 1% bovine serum albumin in phosphate-buffered saline. We considered samples with less than 10 ng/ml of TTX to have no TTX (n = 1). We extrapolated measures of TTX in our skin samples to the whole animal using the calculation from Hanifin, Brodie, and Brodie (2004) to yield estimates of whole newt TTX levels for each individual (mg of TTX/newt).

We mapped the ranges of newt phenotypes across *Ta. granulosa*, *Ta. sierrae* and *Ta. torosa* distributions in the Sierra Nevada and Lower Cascade Ranges using the inverse distance weighted (IDW) interpolation in ArcMap (v10.3.1 ESRI). We used the IUCN database to obtain ranges for *Ta. sierrae* and *Ta. torosa* (which come from Kuchta & Tan, 2006; Stebbins, 2003). Because newt phenotypes did not appear normally distributed, we examined differences in mean phenotypes among populations using nonparametric Kruskal–Wallis tests in R v3.5.1 (R Core Team, 2018). We also tested for correlations between phenotype and latitude using linear regression.

2.2 | Predator phenotype assays

To assess predator phenotypes, we assayed TTX resistance in 293 *Th. couchii* from 35 localities, representing 12 distinct watersheds used as our population-level samples for snakes (Table S2). Prior to phenotypic measures, we housed snakes individually in either 5 or 10 gallon tanks, depending on their size. We provided each tank with a water dish, hide box (Reptile Basics Inc), newspaper or sani-chip bedding (Harlan Teklad), full-spectrum lighting (Reptisun, 10.0 UVA/UVB, Exo Terra) and heat-tape placed under one end of the tank

to generate a thermal gradient from roughly 24 to 30°C. We kept snakes in a room on a 12L:12D cycle with a constant temperature of 26°C, and fed snakes fish (live guppies or frozen trout) or feeder mice (frozen mice from a vendor) once per week.

We measured TTX resistance using a well-established and highly repeatable bioassay of whole-animal performance (Brodie & Brodie, 1990; Ridenhour, Brodie, & Brodie, 2004). We placed snakes on a 4 m track lined with infrared sensors each 0.5 m (and a mounted video camera) to record sprint speed pre- and post-injection with TTX. We used the mean of the quickest two interval times as a snake's speed, which appears to represent an individual's maximal effort and yields repeatable measures over time. After measuring the pre-injection baseline speed of each snake, we rested snakes for 48 hr and then gave each snake an intraperitoneal (IP) injection of TTX diluted with Ringer's solution, starting at 1 mass-adjusted mouse unit (MAMU), where 1 MAMU is the amount of TTX needed to kill a 20-g mouse in 10 min, which corresponds to 0.01429 μg of TTX per gram of snake (Brodie & Brodie, 1990; Brown & Mosher, 1963; Ridenhour et al., 2004). We measured post-injection speeds 30 min after TTX injections (Brodie et al., 2002; Ridenhour et al., 2004). We then rested snakes for 48 hr and subsequently injected them with serially increasing doses (5, 10, 25, 50, 100 or higher MAMUs as needed) and recorded post-injection speeds. Note, however, that due to the prohibitive cost of TTX, we stopped increasing the doses of TTX before a 50% reduction in speed could be estimated in 65 highly resistant snakes, resulting in measurements that underestimate true TTX resistance.

We scored resistance as the dose required to slow a snake to 50% of its pre-injection baseline speed (50% MAMU). We estimated this 50% dose using curvilinear regression on log-transformed dosages; doses equal to 1 we converted to 0.999, and those that were zero we converted to 0.001 (Brodie et al., 2002; Ridenhour et al., 2004). We calculated the curvilinear regression using the linear regression $y' = \alpha + \beta x'$, where y is TTX resistance and calculated as $y' = \ln(1/y - 1)$, and x is the TTX dose calculated as $x' = \ln(x)$, α and β are the estimated regression parameters (Ridenhour et al., 2004).

Using this regression approach, we also calculated the doses required to slow a snake to 85% of its normal crawl speed, and to 15% of its normal speed. Between these values, the dose response curve appears linear, while above 85% and below 15%, TTX resistance appears asymptotic (Hanifin et al., 2008; Ridenhour et al., 2004). In other words, doses of TTX above 85% have little to no effect on snake performance, whereas doses of TTX below 15% severely incapacitate snakes (Hanifin et al., 2008). We then used these 85% and 15% values as approximate thresholds for understanding phenotypic mismatches between newts and snakes (see below).

We mapped the distribution of predator phenotypes (50% doses) across the range of *Th. couchii* in ArcMap as above; we obtained the distribution *Th. couchii* from the IUCN database (which comes from Rossman et al., 1996; Stebbins, 2003). As with newt phenotypes, we examined differences in mean predator phenotypes among populations using nonparametric, Kruskal–Wallis tests in R, and tested for correlations between phenotype and latitude using linear regression.

2.3 | Phenotype matching and mismatching

To determine relationships between prey TTX levels and predator TTX-resistant phenotypes, we first adjusted predator TTX-resistant units from 50% MAMUs (based on IP injections) to mg of TTX in oral doses because snakes in the wild ingest newts whole. We converted 50% MAMU to 50% IP dose using the following equations (Hanifin et al., 2008):

$$\text{IP dose (mg)} = (\theta \times 0.00001429) \times \text{snake mass (g)},$$

where θ is the 50% MAMU and 0.0001429 is the conversion factor (1 MAMU = 0.01429 μg TTX per g of snake; Brodie & Brodie, 1990, 1991; Brodie et al., 2002; Brown & Mosher, 1963; Ridenhour et al., 2004). The effects of TTX are dependent on body size (Abal et al., 2017; Brodie et al., 2002; Hanifin et al., 2008; Ridenhour et al., 2004; Williams, Brodie, & Brodie, 2002); therefore, we estimated the oral dose of TTX required to slow the average adult *Th. couchii* (mean adult mass = 38 g). We then converted IP dose (mg) to the oral dose required to achieve the same performance reduction by multiplying the IP dose by 40 (Abal et al., 2017; Hanifin et al., 2008; Williams et al., 2002). By quantifying TTX resistance as the estimated dose of orally ingested TTX (in mg) needed to reduce snake performance by 50%, we have an ecologically relevant metric to compare predator and prey phenotypes.

