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SHORT COMMUNICATION

Tetrodotoxin affects survival probability of rough-skinned newts (*Taricha granulosa*) faced with TTX-resistant garter snake predators (*Thamnophis sirtalis*)

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Abstract Lethal chemical defenses in prey species can have profound effects on interactions with predators. The presence of lethal defenses in prey can correct the selective imbalance suggested by the life-dinner principle in which the fitness consequences of an encounter between predator and prey should be much greater for the prey species than the predator. Despite the apparent adaptive advantages of lethality the evolution of deadly prey presents a fundamental dilemma. How might lethal defenses confer an individual fitness advantage if both predators and prey die during interactions? We examined the interaction between the rough-skinned newt (*Taricha granulosa*), which contains a powerful neurotoxin called tetrodotoxin (TTX), and the common garter snake (*Thamnophis sirtalis*). In some sympatric populations, *Th. sirtalis* have evolved physiological resistance to TTX. Whether the newts' toxin confers protection from snake predators or has been disarmed by the snakes' physiological resistance has not yet been directly tested. In predator–prey trials, newts that were rejected by snakes had greater concentrations of TTX in their skin (4.52 ± 3.49 mg TTX/g skin) than those that were eaten (1.72 ± 1.53 mg TTX/g skin). Despite the plethora of taxa that appear to use TTX defensively, this is the first direct and quantitative demonstration of the antipredator efficacy of TTX. Because the survival probability of a newt (and thus fitness) is affected by individual TTX concentration, selection can drive the escalation of toxin levels in newts. The variable fitness consequences associated with both TTX levels of newts and resistance to TTX in snakes that may promote a strong and symmetrical coevolutionary relationship have now been demonstrated.

Keywords *Thamnophis sirtalis* · *Taricha granulosa* · Coevolution · Predator–prey · Tetrodotoxin · Antipredator · Defense

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Introduction

The evolution of lethal defenses in prey species represents a fundamental paradox in evolutionary biology. Lethality in prey can ameliorate an innate imbalance associated with predator–prey interactions. Under this model, the potential fitness costs associated with being eaten (i.e., death) are much greater than those associated with failure to eat (e.g., loss of a meal). These costs, in turn, generate asymmetrical selective pressures in the interaction (e.g., Abrams 2000; Brodie and Brodie 1999; Dawkins and Krebs 1979). When prey are lethal, this asymmetry is lessened (or erased) and fitness consequences of an interaction between predator and prey are potentially balanced. However, the evolution of lethality in prey

superficially appears paradoxical because there is no individual advantage to "lethal" defenses if both prey and predators die during a predatory interaction (e.g., Brodie 2010; Brodie and Brodie 1999).

This apparent puzzle can be explained through kin selection in some cases (e.g., Benson 1971; Fisher 1930; Ruxton and Sherratt 2006), or through individual selection when mechanisms exist to allow escape from the full consequences (death) of the interaction (e.g., Ruxton and Sherratt 2006). Taste-rejection is one of many mechanisms used by predators to abate lethal consequences (e.g., Skelhorn and Rowe 2006), and individual prey may survive such sampling. For example, individual toxic insect larvae may survive "tasting" by various bird predators (e.g., Exnerová et al. 2006; Järvi et al. 1981; Wiklund and Järvi 1982), and some ascidian, coral, sponge, gorgonian, hydroid, and bryozoan larvae survive "tasting" by fish, coral, and anenome predators (e.g., Lindquist 1996; Young and Bingham 1987).

Newts of the genus *Taricha* possess tetrodotoxin (TTX) and related analogs (reviewed by Hanifin 2010), which block ion conductance through voltage-gated sodium channels (e.g., $Na_v1.4$; Kao and Levinson 1986; Hille 2001). Despite the fact that other predators such as birds and fish succumb to the newts' deadly neurotoxin (e.g., Brodie 1968; McAllister et al. 1997; Mobley and Stidham 2000), at least three species of gartersnakes (*Thamnophis sirtalis*, *Th. couchi*, and *Th. atratus*) circumvent this defense and consume all four species of newts (*Taricha granulosa*, *Ta. torosa*, *Ta. rivularis*, and *Ta. sierrae*; e.g., Brodie 1968; Brodie and Brodie 1990; Brodie et al. 2005; Edgehouse 2008; Feldman et al. 2009; Greene and Feldman 2009; Gregory and Nelson 1991; Twitty 1966; Williams et al. 2004; Wiseman and Pool 2007).

