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Of poisons and parasites—the defensive role of tetrodotoxin against infections in newts

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Abstract

1. Classical research on animal toxicity has focused on the role of toxins in protection against predators, but recent studies suggest these same compounds can offer a powerful defense against parasites and infectious diseases.
2. Newts in the genus *Taricha* are brightly coloured and contain the potent neurotoxin, tetrodotoxin (TTX), which is hypothesized to have evolved as a defense against vertebrate predators such as garter snakes. However, newt populations often vary dramatically in toxicity, which is only partially explained by predation pressure.
3. The primary aim of this study was to evaluate the relationships between TTX concentration and infection by parasites. By systematically assessing micro- and macroparasite infections among 345 adult newts (sympatric populations of *Taricha granulosa* and *T. torosa*), we detected 18 unique taxa of helminths, fungi, viruses and protozoans.
4. For both newt species, per-host concentrations of TTX, which varied from undetectable to >60 μg/cm^2 skin, negatively predicted overall parasite richness as well as the likelihood of infection by the chytrid fungus, *Batrachochytrium dendrobatidis*, and ranavirus. No such effect was found on infection load among infected hosts. Despite commonly occurring at the same wetlands, *T. torosa* supported higher parasite richness and average infection load than *T. granulosa*. Host body size and sex (females > males) tended to positively predict infection levels in both species. For hosts in which we quantified leucocyte profiles, total white blood cell count correlated positively with both parasite richness and total infection load.
5. By coupling data on host toxicity and infection by a broad range of micro- and macroparasites, these results suggest that—alongside its effects on predators—tetrodotoxin may help protect newts against parasitic infections, highlighting the importance of integrative research on animal chemistry, immunological defenses and natural enemy ecology.

KEYWORDS
amphibian decline, animal toxicity, aposematism, disease ecology, host–parasite interactions, immunity, natural enemy ecology

1 | INTRODUCTION

Numerous animal species endogenously produce toxins or sequester toxins from their diets, which is frequently interpreted as evidence that prey evolves chemical protection against their predators (Brower & Fink, 1985; Daly, 1995). However, many toxins also have negative effects on parasites and pathogens (Cory & Hoover, 2006). For example, monarch butterflies use cardenolides from host plants to deter predators, but these chemicals also provide a potent defense against pathogenic protozoans (Sternberg et al., 2012; Tao, Hoang, Hunter, & Roode, 2016). Thus, alongside more classical forms of immune defenses, host toxicity may represent a complementary strategy to prevent infection or reduce pathology (Otti, Tragust, & Feldhaar, 2014; de Roode, Lefèvre, & Hunter, 2013). Growing evidence indicates that toxins are used against parasites by a wide range of animals, including moths, butterflies, ants and apes (Bos, Sundström, Fuchs, & Freitak, 2015; Huffman, 2003; Lefèvre, Oliver, Hunter, & De Roode, 2010; Singer, Mace, & Bernays, 2009), and the ubiquity of parasitism suggests that it may be influential in the evolution of host toxicity.

Among the amphibians, many taxa of frogs, toads and salamanders have toxic compounds in their skin, including neurotoxins, cardiotoxins and hallucinogens (Colombo, Scalvenzi, Benlamara, & Pollet, 2015; Daly, Spande, & Garraffo, 2005). Poison dart frogs (Dendrobatidae) secrete lipophilic alkaloids that are deadly to many potential predators, while toads (Bufonidae) have bufotoxins in their parotid glands (Clarke, 1997; Jared et al., 2009). The roles of such toxins in limiting parasites and disease are relatively unexplored (Macfoy et al., 2005). Alkaloid-based toxins in the skin of strawberry poison dart frogs (*Oophaga pumilio*) help limit bacterial and fungal infections (Mina, Ponti, Woodcraft, Johnson, & Saporito, 2015). Granular glands in the skin of many amphibians secrete antimicrobial peptides (AMPs) that function as a component of innate immune defense against potential pathogens (Conlon, 2011). Woodhams, Rollins-Smith, et al. (2006) reported that central American and Australian amphibians that naturally produced more potent AMPs were also less affected by exposure to the chytrid fungus, *Batrachochytrium dendrobatidis*, which has been linked to declines of numerous species (Rollins-Smith et al., 2002). Pinto, Pimenta, Antoniazzi, Jared, and Tempone (2013) also found that AMPs isolated from the frog *Phyllomedusa nordestina* were effective against several human parasites.

Tetrodotoxin (TTX) is an exceptionally potent neurotoxin found in taxa ranging from bacteria to pufferfishes (Brodie & Brodie, 1990; Geffeney, Fujimoto, Brodie, & Ruben, 2005; Lorentz, Stokes, Rössler, & Lötters, 2016). By binding to a charged amino acid on the outer pore of sodium channels, TTX prevents the propagation of action potentials through excitable tissues leading to paralysis, respiratory impairment or even death (Brodie & Brodie, 1990). Newts in the genus *Taricha* have especially high concentrations of tetrodotoxin in their skin and eggs (Mosher, Fuhrman, Buchwald, & Fischer, 1964), which is hypothesized to have evolved as a chemical defense against predators (Bucciarelli & Kats, 2015; Hanifin, Yotsu-Yamashita, Yasumoto, Brodie, & Brodie, 1999). Indeed, evidence suggests that TTX deters many predators of adult newts, including fishes, raccoons, birds and snakes (Brodie & Brodie, 1990; Brodie, Ridenhour, Brodie, & Wiens, 2002; Mobley & Stidham, 2000). Although all newts appear to possess TTX, whether they produce the toxin endogenously or through the contributions of bacterial endosymbionts remains an open question (Kotaki & Shimizu, 1993; Lehman & Brodie, 2004). Among newt populations, TTX concentrations vary broadly even across short geographic distances (Hague et al., 2016; Hanifin et al., 1999)—a pattern presumed to be the result of a coevolutionary arms race between newts and TTX-resistant garter snakes (*Thamnophis* spp.) (Brodie & Brodie, 1991; Brodie et al., 2005; Hanifin, Brodie, & Brodie, 2008). However, a functional assessment of resistance and TTX concentrations within newt and snake populations found mismatches in more than one-third of all sampled sites (Hanifin et al., 2008), suggesting that reciprocal selection cannot fully explain variation among newt populations. Whether other natural enemies, such as parasites, contribute to this heterogeneity remains largely unexplored.