We examined the relationship between newt TTX levels (mg of TTX) and snake resistance (mg of TTX) for nine sympatric populations using linear regression in R. We then quantified the relationship of each sympatric interaction by calculating the functional mismatch of each point in our regression. Hanifin et al. (2008) defined a functional mismatch as an ecological interaction between sympatric predator and prey that does not result in comparative fitness consequences. A matched population consists of a sympatric interaction expected to result in similar fitness outcomes (i.e. the average newt in a population contains enough TTX to reduce the average snake performance by 50% in that location; Hanifin et al., 2008). We estimated population mismatches by calculating (d) which is simply the deviation of observed values from the expected matching values described by a linear regression between mean prey and mean predator traits in sympatric populations. We calculated d by modifying the equation in Hanifin et al. (2008) for measuring the shortest distance from a point to a line:

$$d = x_1(A) + y_1(B) + C/\sqrt{(A^2 + B^2)}.$$

Because the expected TTX resistance assumes a matched interaction between snake and newt phenotypes (i.e. total TTX in newt skin = 50% dose of snakes in sympatric population), the line describing a perfect phenotypic match has a slope of 1 (A and B) and an

intercept of 0 (*C*), assuming the traits occur on the same scale. Our equation then reduces to:

$$d = (x_i - y_i)/\sqrt{2},$$

where x_i = average 50% dose of snakes from a given population and y_i = average total TTX in newt skin from the same population, that is, linear match in phenotypes between predator and prey (dashed line in Figure 2b). Following Hanifin et al. (2008), we deemed predator–prey populations with *d* calculations >0.6 or <−0.6 ($d < -0.6$, $d > 0.6$) as showing high phenotypic mismatch (Hanifin et al., 2008); these values correspond to a 15% and 85% reduction in predator crawl speed, respectively, which suggest that reciprocal selection is low or absent. We considered locations where *d* ranges between 0.6 and −0.6 as sites where both prey and predator populations are likely experiencing reciprocal selection.

Finally, we found substantial individual variation in prey and predator phenotypes within populations (see Section 3). Thus, we attempted to quantify phenotypic matching and mismatching at the level of individual newts and snakes from sympatric populations. We simulated chance encounters between prey and predator by randomly drawing one newt and one snake from the same locality and estimating their phenotypic mismatch: snake TTX resistance (50% oral dose in mg TTX) minus newt TTX (total mg of TTX). We then evaluated the distribution of estimated mismatches by randomly sampling newt–snake pairs 10,000 times. Perfectly matched pairs result in no mismatch (0 mg of TTX), while a snake with a 50% dose of TTX that exceeds the amount of TTX in a sympatric newt results in a 'predator mismatch' (excess TTX resistance, reported in + mg of TTX), and a snake with less TTX resistance than a sympatric newt yields a 'prey mismatch' (deficient TTX resistance, reported in − mg of TTX). To put these values of TTX into context, we plotted the 85% and 15% doses of TTX resistance for snakes. These provide an indication of the proportion of sympatric newt–snake pairs that can potentially impose fitness costs on one another, and proportion of interactions that cannot result in reciprocal selection (mismatches that lie outside the zone of 85% and 15% doses).

We performed simulations on three populations of newts and snakes that span the geographic distribution and species involved in the interaction, as well as the range of phenotypes: *Ta. granulosa* (*n* = 13) v. *Th. couchii* (*n* = 11) from Battle Creek; *Ta. sierrae* (*n* = 10) v. *Th. couchii* (*n* = 11) from Battle Creek; *Ta. sierrae* (*n* = 10) v. *Th. couchii* (*n* = 61) from Upper Yuba River; *Ta. torosa* (*n* = 15) v. *Th. couchii* (*n* = 94) from Upper Tule River. We conducted all simulations and mismatch calculations in R.

3 | RESULTS

3.1 | Prey phenotypes

We found extensive variation in TTX levels among species, within species and even within newt populations across the lower Cascade Range and Sierra Nevada Range (Table 1). Among the three *Taricha* species, there are significant differences in levels of TTX (Kruskal–Wallis = 18.597, *df* = 2, $p < 0.001$). The rough-skinned newt, *Ta. granulosa*, possessed the lowest levels of TTX (0.06 mg of TTX/newt), significantly less TTX than the sympatric Sierra newt, *Ta. sierrae* (Dunn's post hoc test, $p < 0.001$), which contained nearly three times more TTX (0.17 mg of TTX/newt) at the same location in North Battle Creek. On the other hand, the California newt, *Ta. torosa*, possessed the most toxic individuals of all three species, although average TTX levels for our single population were on par with those found in populations of *Ta. sierrae* across their range (Dunn's post hoc test, $p = 0.23$).

We also found significant differences in amounts of TTX between populations across the nine sampled watersheds, with mean TTX levels ranging from 0.06 to 1.25 mg/newt (Table 1, Kruskal–Wallis = 30.71, *df* = 7, $p < 0.001$); post-hoc comparisons revealed significant pairwise differences in TTX between the northernmost population (Battle Creek) and three southern populations (Upper Tuolumne River, Upper Kings River and Upper Tule River). In addition, the variation in TTX levels appears to follow a latitudinal gradient, with populations at the higher latitudes possessing less TTX than those at the southern latitudes (Figure 1a; $r = -0.47$, $r^2 = 0.25$, $F = 35.16$, *df* = 106, $p = <0.001$).