Resistance in snakes occurs through modifications of the amino acid sequence at the channel pore that decrease the binding affinity for the TTX molecule (Feldman et al. 2009; Geffeney et al. 2002, 2005). Thus, the impairment of action potentials in nerve and muscle tissue, which eventually leads to asphyxiation and death in susceptible organisms (Kao 1966), is greatly reduced in resistant snakes. Snakes with high individual resistance are more likely to survive interactions with and successfully consume the otherwise deadly newts (Williams et al. 2003). Presumably, an assessment of resistance is made relative to newt toxicity; however, a non-destructive individual assay for newt TTX levels was not available at the time of that experiment (Williams et al. 2003). Thus, fitness (or survival as the proxy thereof) varies with respect to predator exploitive abilities (resistance), but whether prey defenses likewise affect prey fitness is unknown. Here, we directly test whether newt TTX levels affect newt survival probability.

Materials and methods

Only empirical tests with predators and prey that interact ecologically can demonstrate the realized antipredator efficacy of a defensive trait. Thus, we tested antipredator efficacy of TTX in Benton Co., OR, where snake predators (*Th. sirtalis*) are known to consume newts (*Ta. granulosa*; Brodie and Brodie 1990; Williams et al. 2004). Adult *Ta. granulosa* and *Th. sirtalis* were collected during the spring and summer of 1998–2000. Animals were housed at Utah State University with a 14:10 light–dark cycle. Snakes were maintained at an ambient temperature of 24°C and offered a thermal gradient (24–30°C), a moist hide box, fresh water, and weekly fish meals. Newts were housed at 14°C in filtered water and fed crickets and worms weekly.

Assaying TTX levels in newts

Newt TTX content was assessed via comparison to a commercial standard with high performance liquid chromatography (HPLC). Our methods were the same as Williams et al. (2004), which were a modification of methods previously reported (Hanifin et al. 1999; Yotsu et al. 1989), with the following exceptions. A small section ($\sim 1 \times 2$ mm) of newt skin mid-dorsum was removed from anesthetized animals prior to feeding trials and weighed to the nearest 0.001 g and extracted as per the methods of Williams et al. (2004) for newts. Parameters and equipment for HPLC quantification were the same as in Williams et al. (2004). Concentration of TTX in the skin was reported in mg TTX per g skin. Mean concentrations of TTX in the skin that were consumed versus those that were rejected were compared with *t* tests. Newt TTX concentrations were regressed on newt mass via ANOVA.

Assaying TTX resistance in snakes

Resistance of snakes to TTX also affects predator–prey interactions between snakes and newts (Williams et al. 2003). Thus, we assessed resistance to confirm that it did not vary between our treatment groups and confound the effect of newt TTX concentration on newt survival. Resistance was assessed by the methods of Brodie and Brodie (1990) as data were collected for other ongoing projects (Brodie et al. 2002; Hanifin et al. 2008). Briefly, snake crawl speed is reduced after an intraperitoneal injection of TTX and this reduction, expressed as a percentage, offers a quantitative measure of resistance. Snakes with high resistance may crawl at 100% of their original crawl speed whereas snakes with low resistance crawl only at a small percentage of their original speed or not at all (resistance = 0%). During this experimental period, the amount of TTX necessary to assess resistance in adult

snakes was prohibitive. Thus, resistance was directly assessed in five adult snakes but assessed indirectly for eight females that bore litters. Because resistance is heritable ($h^2 = 0.65-0.80$; Brodie and Brodie 1990, 1991), we estimated these eight females' resistance as simply the mean resistance of their offspring. We did not assess resistance in seven of the snakes. We tested whether groups of snakes that either rejected or consumed newts differed in resistance with a *t* test.

Assaying predator–prey interactions in snakes and newts

Gravid females may forgo feeding late in gestation. The majority of snakes used in this study were female, but were not gravid. All the snakes were consuming weekly meals of fish regularly and to satiation before commencement of this experiment. Previous to behavioral trials, snakes' weekly offering of fish was withheld. Thus, snakes were deprived of food for 8–13 days before testing.

During feeding trials, an adult newt was introduced into an adult snake's home enclosure. Newt size was qualitatively matched to snake size to emphasize whether rejection or consumption was based on newt TTX levels rather than relative size of predator and prey. The assumption of equal size ratios between snakes that either ate or rejected newts was examined after behavioral trials with a *t* test. Because we were primarily interested in the outcome of the predation sequence after contact, each newt was placed within the visual field of a snake. When snakes oriented toward the newt, the newt was released and the predation sequence progressed without further observer interference. The observation was terminated when newts had been consumed, as determined by the snake closing its mouth after the newt had moved down the gullet, or when a snake had rejected a newt as determined by a strike, consequent release of the newt, and no further strikes for a period of 5 min. All snakes struck at newts and were thus presumably hungry.