The goal of this study was to investigate the relationship between parasitic infection and phenotypic variation in toxicity among two congeneric newt species. We had two specific objectives: (1) to broadly census the diversity of parasites in newts and (2) to evaluate the association between infection and TTX concentrations. In portions of central and northern California, rough-skinned newts (*Taricha granulosa*) and California newts (*T. torosa*) occur sympatrically and often use the same ponds. Relatively little is known about parasitic infections in adult newts or how they differ between species, although past studies have reported infections by several nematodes, trematodes, protozoans and an acanthocephalan (Kuchta, 2005; Marks & Doyle, 2005). Accordingly, we sampled 345 adult newts (*T. torosa* and *T. granulosa*) from breeding ponds and used microscopy, blood smears, DNA-sequencing and qPCR to systematically quantify their micro- and macroparasitic infections, including blood-borne infections (trypanosomes and filarial worms), larval and adult helminths (nematodes, trematodes and acanthocephalans), chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus (Table 1). We then tested how parasite richness, composition and infection load varied between host species and in response to among-host differences in TTX, with the hypothesis that high levels of TTX would inhibit the likelihood of parasite infection or parasite load.

2 | MATERIALS AND METHODS

2.1 | Field sampling

Between May and June of 2015, we collected adult newts from 42 ponds in the eastern San Francisco Bay Area region of California (Contra Costa and Alameda counties). Ponds were distributed across watershed lands, publicly accessible parks and biological preserves (Pleasanton Ridge Regional Park, Garin/Dry Creek Regional Park, Five Canyons Regional Park, Vargas Regional Park, Briones Regional Park, East Bay Municipal Utility District). This portion of the Bay Area is a zone of sympatry for *Taricha*, representing the southern boundary of the rough-skinned newt, *T. granulosa*, and within the northern range of the California newt, *T. torosa*, which continues northward to Mendocino County (Stebbins & McGinnis, 2012). At each site, we

TABLE 1 Patterns of micro- and macroparasite infections in adult California newts (*Taricha torosa*, *n* = 182) and rough-skinned newts (*T. granulosa*, *n* = 163) from wetlands in California. Presented for each parasite taxon is the life stage found in newts, its primary infection location and mode of entry into the host. For each of the two newt host species, we also indicate the proportion of sites that supported a given parasite, the average infection prevalence at sites with infection (i.e. omitting sites without the parasite) and the average infection load ± 1 *SE* and range of infection values among animals from sites where the parasite was detected in at least one host (including uninfected individuals)

				Taricha torosa			*Taricha granulosa*		
	Stage	Location	Mode of entry	Sites with infection	Average prevalence (%)	Average load ± 1 *SE* (range)	Sites with infection	Average prevalence (%)	Average load ± 1 *SE* (range)
Acanthocephalan									
Unidentified larval acanthocephalan	Larval	Intestine	Trophic	4/36	2.29	0.29 ± 0.17 (0–5)	—	—	—
Digenea									
Clinostomum spp.	Metacercaria	Dermis, subcutaneous, body cavity	Penetrate	6/36	4.95	0.25 ± 0.86 (0–3)	7/25	20.25	1.16 ± 0.23 (0–11)
Ribeiroia ondatrae	Metacercaria	Subcutaneous	Penetrate	1/36	0.55	0.14 ± 0.14 (0–1)	1/25	1.84	0.05 ± 0.07 (0–1)
Nematodes									
Microfilaria	Adult	Plasma	Diet	1/20	0.55	0.14 ± .014 (0–1)	2/18	1.23	0.11 ± 0.08 (0–2)
Chabaudgolvania sp.	Adult	Small intestine	Trophic	10/36	9.34	0.50 ± 0.12 (0–4)	7/25	12.27	1.78 ± 0.56 (0–27)
Rhabdias tarichae	Adult	Lung	Penetrate	28/36	47.25	11.85 ± 4.83 (0–727)	13/25	11.66	3.23 ± 2.76 (0–282)
Cosmocercodies variabilis	Adult	Small intestine	Diet	17/36	31.32	34.60 ± 19.16 (0–2160)	4/25	3.68	1.96 ± 1.67 (0–88)
Unidentified larval nematode	Larval	Small intestine	Diet	3/36	1.65	0.42 ± 0.23 (0–2)	6/25	4.29	0.24 ± 0.11 (0–7)
Protozoan									
Eimeria tarichae	Adult	Small intestine	Diet	3/36	6.59		3/25	6.78	
Tritrichomonas augusta	Adult	Small intestine	Diet	6/36	3.30		6/25	5.52	
Nyctotherus cordiformis	Adult	Rectum	Diet	—	—		1/25	0.61	
Opalina ranarum	Adult	Rectum	Diet	—	—		1/25	0.61	
Trypanosoma granulosae	Adult	Plasma	Vector-borne	—	—		2/18	2.45	0.39 ± 0.19 (0–4)
Trypanosoma barbari	Adult	Plasma	Vector-borne	1/20	0.55	0.43 ± 43 (0–3)	—	—	—
Lankesterella spp.	Adult	Erythrocyte	Vector-borne	—	—	—	3/18	1.84	0.27 ± 0.15 (0–2)
Balantidium spp.	Adult	Rectum	Diet	6/36	3.85		6/25	20.86	
Virus									
Ranavirus		Epidermis	Contact	12/36	9.89		6/25	17.79	
Fungus									
Batrachochytrium dendrobatidis	Sporangium	Epidermis	Contact	18/36	21.00		12/25	4.91	

used visual encounter surveys, standardized dipnet sweeps and 3–4 habitat-stratified seine hauls to collect adult newts, of which a subset (~10) were collected for further examination. Individuals of both species often co-occur in the same system, and we identified them to species based on the position of the eyes (from ventral view, the eyes of *T. granulosa* do not extend past the margin of the head whereas those of *T. torosa* do) and coloration of the lower eyelids (those of *T. torosa* are light in colour whereas those of *T. granulosa* are darker) (Marks & Doyle, 2005; Powell, Collins, Hooper, & Powell, 2012). Immediately after collection, adult newts were sampled for *B. dendrobatidis* (Bd) using a sterile swab to gently swab the chin, forelimbs, abdomen, hindlimbs and cloaca followed by qPCR (Briggs, Knapp, & Vredenburg, 2010).