Lastly, we noted substantial variation in the amounts of TTX in the skin of individual *Ta. sierrae* and *Ta. torosa* within populations (Table 1). Intrapopulation-level variation appears most pronounced in the southern half of the range; in the four southernmost locations (Upper Cosumnes River, Upper Tuolumne River, Upper Kings River, Upper Tule River), three newt populations have TTX levels above 1 mg, and the southernmost population (*Ta. torosa*, Upper Tule River watershed) contains tremendous TTX variation with individuals that possess low levels of TTX (0.02 mg) to those with 5.48 mg (Table 1).

3.2 | Predator phenotypes

We documented extensive phenotypic variation in the predator *Th. couchii*, with significant differences in TTX resistance across the 12 sampled watersheds (Table 1, Kruskal–Wallis = 210.86, *df* = 11, $p < 0.001$). Post-hoc comparisons revealed significant pairwise differences between oral dose of TTX (mg) in 29 of the 66 watershed comparisons. Phenotypic variation in snakes follows a similar geographic trend as observed in sympatric newts, with populations of *Th. couchii* in the north displaying low TTX resistance, while those in the south show elevated TTX resistance (Figure 1b), generating a strong correlation between TTX resistance and latitude ($r = -0.77$, $r^2 = 0.59$, $F = 429$, *df* = 291, $p < 0.001$). Further, populations of *Th. couchii* at the southern end of the species range (Upper Tuolumne River, Upper Kings River, Upper Tule River watersheds) possess individuals that function above 50% of their baseline sprint speed at well over 100 MAMUs. Multiple populations (especially in the middle Sierra Nevada) contain high levels of phenotypic variation with 50% oral doses ranging from 0.40

TABLE 1 Sample locations and summary statistics for prey (*Taricha* sp.) and predator (*Thamnophis couchii*) phenotypes, as well as degree to which phenotypes match (*d*). Tetrodotoxin (TTX) resistance is given in oral doses of TTX (mg) for ease of comparison to matching newt TTX levels, and intraperitoneal injections (IP) of MAMUs (Mass Adjusted Mouse Units of TTX) to allow direct comparison to prior work on *Thamnophis* using the latter measure (e.g. Brodie et al., 2002)

Watershed (latitude, longitude)	Newt species	Newt sample size (*n*)	Mean TTX; mg TTX/newt	Range TTX; mg TTX/newt	Snake sample size (*n*)	Mean TTX resist; oral dose mg TTX (IP 50% MAMU)	Range TTX-resist; oral dose mg TTX (IP 50% MAMU)	Phenotypic mismatch (*d*)
1. Battle Creek (40.44, −121.76)	*Ta. granulosa* *Ta. sierrae*	All newts: 23 *Ta. granulosa*: 13 *Ta. sierrae:* 10	All: 0.11 ± 0.08 *Ta. granulosa:* 0.06 ± 0.03 *Ta. sierrae:* 0.17 ± 0.08	All: 0.02–0.29 *Ta. granulosa:* 0.02–1.20 *Ta. sierrae:* 0.07–0.28	11	0.06 ± 0.02 (2.58 ± 0.70)	0.03–0.08 (1.28–3.81)	All: −0.02 *Ta. granulosa:* 0.01 *Ta. sierrae:* −0.06
2. Honey Eagle Lakes (40.31, −120.35)	*Ta. sierrae*	8	0.14 ± 0.10	0.01–0.31	20	0.11 ± 0.05 (4.83 ± 2.36)	0.00–0.21 (0.1–9.5)	0.002
3. North Fork Feather River (40.12, −121.27)	*Ta. sierrae*	5	0.13 ± 0.08	0.05–0.25	3	0.14 ± 0.04 (6.41 ± 1.70)	0.10–0.18 (4.73–8.12)	0.05
4. Upper Yuba River (39.44, −120.89)	*Ta. sierrae*	10	0.23 ± 0.17	0.08–0.57	60	0.32 ± 0.37 (14.49 ± 17.16)	0.04–2.17 (1.7–82.5)	0.15
5. South Fork American River (38.79, −120.54)	*Ta. sierrae*	10	0.27 ± 0.13	0.09–0.44	11	1.14 ± 0.72 (52.54 ± 32.97)	0.14–2.17 (6.3–100)	0.95
6. Upper Cosumnes River (38.53, −120.86)	*Ta. sierrae*	18	0.38 ± 0.47	0.01–1.56	47	1.18 ± 0.52 (54.20 ± 24.03	0.40–2.22 (18.6–102)	0.91
7. Upper Mokelumne River (38.37, −120.72)	—	—	—	—	10	0.40 ± 0.38 (18.42 ± 17.36)	0.05–1.09 (2.47–50.00)	—
8. West Walker River (38.366, −19.48)	—	—	—	—	1	0.07 (3.04)	—	—
9. Upper Stanislaus River (38.20, −119.97)	—	—	—	—	2	0.38 ± 0.44 (17.64 ± 20.31)	0.07–0.70 (3.28–32.0)	—
10. Upper Tuolumne River (37.91, −120.02)	*Ta. sierrae*	9	0.69 ± 0.26	0.38–1.04	19	1.31 ± 0.86 (60.40 ± 39.68)	0.18–2.99 (8.10–137.60)	0.82
11. Upper Kings River (36.92, −118.84)	*Ta. sierrae*	10	0.72 ± 0.57	0.10–1.63	15	2.19 ± 0.52 (100.36 ± 24.40)	1.09–3.80 (50.0–175.0)	1.68
12. Upper Tule River (36.17, −118.97)	*Ta. torosa*	15	1.25 ± 1.58	0.02–5.48	94	1.846 ± 0.53 (85.63 ± 24.54)	0.79–3.51 (36.5–161.5)	0.97

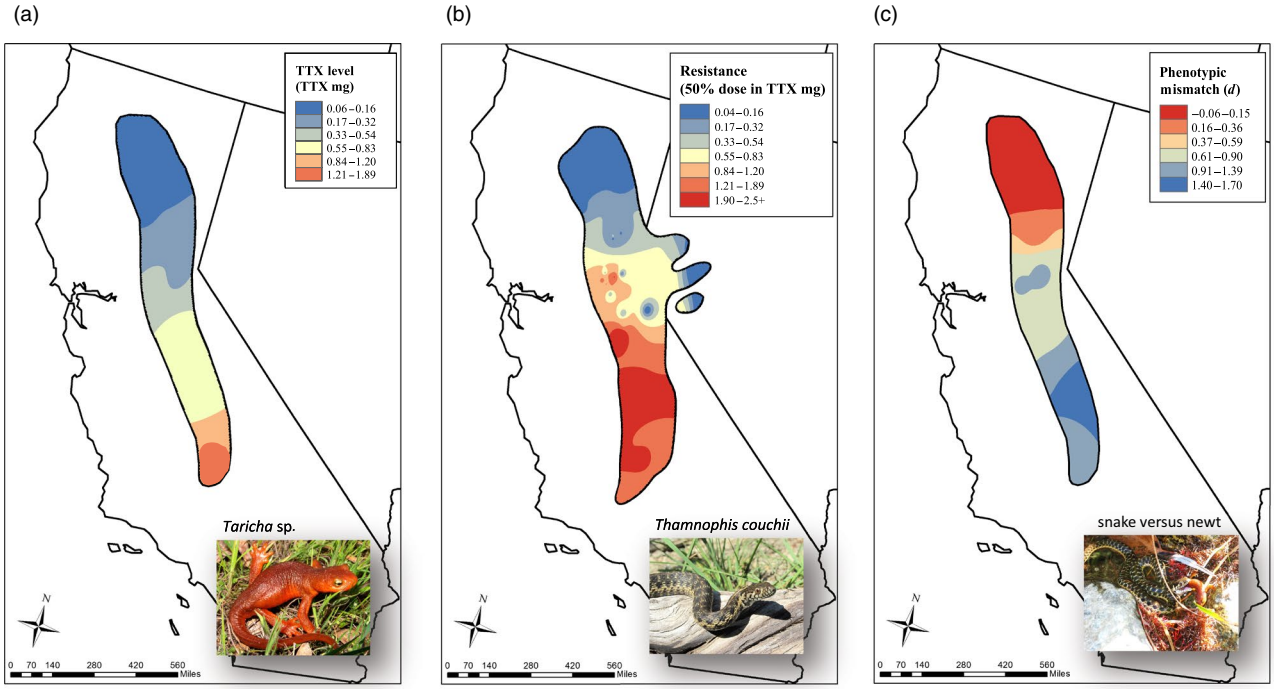

FIGURE 1 Geographic distribution of phenotypes for newt prey (*Taricha granulosa*, *Ta. sierrae* and *Ta. torosa*) and snake predator (*Thamnophis couchii*) in California, as well as hotspots and coldspots. (a) Distribution of tetrodotoxin (TTX) levels (TTX mg/newt) across newt range in the Sierra Nevada and Lower Cascade Ranges, based on interpolation of samples from 10 localities (Table 1). (b) Distribution of TTX resistance (oral dose of TTX in mg required to slow a snake to 50% of its baseline speed) across *Th. couchii* range, based on interpolation of samples from 35 localities. (c) Phenotypic mismatch (*d*) among prey and predator phenotypes at paired locations representing hot and cold spots. Note that colours on panels (a) and (b) are on the same scale (in mg of TTX), while colours on panel (c) represent degree of prey–predator matching, with red corresponding to high phenotypic matching (hotspots) and blue representing phenotypic mismatch (coldspots). Photos: J. Vindum, G. Nafis, A. Pool

to 2.21 mg of TTX (18–102 MAMUs) in Upper Cosumnes River, and 0.18 to 2.99 mg of TTX (8–137 MAMUs) in Upper Tuolumne River watersheds.

3.3 | Phenotype matching and mismatching

We found a strong linear relationship between prey and predator phenotypes across their shared ranges (Figure 2; $r = 0.86$, $r^2 = 0.74$, $F = 25.59$, $df = 8$, $p < 0.001$). Using our metric of functional trait matching (*d*), we found both areas of tight phenotypic matching ($d < 0.6$) and zones of mismatching ($d > 0.6$) across the landscape (Figures 1 and 2). A *d* of 0 indicates the average amount of TTX in a newt exactly matches the oral dose of TTX required to slow the average snake to 50% of its baseline speed in that locality. Negative values of *d* indicate the average amount of TTX in sympatric newts is higher than average 50% oral dose of TTX in sympatric snakes, and conversely, a positive *d* suggests that mean newt TTX levels are lower than the 50% dose of snakes. The four northernmost populations of predators and prey are especially well matched, with low values of *d* (0 to 0.15) that reveal coevolutionary hotspots (Table 1; Figures 1 and 2). The five southernmost populations display relatively high phenotypic mismatch, with *d* values all greater than 0.6 that reveal coldspots (Table 1; Figures 1 and 2). In three populations, in particular (South Fork American River, Upper Cosumnes River and Upper Kings River), the average newt does not contain enough TTX to slow the average snake to even 85% of baseline speed. However, individual variation in prey and predator traits appears high in these southern populations (Table 1; Figure 2).