2.2 | Tetrodotoxin quantification

From each collected newt, we used a skin biopsy punch (AcuPunch™, Acuderm Inc.) to remove a 5-mm-diameter disc of skin from the dorsal surface to the right of the pelvic girdle (Hanifin, Brodie, & Brodie, 2002). This region of skin has a more uniform distribution of skin glands and shows little within-individual variation (Hanifin et al., 2002). Skin biopsies were ground by hand in 600 μl of 0.1 M acetic acid before extracting TTX following Hanifin et al. (2002). We quantified TTX ng/cm^2 of skin using a Competitive Inhibition Enzymatic Immunoassay (CIEIA) procedure, which provides a highly repeatable, sensitive and accurate means of quantifying TTX (see Stokes, Williams, & French, 2012). Standards were prepared using 1 mg TTX lyophilized in citrate buffer (Abcam; 120055), diluted within the linear range of the standard curve (10 and 500 ng/ml) in 1% bovine serum albumin (BSA; Sigma; A7906-50G) diluted in phosphate-buffered saline (PBS). Values below 10 ng/ml are below detectable limits and treated as zero. Newt samples that fell outside of the standard curve were further diluted until within the appropriate range. The average interplate coefficient of variation was 10.9%.

2.3 | Parasite assessment

Following removal of the skin punch, we necropsied newts to systematically identify parasites and quantify their abundance. We measured the snout-vent length of each individual using digital calipers. All major organs and tissues (e.g. heart, liver, intestines, rectum, stomach, tongue, mandible, kidneys and fat bodies) were examined for macroparasites under an Olympus SZX10 stereodissecting microscope. Isolated live parasites were counted and identified using Schell (1985), Sleigh (1991), Gibson, Jones, and Bray (2002), Duszynski, Bolek, and Upton (2007), and Anderson, Chabaud, and Willmott (2009). We closely inspected the body cavity, muscles and skin for larval parasites, such as encysted helminths. A subsample of liver and kidney tissue from each host was frozen to test for ranavirus infection using qPCR (see below).

2.4 | Quantitative PCR analysis

We used standardized protocols to extract and quantify DNA from Bd and ranavirus (Rv) (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004; Forson & Storfer, 2006; Pallister et al., 2007). For Bd, swabs were considered positive if they amplified in two of three runs, which were conducted alongside an internal positive control (IPC). For Rv, each pPCR run included a standard curve (log-based dilution series of 4.014×10^9 viral copies/μl to 4.014×10^6 viral copies/μl) and negative control (water). The standard was a synthetic double-stranded 250-bp fragment of the major capsid protein (MCP) gene (gBlocks Gene Fragments; Integrated DNA Technologies), which is conserved among ranaviruses (Forson & Storfer, 2006). All samples were run in duplicate. The concentration of genomic DNA (ng of DNA/μl) was measured using a NanoDrop 2000c (Thermo Scientific) (Pochini & Hoverman, 2016).

2.5 | DNA sequence analysis

To confirm morphological identifications and verify conspecificity of lung nematodes parasitic in two newt species examined in this study, genomic DNA was extracted from six specimens of *Rhabdias* obtained from *T. torosa* and *T. granulosa* according to Tkach and Pawlowski (1999). Two DNA regions were targeted as follows: (1) a fragment spanning the 3′ end of nuclear 18S ribosomal DNA (rDNA) gene, internal transcribed spacer (ITS) ITS1, 5.8S rDNA, ITS2 and 5′ end of the 28S rDNA gene (including variable domains D1–D3), and (2) partial mitochondrial cytochrome oxidase 1 (CO1) gene. Nuclear ribosomal DNA fragment was amplified using Rhabdiasidae-specific forward primer ritf (5′-GCGGCTTA ATTTGACTCAACACGG-3′) and universal reverse primer 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) and annealing temperature of 53°C. Both PCR primers and internal primer 300R (5′-CAACTTTCCCTCACGGTACTTG-3′) were used for sequencing (Tkach, Kuzmin, & Snyder, 2014). CO1 region was amplified using forward primer LCO1490 (5′-GGTCAACAAATC ATAAAGATATTGG-3′) and reverse primer HCO2198 (5′-TAAAC TTCAGGGTGACCAAAAAATCA-3′) with annealing temperature 45°C; the same primers were used for sequencing. PCR products were purified using Exo-Sap IT PCR Product Clean-up kit from Affymetrix (USA) and sequenced directly on an ABI Prism 3100™ automated capillary sequencer using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) according to the manufacturer's protocols. Contiguous sequences were assembled using Sequencher™ ver. 4.1.1 (GeneCodes Corp., USA) and submitted to GenBank under accession numbers MH023521-MH023523 (rDNA sequences) and MH021878-MH021883 (CO1 sequences). Sequences were aligned and compared using BIOEDIT software, version 7.0.1 (Tkach et al., 2014).

2.6 | Blood collection and examination

We used a 25-gauge needle with a heparinized syringe to collect a 0.5–1 ml sample of cardiac blood from a subset of hosts for examination of blood parasites. We made two blood smears for each animal: one for parasite examination and one for both parasite examination and quantification of white blood cells (leucocyte profile).

We stained slides with Dip Quick Stain (JorVet J-322) and examined them using a BX41 Olympus microscope under 1000× magnification. Following LaFonte and Johnson (2013) and Calhoun, Schaffer, Gregory, Hardy, and Johnson (2015), we quantified the number of white blood cells (WBCs; neutrophils, eosinophils, lymphocytes, basophils and monocytes) encountered across a sample of ~2,000 erythrocytes. We identified intracellular and extracellular parasites using the taxonomic keys in Hadji-Azimi, Coosemans, and Canicatti (1987) and additional sources (Allender & Fry, 2008; Clarke, 1997; Sailasuta, Satetasit, & Chutmongkonkul, 2011).