To further explore phenotypic mismatching within populations, we randomly paired newts and snakes (10,000 times) from sympatric sites, and then calculated trait matching (in units of mg of TTX), allowing us to create histograms of individual phenotypic mismatches expected to occur within populations. We conduced these simulations for newt–snake pairs at three sites that represent the full range of phenotypes, as well as the entire geographic and taxonomic breadth of the interaction. Simulations (Figure 3) reveal that even at a single site, the full range of predator–prey outcomes are possible, from newts that are too toxic for some sympatric snakes to survive, to predator–prey pairs that fall within a phenotypic interaction space in which both species could experience reciprocal selection (between 85% and 15% doses of TTX for snakes), to snakes that are so resistant to TTX that they can handle most sympatric newts with little or no ill effects (snakes not slowed to even 85% of normal speed). The distributions of individual mismatches show that this range of potential outcomes is possible, even at sites where the mean values of newt and snake traits suggest that either the prey or predator are 'winning' (e.g. Upper Tule River).

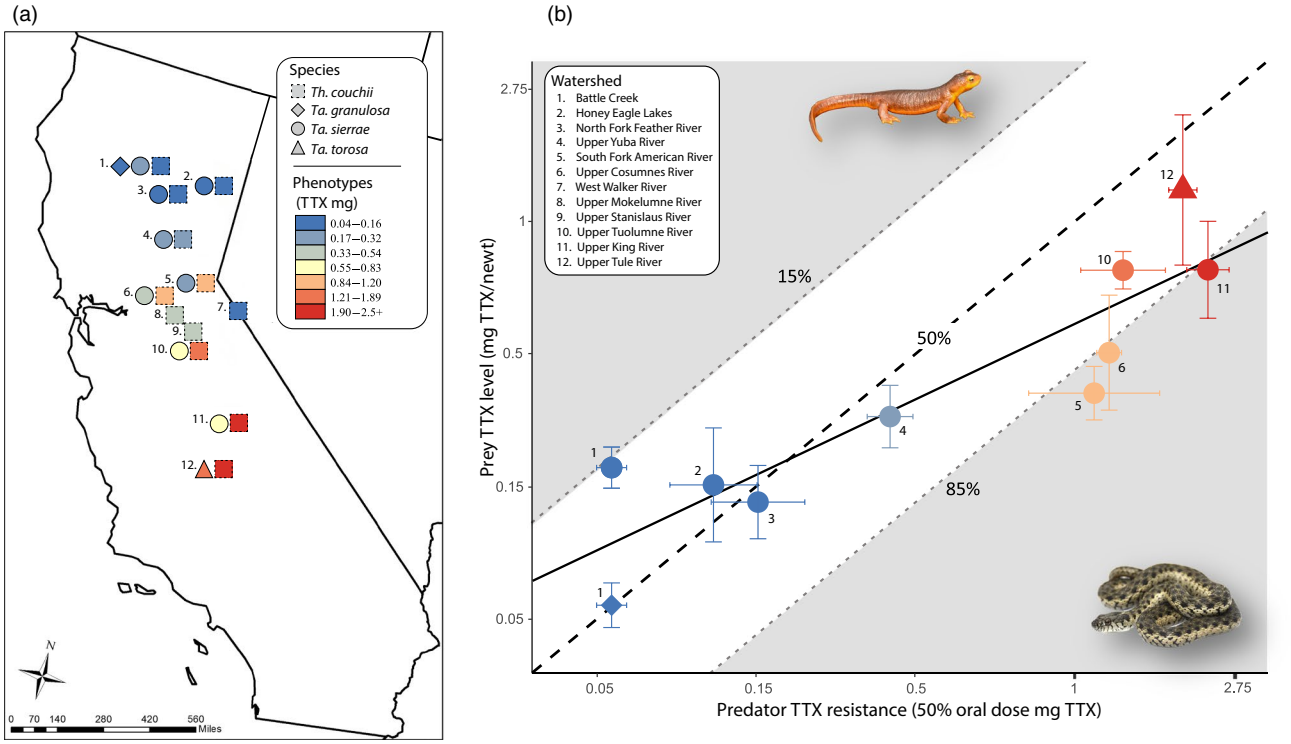

FIGURE 2 Distribution of phenotypic matching between newt prey and garter snake predator at sympatric sites, as well as the relationship between prey and predator phenotypes. (a) Mean tetrodotoxin (TTX) levels of newts (*Taricha granulosa*, diamond; *Ta. sierrae*, circle; *Ta. torosa* triangle) and mean TTX resistance of snakes (*Thamnophis couchii*, dashed square) of the nine paired populations. (b) Linear relationship (r = 0.86, r^2 = 0.74, F = 25.59, df = 8, p < 0.001) between mean prey (TTX mg/newt) and mean predator phenotypes (50% oral dose of TTX in mg) at paired locations. Symbols represent newt species, and show mean and *SE* (vertical bars) of newt populations, while colours correspond to mean snake resistance values with *SE* (horizontal bars) in each matching population. Black dashed line shows the expected 1:1 relationship of perfectly matched phenotypes across populations (i.e. mean total TTX in newt skin would reduce mean sympatric snake to 50% of its normal sprint speed); solid line shows best fit regression of actual mean newt TTX levels and mean snake 50% doses of TTX; dashed grey lines represent the 15% and 85% doses of TTX resistance estimated for each snake population. Populations that fall outside the 15% and 85% doses of TTX resistance (grey areas) are considered mismatched; left of the 15% line, the average newt contains more TTX than the average sympatric snake can safely handle, while right of the 85% line the average snake would be unaffected by the TTX of the average sympatric newt. Regression performed on raw phenotypic values, but displayed on log scale (ln) with back-transformed units of TTX for ease of viewing. Photos: D. Picklum

4 | DISCUSSION

We found strong patterns of trait matching between sympatric newt prey (*Ta. granulosa*, *Ta. sierrae* and *Ta. torosa*) and snake predators (*Th. couchii*) but also trait mismatching that suggests a potential 'escape' from the arms race in some snake populations. These patterns largely mirror those seen in the well-characterized *Ta. granulosa* and *Th. sirtalis* system (Brodie et al., 2002; Hanifin et al., 2008), indicating these separate arms races between newts and their snake predators have experienced similar dynamics across time and space. The next steps will be uncovering the ecological determinants and evolutionary constraints that lead to such repeatable patterns of coevolution.