2.7 | Analysis

We used generalized linear- and generalized linear mixed models (GLM and GLMM) to evaluate differences in parasite infection as a function of host species, host body size and TTX concentration. We calculated per-host parasite richness as the count of unique parasite taxa (micro- and macroparasites), which was modelled as a Poisson distribution with a log link. We did not include blood-borne infections in this analysis owing to the lower sample size of examined hosts (345 vs. 116). Total parasite load (summed among macroparasite taxa) was modelled as an overdispersed Poisson distribution by including an observation-level random effect, which functionally approximates a negative binomial response (Elston, Moss, Boulinier, Arrowsmith, & Lambin, 2001; Harrison, 2014). For the microparasites Rv and Bd, we evaluated variables that affected their prevalence (yes or no, as a Bernoulli distribution with complementary log–log link function). Because these infections were not detected at all wetlands, we performed these analyses both using all sampled sites (including wetlands at which the parasite was not detected, for which wetland identity was a random intercept term) and by restricting the analysis only to sites at which at least one host tested positive. We performed the analyses both ways because omitting sites without infection ignores the potential for TTX to inhibit transmission and lower the likelihood of detection, particularly in cases when the number of sampled hosts is low.

Across all models, we included fixed effects for host species, sex, body size (snout-vent length; mm) and TTX concentration ($\log_{10}$[x+1]-transformed), and random intercept term for wetland identity to account for autocorrelation among hosts collected from the same site. All continuous predictors were scaled prior to inclusion to obtain comparable coefficients. For the subset of hosts on which blood samples were collected, we evaluated how the proportion of white blood cells (WBCs divided by the total number of red blood cells [RBCs] and then arcsine-square-root-transformed), host species, sex, body size and TTX influenced parasite taxonomic richness (here including blood-borne parasites) and total parasite load. Models were fit using lme4 and lmerTest in the R computing environment (Bates, Maechler, Bolker, & Walker, 2013; Bolker, Skaug, Magnusson, & Nielsen, 2012). For all models, we present the sample sizes, model structures, standardized coefficient estimates ± 1 *SE* and *p*-values in Table S1.

3 | RESULTS

In total, we sampled 345 adult newts from 42 wetlands (182 *T. torosa* and 163 *T. granulosa*). The number of individuals collected per site ranged from 1 to 27 ($M \pm 1\ SE = 7.84 \pm 1.02$), with both species co-occurring at 17 sites (38.6%). Most examined individuals were male (57.7% *T. torosa*, 57.1% *T. granulosa*). On average, *T. granulosa* were significantly larger in SVL than *T. torosa*, while males were larger than females for both species (GLMM with Gaussian distribution, species[TATO]: −2.0358 ± 0.82, $p = .014$; sex[male]: 4.196 ± 0.764, $p < .00001$; $n = 345$ newts). Across both species, TTX concentrations varied from below detectable limits (~0) to 61.14 μg/cm^2. TTX concentrations were higher in *T. granulosa* ($M \pm 1\ SE$: 7.64 ± 0.85) relative to *T. torosa* (5.80 ± 0.49) and in females relative to males, with a marginally positive effect of body size (GLMM with Gamma distribution and log link, species[TATO]: −0.233 ± 0.11, $p = .03$; scaled[SVL]: 0.10 ± 0.051, $p = .057$; sex[male]: −0.214 ± 0.11, $p = .04$; $n = 300$ newts; Figure 1). The variance associated with the random effect of pond identity was 0.234, while the residual variance was 0.807, suggesting that ~22.5% of the variance in TTX between the two species (the fixed-effect) was associated with site-level differences.

3.1 | Parasite assessment

We detected 18 unique parasite taxa, including one acanthocephalan, two trematodes, five nematodes, eight protists, one virus and one chytrid fungus (Table 1). We found at least one infection in 79% of hosts and 95% of wetlands. The most common infections were *Rhabdias tarichae* (28.6% of sampled hosts), *Batrachochytrium dendrobatidis* (21.9%), *Cosmocercodies variabilis* (18.1%), *Balantidium* sp. (17.6%), *Chabaudgolvania* sp. (11.0%), *Clinostomum* spp. (11.0%) and ranavirus (7.4%). Comparison of a 1595-base pair fragment of nuclear rDNA indicated that sequences of *Rhabdias tarichae* among sites and between newt species were identical, while 557-bp sequences of the CO1 gene showed only 0.8%–1.1% divergence. The highest infection loads were associated with the nematodes *R. tarichae* and *C. variabilis*, for which worms per host reached as high as 727 and 2,160, respectively. On average, individuals of *T. torosa* supported higher parasite richness relative to *T. granulosa*, with no added effects of body size or sex (GLMM with Poisson distribution, species[TATO]: 0.407 ± 0.114, $p = .0004$; scaled[SVL]: 0.047 ± 0.054, $p = .379$; sex[male]: 0.012 ± 0.104, $p = .912$; $n = 345$ newts; Figure 2a). Across all sites, we recorded 14 parasite taxa from *T. torosa* and 16 from *T. granulosa*, with mostly shared distributions. The acanthocephalan parasite was unique to *T. torosa* (four different sites), whereas all three records of the apicomplexan *Lankesterella* spp. and isolated observations of the protozoans *Opalina ranarum* and *Nyctotherus cordiformis* were from *T. granulosa* (Table 1).

Taricha torosa also supported a higher total abundance of macroparasites relative to *T. granulosa*, with additional effects of body size and host sex (GLMM with overdispersed Poisson distribution, host species[TATO]: 1.783 ± 0.316, $p < .0001$; scaled[SVL]:

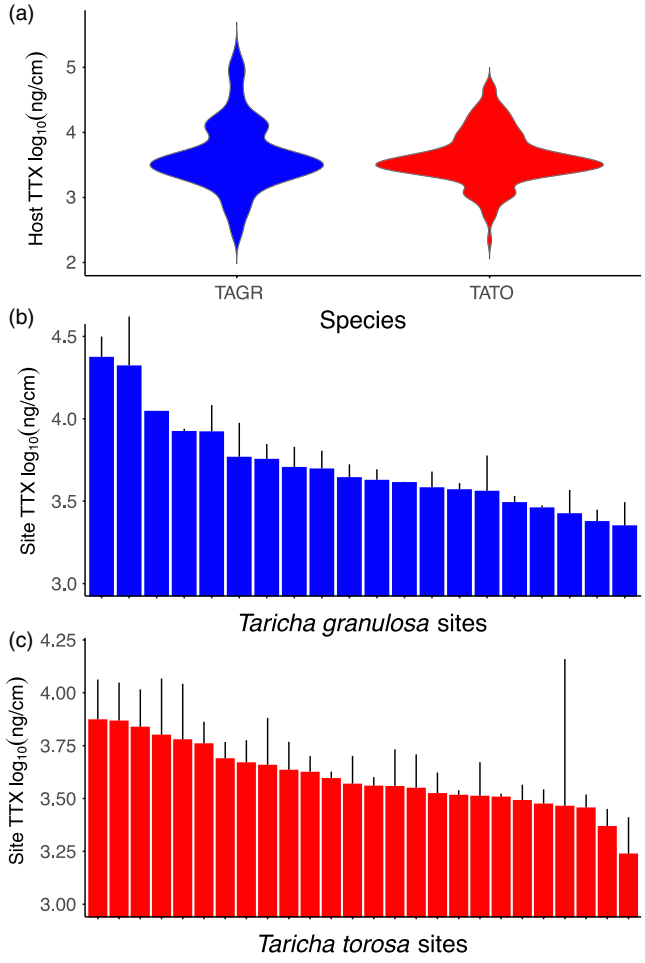

FIGURE 1 Distribution of tetrodotoxin concentrations ($\log_{10}$(ng/cm^2)) in adult newts (*Taricha torosa* [TATO] and *T. granulosa* [TAGR]). (a) Violin plots illustrating variation in TTX concentrations among individuals of each species, for which the position along the *y*-axis indicates the TTX concentration, while the width of the distribution reflects the frequency of individuals corresponding to a particular value. We controlled for site-level variation by adjusting each individual newt's TTX concentration by the pond-level random effect and then plotting the corrected values. Sample sizes: *T. torosa* (*n* = 182), *T. granulosa* (*n* = 163). Variation in site-level TTX concentrations across sites with (b) *T. granulosa* (*n* = 20) and (c) *T. torosa* (*n* = 26); heights of the bars represent the site-level mean TTX concentration + 1 *SE* [Colour figure can be viewed at wileyonlinelibrary.com]

0.541 ± 0.145, *p* = .0001; sex[male]: −0.219 ± 0.282, *p* = .436; *n* = 345 newts; Figure 2b). This pattern was driven primarily by the nematodes *R. tarichae* and *C. variabilis*, both of which had higher average loads in *T. torosa* than in *T. granulosa* (Table 1), whereas other helminth taxa were similarly abundant between the two newt species (e.g. *Clinostomum* spp. and *Chabaudgolvania* sp., see Table 1). For microparasitic infections, *T. torosa* was marginally more likely to be infected with ranavirus relative to *T. granulosa* (GLMM with binomial distribution, species[TATO]: 0.758 ± 0.429, *p* = .077; scale[SVL]: 0.148 ± 0.209, *p* = .479; sex[male]: 0.454 ± 0.44, *p* = .303; *n* = 344 newts), whereas Bd was negatively related to host size with no effects of sex or species (species[TATO]: 0.119 ± 0.295, *p* = .685; scale[SVL]: −0.498 ± 0.153, *p* = .001; sex-[male]: −0.040 ± 0.289, *p* = .888; *n* = 277 newts). Constraining the analysis only to wetlands at which at least one host was infected with each parasite, Rv infection was marginally associated only with host sex (species[TATO]: 0.359 ± 0.466, *p* = .44; scale[SVL]: 0.123 ± 0.225, *p* = .583; sex[male]: 1.107 ± 0.577, *p* = .055; *n* = 160 newts), whereas Bd continued to exhibit a main effect for host body size only (species[TATO]: 0.399 ± 0.299, *p* = .181; scale[SVL]: −0.402 ± 0.163, *p* = .0135; sex[male]: 0.264 ± 0.297, *p* = .889; *n* = 207 newts).

3.2 | Effects of TTX on infection

TTX concentration correlated negatively with parasite richness per host, with an additional effect of host species (TATO > TAGR) but no influence of host sex or size (GLMM with Poisson distribution, host species[TATO]: 0.332 ± 0.123, *p* = .007; $\log_{10}$[TTX+1]: −0.119 ± 0.056, *p* = .034; scale[SVL]: 0.039 ± 0.059, *p* = .516; sex[male]: 0.04 ± 0.116, *p* = .729; *n* = 300 newts) (Figure 3a). For Bd and ranavirus, TTX correlated negatively with the likelihood of infection (Figure 3b,c): the model for Bd included negative coefficients for TTX and body size (GLM with binomial distribution, $\log_{10}$[TTX+1]: −0.337 ± 0.144, *p* = .019; host species[TATO]: 0.109 ± 0.299, *p* = .715; scale[SVL]: −0.456 ± 0.157, *p* = .0035; sex[male]: −0.157 ± 0.295, *p* = .594; *n* = 277 newts). Similarly, ranavirus infection was negatively associated TTX ($\log_{10}$[TTX+1]: −0.464 ± 0.221, *p* = .035; host species[TATO]: 0.557 ± 0.449, *p* = .215; scale[SVL]: 0.355 ± 0.234, *p* = .129; sex[male]: 0.596 ± 0.535, *p* = .264; *n* = 299 newts). Restricting the dataset to wetlands at which at least one host was infected with the parasite caused the effect of TTX on ranavirus to become marginally significant ($\log_{10}$[TTX+1]: −0.368 ± 0.208, *p* = .076; host species[TATO]: 0.337 ± 0.466, *p* = .469; scale[SVL]: 0.237 ± 0.235, *p* = .313; sex[male]: 0.90 ± 0.59, *p* = .127; *n* = 160 newts), while having little change in the results for Bd infection ($\log_{10}$[TTX+1]: −0.324 ± 0.143, *p* = .023; host species[TATO]: 0.375 ± 0.304, *p* = .217; scale[SVL]: −0.345 ± 0.167, *p* = .039; sex[male]: 0.115 ± 0.307, *p* = .708; *n* = 207 newts) (see Table S1). There was no correlation between TTX and total infection load by helminths, despite persistent differences between the two host species (GLMM with negative binomial distribution, host species[TATO]: 1.524 ± 0.346, *p* < .0001; $\log_{10}$[TTX+1]: −0.109 ± 0.156, *p* = .484; scale[SVL]: 0.572 ± 0.163, *p* = .0005; sex[male]: −0.081 ± 0.321, *p* = .800; *n* = 300 newts).