4.1 | Prey and predator phenotypes

Both prey and predator demonstrate substantial variation in phenotypes across their ranges in the Sierra Nevada and Lower Cascade, consistent with a latitudinal gradient. The variation in TTX levels among populations of *Ta. granulosa*, *Ta. sierrae* and *Ta. torosa* is similar to that seen among coastal populations of *Ta. granulosa* and *Ta. torosa* (Hanifin et al., 2008). Some populations of Sierran *Ta. sierrae* and *Ta. torosa* possess amounts of TTX that would be lethal to nearly any potential vertebrate predator, as is the case for coastal *Ta. granulosa* (Brodie, 1968; Hanifin et al., 2002). Our most toxic newt population (*Ta. torosa*, Upper Tule watershed) included individual newts with over 5 mg of TTX (i.e. animals weighing less than 15 g with enough poison to kill 2–5 humans). Newts with higher TTX levels have only been found in a handful of sites (Hanifin et al., 2008; Stokes et al., 2015). Surprisingly, these southern watersheds, which harbour our most toxic newts, also contain some of our least toxic individuals (range from 0.02 to 5.48 mg TTX). This extreme variability in newt phenotypes at a single location is rare, and it has only been documented in a few other locations (Hague et al., 2016; Stokes et al., 2015; Williams, Hanifin, Brodie, & Brodie, 2010). Such within population variation in prey phenotypes warrants further investigation into the genetic and

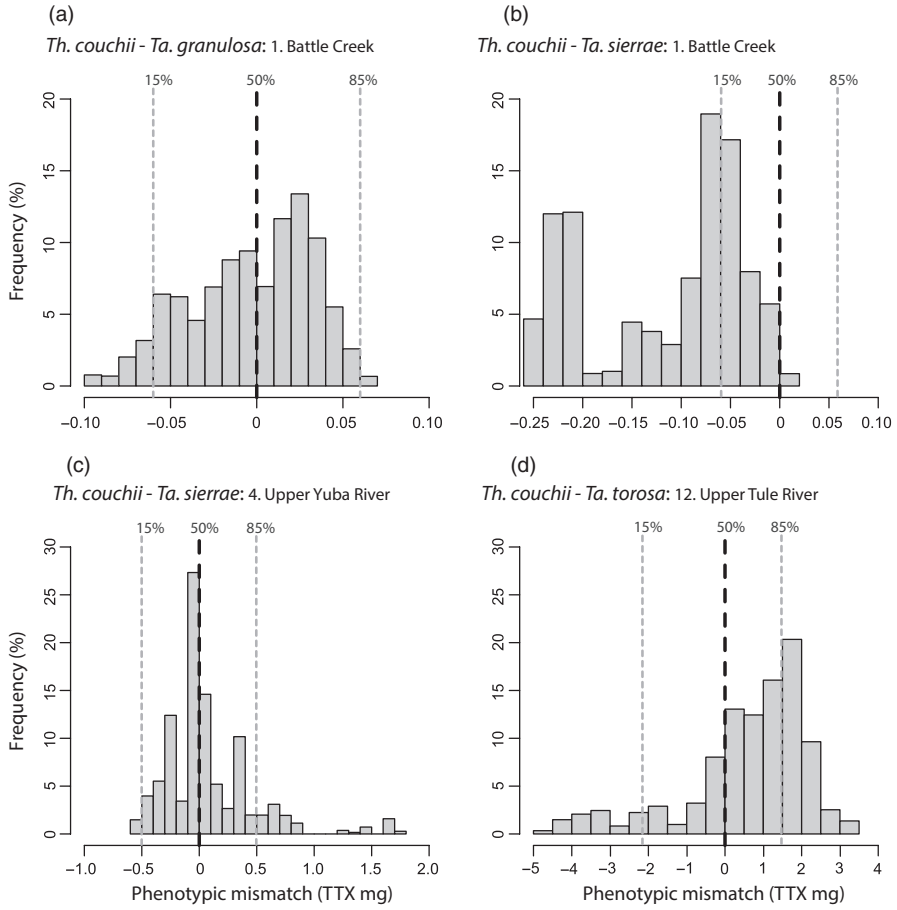

FIGURE 3 Histograms of simulated interaction mismatches for randomly drawn pairs of newts and snakes from three sympatric localities. (a) Distribution of mismatches between pairs of *Thamnophis couchii* and *Taricha granulosa* from Battle Creek. (b) Distribution of mismatches between pairs of *Th. couchii* and *Ta. sierrae* from Battle Creek. (c) Distribution of mismatches between pairs of *Th. couchii* and *Ta. sierrae* from Upper Yuba River. (d) Distribution of mismatches between pairs of *Th. couchii* and *Ta. torosa* from Upper Tule River. Mismatches calculated by taking the difference in a newt phenotype (total newt tetrodotoxin [TTX] in mg) from a paired snake phenotype (50% oral dose of TTX in mg); perfect matching between newt and snake yield no mismatch (0 mg TTX), while excess TTX resistance in a snake nets positive amounts of TTX, and deficient levels of TTX resistance in a snake results in negative amounts of TTX. Black dashed lines represent the expected 1:1 match between newt and snake phenotypes, and dashed grey lines represent the 15% and 85% doses of TTX resistance estimated for each snake population (as in Figure 2). Pairs that fall outside these boundaries are considered mismatched; left of the 15% line, a newt could severely incapacitate or kill its paired snake predator, while right of the 85% line a snake is essentially unaffected by the TTX of its paired newt prey

physiological underpinnings of TTX levels in newts (e.g. Bucciarelli, Shaffer, Green, & Kats, 2017; Mailho-Fontana et al., 2019).