3.3 | Blood parasites and leucocyte profiles

Among the 116 hosts with blood samples, blood-borne infections were rare (*n* = 10 infected hosts, or 8.6%). Five hosts were infected with trypanosomes (likely *Trypanosoma barbari* and *T. granulosae*), two hosts were infected with apicomplexans (*Lankesterella* spp.), two were infected with microfilaria and one host was co-infected with *Lankesterella* spp. and microfilaria. Eight of the 10 infected hosts were *T. granulosa*. The total count of white blood cells (WBCs) correlated

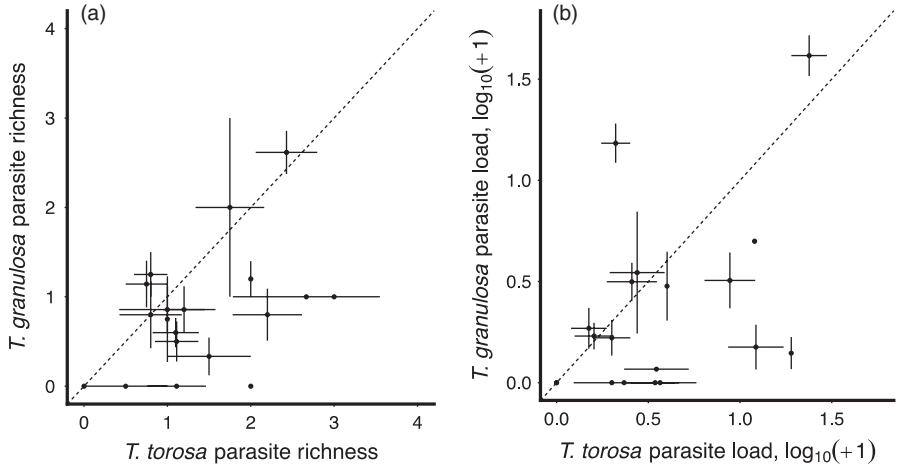

FIGURE 2 Comparisons of parasite infection among adult newts (*Taricha torosa* and *T. granulosa*) within the Bay Area region of California for ponds where both newt species co-occurred. (a) Average parasite richness per individual host, including micro- and macroparasites. (b) Total macroparasite load per host ($\log_{10}$+1-transformed). For both plots, the blacked dotted line is the 1:1 relationship expected if both newt species had equivalent parasite richness or load. Error bars represent ± 1 *SE* in each direction. Only infections detected in both host species and found in at least 5% of sampled hosts were included here

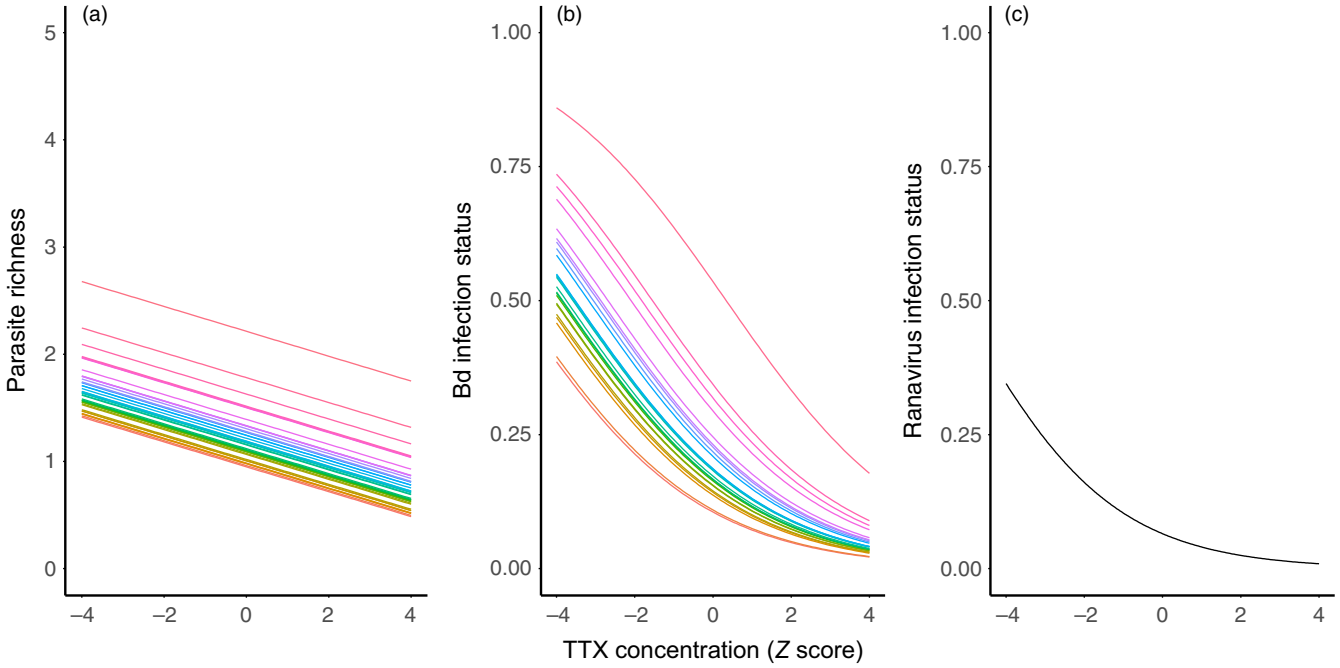

FIGURE 3 Associations between tetrodotoxin concentration per newt and patterns of (a) total parasites richness, (b) infection by the chytrid fungus, *Batrachochytrium dendrobatidis* [Bd] and (c) infection by ranavirus (Rv). Tetrodotoxin concentrations (ng/cm^2) were $\log_{10}$-transformed + 1, scaled and centred. Parasite richness includes all surveyed micro- and macroparasite species, whereas Bd and ranavirus prevalence are the number of infected hosts relative to the total number of surveyed individuals. Lines represent the best-fit relationships between TTX and parasite richness or infection prevalence at each sampled site (i.e. the random intercept terms for wetland identity). For ranavirus, the site-level random effect was effectively zero, leaving only a single best-fit relationship [Colour figure can be viewed at wileyonlinelibrary.com]

positively with both parasite richness (here including blood-borne infections) (GLMM with a Poisson distribution, WBC/RBC: 0.233 ± 0.085, p = .006; host species[TATO]: 0.238 ± 0.166, p = .152; $\log_{10}$[TTX+1]: −0.087 ± 0.087, p = .319; scale[SVL]: 0.045 ± 0.085, p = .599; sex[male]: 0.127 ± 0.177, p = .472; n = 116 newts) and total parasite load (sum of all macroparasitic infections) (GLMM with overdispersed Poisson distribution, WBC/RBC: 0.594 ± 0.273, p = .029; host species[TATO]: 1.735 ± 0.612, p = .0046; $\log_{10}$[TTX+1]: 0.105 ± 0.259, p = .686; scale[SVL]: 0.473 ± 0.263, p = .073; sex[male]: 0.585 ± 0.532, p = .271; n = 116 newts).