Similar to the variability across newt populations, we observed wide phenotypic variation in *Th. couchii*, with average oral doses of TTX resistance (50% dose) ranging from 0.06 mg of TTX to 2.19 mg of TTX (2.6–100 MAMUs). Populations of *Th. couchii* in the southern end of their range are most resistant to TTX, and possess individuals that function at 50% of their baseline sprint speed at oral doses well over 3 mg of TTX (over 150 MAMUs). These measures of TTX resistance place southern populations of *Th. couchii* among the most resistant *Thamnophis* recorded (Brodie et al., 2002, 2005; Feldman et al., 2009, 2010; Hague et al., 2017). In addition, the range of phenotypes seen within several populations of *Th. couchii* is uncommon, with only a few populations of *Th. atratus* and *Th. sirtalis* possessing such extreme within-population variation (Brodie et al., 2002; Feldman et al., 2010; Hague et al., 2017).

4.2 | Hotspots and coldspots

The strong linear relationship between TTX levels in *Taricha* and TTX resistance in sympatric *Th. couchii* reveals hotspots of reciprocal selection (Figure 2). In other words, prey and predator traits appear functionally well matched across the landscape (i.e. mean newt TTX level and dose of TTX required to slow the average sympatric snake to 50% of normal crawl speed), suggesting a history of ever-increasing phenotypic evolution between newts and Sierra garter snakes.

Despite overall trait matching between sympatric *Taricha* and *Th. couchii*, we also uncovered coevolutionary coldspots, or areas of weak (or absent) reciprocal selection. Hanifin et al. (2008) classified roughly one-third of sympatric populations of *Ta. granulosa* and *Th. sirtalis* as functional mismatches, where predator resistance was so extreme that the average newt TTX would have little to no effect

on co-occurring snakes. We observed similar relationships in the southern end of the Sierra Nevada between the newt prey *Ta. sierrae* and *Ta. torosa*, and sympatric *Th. couchii* predators. Areas of functional trait mismatch show exaggerated TTX resistance in southern populations of *Th. couchii* that far exceed the levels necessary to eat toxic newts at those locations (Figures 1 and 2). Thus, in roughly half the populations, *Th. couchii* may be imposing intense selection on *Ta. sierrae* and *Ta. torosa*, but this interaction may not be currently reciprocal. The mechanisms driving local patterns of hotspots and coldspots require investigation (see below), particularly the notable finding that two phylogenetically independent snake predators may have evolutionarily 'escaped' the arms race with toxic newts.

4.3 | Understanding trait matching and mismatching

Determining the ecological conditions and evolutionary forces that drive spatial patterns of phenotypic matching and mismatching is the next step in understanding the GMTC. Differences in ecology among populations may explain the presence or absence of reciprocal selection (Thompson, 1994, 1997, 1999, 2005). Selection promoting predation on newts might vary across the Lower Cascade and Sierra Nevada. Differences in the abundance, availability and seasonal importance of various prey items, especially newts, might permit northern populations of *Th. couchii* to generalize on other aquatic species, whereas southern populations of *Th. couchii* may be forced to specialize on *Taricha* and their larvae at certain times of year. If *Th. couchii* from southern populations of are more likely to come across *Taricha* or are forced to rely on newts as a critical food resource, then there may be stronger selection on those snake populations to exploit toxic prey. Diet analysis of *Th. couchii* across their range could help clarify some of these questions and provide further hypotheses to explain why TTX resistance is so elevated in the southern part of the range.

Besides quantifying the interactions between prey and predator, and obtaining a more complete view of the ecological communities they occupy, elucidating the genetic basis of the co-evolved traits would help us understand patterns of hotspots and coldspots. Unfortunately, the genetic basis of TTX synthesis in newts remains unknown (Hanifin, 2010; Jal & Khora, 2015). On the other hand, TTX resistance in garter snakes appears to have a relatively simple genetic basis, involving structural changes in outer pore (P-loops) of the sodium channels expressed in muscles and nerves that are the molecular targets of TTX (Feldman, Brodie, Brodie, & Pfrender, 2012; Geffeney, Fujimoto, Brodie, Brodie, & Ruben, 2005; McGlothlin et al., 2014, 2016). Functional variation in the skeletal muscle sodium channel ($Na_v1.4$) appears especially important in contributing to whole-animal TTX resistance (Feldman et al., 2010; Geffeney et al., 2005; McGlothlin et al., 2016), and allelic variation in the gene that encodes this protein (*SCN4A*) appears responsible for drastic differences in phenotypes within and among populations of *Th. sirtalis* (Feldman et al., 2010; Geffeney et al., 2005; Hague et al., 2017) and *Th. atratus* (Feldman et al., 2010). Geographic variation in TTX resistance in *Th. couchii* may be due to similar genetic changes. In fact, the southernmost population of *Th. couchii* possess a mutation in *SCN4A* (Feldman et al., 2009) that is found in TTX-bearing pufferfish and in newts (Hanifin & Gilly, 2015; Jost et al., 2008), and is known to confer a 15-fold decrease in TTX ligation to the channel (Jost et al., 2008). Thus, phenotypic variation in *Th. couchii* might be explained by allelic variation at this locus. Work is needed to characterize functional variation in *SCN4A* across the range of *Th. couchii*, and whether rates of gene flow might contribute to the high degree of phenotypic variation in snake populations.