4 | DISCUSSION

While investigations of host–parasite interactions and predator–prey dynamics have evolved largely independently, an emerging focus in ecology seeks to unite studies on "natural enemies" into a more cohesive construct (Lafferty & Kuris, 2002; Raffel, Martin, & Rohr, 2008). For instance, although chemical defenses are a common component of vertebrate and invertebrate immunity, ecological research on organismal toxicity has focused largely on predator–prey interactions. Recent studies suggest that many toxins studied for their effects against predators also offer a powerful defense against parasites and diseases (Otti et al., 2014; de Roode et al., 2013). Results of the current study offer insight into the potential effects of tetrodotoxin—a potent neurotoxin found in newts—on parasitic infections. By assessing the micro- and macroparasitic infections of >300 newts, we found that individual TTX concentration correlated negatively with total parasite richness, infection by two virulent microparasites (Bd and ranavirus) and infection load of the nematode *Cosmocercoides variabilis*. For instance, an individual host was 0.42× as likely to be infected with Bd and 0.35× as likely to be infected with ranavirus for each 10 ng increase in TTX. These effects were evident in both newt species and while accounting for other variables likely to influence parasite exposure, including host traits (e.g. size and sex), location and sampling date.

Given the correlational nature of the reported relationships, multiple mechanistic explanations could contribute to the observed association between host toxicity and infection, either individually or in combination. For instance, healthier newts with greater vigour may exhibit both a more effective immunological defense against infection and higher concentrations or production of TTX. This might occur if resource availability jointly limits both toxicity and immune function (Parker, Barribeau, Laughton, de Roode, & Gerardo, 2011). Bucciarelli, Shaffer, Green, and Kats (2017) provided evidence that at least a portion of the TTX response by larval and adult *Taricha* is inducible. Newts exposed to sustained stressful conditions in the form of simulated predator attacks exhibited increased toxicity over time. Both in this study and in the previous work (e.g. Hanifin et al., 2002), toxicity also tended to increase with time spent in captivity. If forms of environmental stress are associated with a reduction in parasite infection risk to newts, for instance by lowering the abundance or infectivity of infectious stages or intermediate hosts (Lafferty & Holt, 2003), newt toxicity and infection could show negative covariance. Alternatively or additionally, high levels of infection could lead to downregulation in the synthesis of defensive compounds. Many parasites and viruses are known to manipulate their hosts, in some cases causing a weakened immune response (Cecílio et al., 2014; Crow, Lum, Sheng, Song, & Cristea, 2016; Pollard, Knoll, & Mordue, 2009) and raising the possibility that infection could interfere with induction of antiparasitic toxins. In general, understanding the influence of these pathways remains limited by an incomplete understanding of how newts produce TTX, including the potential role of endosymbionts as well as its physiological costs (e.g. Bucciarelli et al., 2017).

Finally, alongside its effects on predators, TTX could protect newt hosts against parasitism more directly. In general, infections of the skin—which is where TTX is often most concentrated (Mosher et al., 1964; Brodie & Brodie, 1999)—were rare compared with parasites in the intestinal lumen or within internal organs. For instance, larval trematodes such as *R. ondatrae* that encyst in the skin were encountered in <2% of hosts, despite being common among larval newts and other amphibians that develop in these ponds (Johnson & Wilber, 2017). Consistent with these field-based observations, Calhoun, Bucciarelli, Kats, Zimmer, and Johnson (2017) recently showed that five species of trematodes exhibited dose-dependent increases in mortality with exposure to TTX. By comparision, aquatic macroinvertebrates that frequently co-occur wiht newts showed little response to exogenous TTX over the same time peroid (Calhoun et al., 2017). However, whether such effects extend to other parasite taxa, including nematodes, fungi and viruses, is unknown. In a study of 30 red-spotted newts (*Notophthalmus viridescens*), for instance, Mebs, Yotsu-Yamashita, Seitz, and Arakawa (2012) reported no negative relationship between infection by intestinal nematodes and TTX concentration, and some evolutionary evidence suggests that nematodes have lost their voltage-gated sodium channels (Bargmann, 1998)—which are often considered the primary target of TTX toxicity. Because the exact pathways through which newts produce TTX also remain the subject of debate (e.g. via endosymbionts or endogenous synthesis; Kotaki & Shimizu, 1993; Lehman & Brodie, 2004), it is difficult to differentiate among these possibilities, particularly in the absence of experimental data.

Interestingly, TTX concentrations in the current study also correlated negatively with infection by acellular or single-celled microparasites, such as ranavirus and Bd. While both parasites likely encounter TTX (Bd occurs primarily in the keratinized skin of amphibians while ranavirus occurs in internal organs such as the liver and kidney), the mechanism underlying this link and whether it was causal is unclear. TTX is traditionally thought to operate on sodium channels within the animal nervous system, which are notably absent in fungi and viruses. Ca^{2+} channel precursors have been identified in both fungi and prokaryotes, and homologues of the ancestral ionotropic purinoceptors protein, P2X, occur in Bd (Verkhratsky & Parpura, 2014). Cation channels have also been found in viruses (Moran, Barzilai, Liebeskind, & Zakon, 2015). A wide variety of toxins, including pyrrolizidine alkaloids, polybrominated diphenyl ethers and iridoid glycosides are biologically active against highly diverged eukaryotes, including vertebrates, bacteria, fungi and protozoans (Modaressi et al., 2009; Neto et al., 2016; Zhang, Skildum, Stromquist, Rose-Hellekant, & Chang, 2008), and it is becoming increasingly clear that these toxic effects are mediated through different pathways. For example, the toxic effects of cardiac glycosides on animals and apicomplexan parasites likely stem from interference with different types of ATPases (Gowler, Leon, Hunter, & de Roode, 2015; Petschenka et al., 2013; Spillman & Kirk, 2015). Importantly, studies have shown inhibition of herpes simplex virus replication by a neurotoxin isolated from cobra venom (Yourist, Haines, & Miller,

1983), demonstrating that chemicals that are traditionally known for their toxic effects on vertebrate neurons can also inhibit the growth of neuron-free viruses.