Our results also stress the importance of understanding the biogeographic history and genetic structure of interacting species. The snakes and newts of this region may have distinct histories of colonization and fragmentation, and thus, different temporal depths of association across the range. In addition, prey and predator populations may have distinct spatial patterns of connectivity, which could contribute to hotpot and coldspot patterns. Indeed, the Sierra Nevada and Lower Cascade Ranges possess a varied topography, geology and climate (Schoenherr, 2017), as well as a complex history of glaciation and orogeny (Moore & Moring, 2013) well known for fragmenting and re-connecting populations of vertebrates in this region through time (Feldman & Spicer, 2006; Kuchta, Parks, Mueller, & Wake, 2009; Kuchta & Tan, 2006; Lavin, Wogan, McGuire, & Feldman, 2018; Rissler, Hijmans, Graham, Moritz, & Wake, 2006; Schierenbeck, 2014). Thus, determining patterns of population structure and occupancy will aid in our interpretation of patterns of trait matching and mismatching.

Lastly, we found substantial variation in prey and predator phenotypes within populations. Intrapopulation variation is most pronounced in the southern watersheds, and includes over half the geographic range of the interaction (from the American River south). Notably, this variation is biologically meaningful, and appears to permit the full range of coevolutionary dynamics in a single population, even at sites where the average snake predator appears to be 'winning' the arms race (Figure 3). How such variation in adaptive traits is maintained within populations remains an open question. Specifically, why are there individual newts with low amounts of TTX and snakes with low TTX resistance in the same (southern) populations that harbour newts and snakes with extreme phenotypes? Presumably natural selection would have culled individuals with lower trait levels out of these populations, simply through generations of encounters with predators or prey possessing more extreme traits. Gene flow from the northern populations is one mechanism that could deliver allelic variation to create the range of phenotypes we observe. However, gene flow alone seems unlikely to connect these populations of small vertebrates across such broad expanses of space. An intriguing possibility is that newts and snakes with extreme trait values experience associated costs and lower fitness outside of the context of the predator–prey interaction (Brodie & Brodie, 1999a, 1999b).

The evidence of a trade-off remains circumstantial in newts, but seems likely. Newts possess specialized structures in their skin

to house and excrete TTX (Hanifin et al., 2004; Mailho-Fontana et al., 2019), and after discharging TTX, it can take months to regenerate TTX (Cardall, Brodie, Brodie, & Hanifin, 2004). Thus, the production, storage and secretion of TTX are likely costly, regardless of whether TTX is the product of endogenous synthesis, bacterial symbionts or bioaccumulation (Hanifin, 2010; Jal & Khora, 2015; Mailho-Fontana et al., 2019). Not surprisingly, newts that are allopatric with garter snakes possess exceptionally low (often undetectable) levels of TTX (Hague et al., 2016; Hanifin et al., 2008; Mebs, Yotsu-Yamashita, Ream, Zajac, & Zehner, 2016). The case for a trade-off in garter snakes is clearer; the most TTX-resistant *Th. sirtalis* display slower crawl speeds (Brodie & Brodie, 1999b; Hague et al., 2018). This performance trade-off is probably the direct result of amino acid replacements in $Na_v1.4$ that not only reduce TTX ligation to the skeletal muscle sodium channel but also impair ion channel function (Feldman et al., 2012; Hague et al., 2018). Understanding the physiological basis of TTX production in newts, and TTX resistance in *Th. couchii* will be necessary to determine the costs associated with these adaptations, and whether countervailing selection puts the brakes on the evolution of extreme phenotypes in this system.

5 | CONCLUSIONS

Our characterization of a phylogenetically independent newt–snake system displaying dramatic prey and predator phenotypes, as well as similar patterns of a latitudinal gradient in phenotypic matching and mismatching, suggests there may be common environmental or ecological determinants that structure the mosaics similar ways in both newt–snake systems. Such parallel phenotypic responses between newts and snakes, particularly the potential 'escape' from the arms race in both *Th. couchii* and *Th. sirtalis*, call for additional work to understand the genetic architecture of resistance in *Th. couchii* and TTX production in species of *Taricha*. Investigating coevolutionary dynamics in these independent predator–prey systems may reveal common mechanisms that lead to parallel responses over space and time, creating some degree of predictability to the geographic mosaic.

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AUTHORS' CONTRIBUTIONS

C.R.F., E.D.B. Jr., E.D.B. III and M.E.P. designed the study; C.R.F., E.D.B. III, E.J.E. and J.S.R. field collected animals; E.D.B. Jr., C.R.F., H.A.M., V.L.T., J.S.R. and A.N.S. generated the data; J.S.R., C.R.F., J.M.H., E.D.B. III and M.E.P. analysed the data; all authors helped interpret the results and draft the manuscript.

DATA AVAILABILITY STATEMENT

We deposited all animals as voucher specimens in museums: herpetology collections of the California Academy Sciences (CAS); University of Texas, Arlington (UTA); University of Nevada, Reno (UNR). Data are available on the Open Science Framework digital repository: https://osf.io/yp9nt/ (Feldman, 2020).

ORCID

Jessica S. Reimche https://orcid.org/0000-0001-6536-7039
Edmund D. Brodie https://orcid.org/0000-0002-5739-4747
Amber N. Stokes https://orcid.org/0000-0001-6935-7794
Erica J. Ely https://orcid.org/0000-0003-2457-0190
Haley A. Moniz https://orcid.org/0000-0003-2838-511X
Vicki L. Thill https://orcid.org/0000-0002-6999-0909
Joshua M. Hallas https://orcid.org/0000-0003-4147-4037
Michael E. Pfrender https://orcid.org/0000-0001-6861-0655
Edmund D. Brodie https://orcid.org/0000-0001-9231-8347
Chris R. Feldman https://orcid.org/0000-0003-2988-3145

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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