The effect of animal toxicity on infections relates closely to research on innate immunity in hosts. Granular glands in the skin of many amphibians secrete antimicrobial peptides (AMPs) (Becker & Harris, 2010; Conlon, Halverson, Dulka, Platz, & Knoop, 1999), which are an important component of host defense against microparasites (Woodhams, Voyles, Lips, Carey, & Rollins-Smith, 2006). However, relatively little is known about how (non-protein) skin secretions of amphibians affect parasitic infections. Tree frogs in the genus *Hyla*, many of which have noxious compounds associated with their skin (Conlon, 2011; Prates et al., 2004), exhibit high resistance to the trematode *Ribeiroia ondatrae*. In experimental exposure trials, LaFonte and Johnson (2013) found that larvae of *Hyla versicolor*, *H. cinerea* and *H. gratiosa* cleared infections within 72 hr, which is 10× faster than in 11 other species of frogs, toads and salamanders (Johnson & Hartson, 2009; Johnson et al., 2012). Although the mechanism underlying this resistance is unknown, it was not driven by differences in host antimicrobial peptides (Calhoun et al., 2016), and immune suppression (via corticosterone) caused only minor increases in infection (LaFonte & Johnson, 2013). An important priority will thus involve identifying the mechanistic pathways through which these and other skin secretions—including TTX—affect parasite infection success and persistence.

Using multilevel modelling approaches, our results also provided new information on the parasite communities of pond-breeding newts and how they vary with ecological variables such as host species, body size and sex while accounting for wetland identity. Compared with studies on the parasite communities of frogs and toads, relatively few studies have examined parasites from salamanders generally and from species of *Taricha* specifically (Aho, 1990; Kuchta, 2005; Prudhoe & Bray, 1982). Consistent with previous research on helminth infections in salamanders (Goater, Esch, & Bush, 1987; Goldberg, Bursey, & Cheam, 1998; Ingles, 1936), per-host parasite richness and intensity were relatively low. In a study of adult *T. torosa* from southern California, for instance, Goldberg et al. (1998) reported infection by four nematode species and characterized the parasite community as "depauperate" (see also Ingles, 1936). Across both newt species, host size tended to positively influence infection or load, as also reported for a range of parasite taxa (Poulin, 2007). Such results could be due to the effects of body size on parasite encounter (including foraging rate for trophically acquired infections) or the influence of age on parasite accumulation, especially given the tendency of amphibians to exhibit indeterminate growth. For Bd, host body size correlated negatively with infection, which has been previously suggested to reflect maturation of immunity with age (Kriger, Pereoglou, & Hero, 2007). Female newts also had higher macroparasite infection loads than males, even after accounting for body size, which might owe to differences in behaviour, diet or reproductive physiology (Grayson, De Lisle, Jackson, Black, & Crespi, 2012).

However, the greatest differences were at the species level: *T. torosa* had higher parasite richness and greater infection loads relative to *T. granulosa*, even when the two host species co-occurred around the same wetland. Although this difference may have been influenced by the greater TTX levels in *T. granulosa* (~1.3× higher), the effect persisted when TTX concentration was incorporated as a covariate, raising intriguing questions about ecological or immunological variation between these two species. This pattern could stem from differences either in parasite exposure or in infection success and persistence between the two newts. While larval newts of both species frequently co-occur in the same wetlands and microhabitats, outside of the breeding season adult *T. torosa* are typically associated with more forested habitats relative to *T. granulosa* (Pimentel, 1960), which could expose them to different parasite types or intensities. *Taricha granulosa* is also considered the most aquatic of the *Taricha* spp. (Twitty, 1942) and may remain aquatic all year around in California (Packer, 1961). In addition, *T. granulosa* within these counties specifically are at the southern end of their range and occur in far fewer wetlands (and usually at lower abundance) relative to *T. torosa* (Marks & Doyle, 2005; Stebbins, 1985). Local adaptation by parasites could, therefore, favour higher infection success in the more widespread and abundant host (*T. torosa*) (Imhoof & Schmid-Hempel, 1998; Pérez-Tris & Bensch, 2005), although we cannot rule out the potential for differential immune responses between the two hosts.

The synthetic field of natural enemy ecology aims to understand concurrent threats faced by animal populations, including predation and parasitism. In some cases, defenses against one threat may trade-off against heightened risk for another (Marino & Werner, 2013); for instance, *Daphnia* that migrate diurnally to deeper waters to avoid fish may be exposed to more infection from the sediment ("spore bank") (Decaestecker, De Meester, & Ebert, 2002). In others, physiological or behavioural strategies may help mitigate multiple threats, such as the use of endogenously produced or acquired toxins against both predators and parasites. Greater integration of natural enemy research may also help address unresolved questions regarding the evolutionary origins for such defenses. Because predation attempts frequently result in prey death, even for highly toxic animals, it remains enigmatic how toxicity and warning coloration evolved initially and became common enough to be effective (Mappes, Marples, & Endler, 2005; Speed, Ruxton, & Pellmyr, 2005). One potential solution to this problem is that prey initially evolved toxicity as a defense against parasites, after which it was co-opted for protection against predators (Santos, Coloma, & Cannatella, 2003). An exciting frontier for future research will therefore involve efforts to determine the relative roles of parasites and predators in driving variation in animal toxicity, including tetrodotoxin in newts. Even if the TTX–infection association is not causal, it could nonetheless offer valuable insight for understanding the functional determinants of heterogeneity in host infection and contributions to population-level transmission, which remains a core challenge in disease ecology.

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AUTHORS' CONTRIBUTIONS

P.T.J.J. and D.M.C. designed the study; A.N.S. and C.B.S. quantified tetrodotoxin; D.M.C., J.T.H., C.J.B. and V.V.T. collected or processed parasite samples; T.M.G. conducted fieldwork; P.T.J.J. and T.M.G. performed statistical analyses; P.T.J.J. wrote an initial draft of the manuscript and all authors contributed to the revisions.

DATA ACCESSIBILITY

Summaries of the data collected as part of this project are available in Table 1 and on Dryad Digital Repository: https://doi.org/10.5061/dryad.61c6n05 (Johnson et al., 2018).

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