We measured snake and newt mass to the nearest 0.01 g. Newts were measured immediately preceding feeding trials, while snakes were measured post-feeding to minimize disturbance before behavioral trials. Snake mass was estimated for snakes that had consumed a newt by subtracting the newt mass from the total mass of the snake with a newt bolus.

Results

During 20 predator–prey trials, each with different individual snakes and newts, 11 newts were rejected and 9 were consumed. Those rejected had higher concentrations of TTX in the skin on average (mean ± SD = 4.523 ± 3.485 mg TTX/g skin) than those that were eaten (1.722 ± 1.531 mg TTX/g skin; $t = 2.232$, $df = 18$, $p = 0.019$; Table 1; Fig. 1). One rejected newt possessed an extremely high TTX concentration (12.817 mg TTX/g skin) and appears to be an outlier (Fig. 1). However, removal of this data point did not affect the statistical conclusion that rejected newts possessed higher TTX concentration than those consumed ($t = 2.201$, $df = 17$, $p = 0.021$). In addition, this concentration is still congruent with TTX amounts in other published accounts of this population (Hanifin et al. 2004); thus, we have no reason to doubt the authenticity of this data point and it was not removed from our dataset. Snake size relative to newt size did not vary between groups that consumed versus those that did not consume newts ($t = 0.881$, $df = 18$, $p = 0.195$), nor did resistance differ for the subsample of 13 snakes for which we had data (7 newts rejected, 6 consumed by these snakes; $t = 0.998$, $df = 11$, $p = 0.170$). Thus, because we controlled for size and resistance of snakes we were able to directly test the effect of TTX concentration on newt survival probability.

Discussion

The concentration of TTX in newt skin affects newt survival probability in interactions with resistant predators. Selection on newt TTX levels requires variation in predator–prey outcomes that depends on variation in TTX levels of individual newts. A fundamental paradox associated with lethal defenses, like TTX, in prey species is how selection acts if an individual dies as a result of delivering its toxin load during a predator–prey interaction. Here we demonstrate a mechanism to explain variable fitness consequences associated with TTX levels of newts in relationship to predation by TTX resistant garter snakes. Snakes mitigate the consequences of attempted ingestions of TTX-bearing newts by behaviorally rejecting newts with high TTX levels (this paper, Williams et al. 2003). Thus, TTX concentration in newt skin is an effective defense.

Despite the plethora of taxa that possess TTX and presumably employ it defensively (e.g., Williams 2010), this is the first direct demonstration that survival probability of any organism depends on the quantity of TTX they possess when faced with a natural predator. However, note that approximately one-third of populations tested are mismatched with snake resistance exceeding newt TTX levels (Hanifin et al. 2008). In these populations we expect the effect of newt TTX levels to be inconsequential on the probability of newt consumption by garter snakes.

Another factor that should influence outcome of interactions between snakes and newts is the relative size of

Table 1 Outcome of predator–prey trials between individual newts (*Taricha granulosa*) and snakes (*Thamnophis sirtalis*)

Outcome	Newt mass (g)	TTX conc. (mg TTX/g skin)	Snake mass (g)	Resistance (%)	Litter size
Consumed	13.5	0.00	98.3	28[a]	6
Consumed	21.0	0.40	115.7	NE	n/a
Consumed	11.2	0.54	110.3	NE	n/a
Consumed	13.2	0.81	102.0	32[a]	22
Consumed	24.8	1.41	149.0	10[a]	8
Consumed	16.8	1.60	101.8	NE	n/a
Consumed	13.4	2.68	85.4	15[a]	15
Consumed	16.9	3.76	117.4	40[a]	19
Consumed	18.4	4.29	130.8	38[a]	19
Rejected	11.6	0.68	91.6	20	n/a
Rejected	18.0	1.61	108.1	70[a]	11
Rejected	17.7	1.65	95.6	57[a]	9
Rejected	11.8	2.31	84.6	23	n/a
Rejected	12.2	2.96	131.9	27	n/a
Rejected	9.3	3.44	76.2	30	n/a
Rejected	5.3	4.98	88.9	NE	n/a
Rejected	12.7	5.69	70.2	NE	n/a
Rejected	11.7	6.24	70.1	NE	n/a
Rejected	20.5	7.38	106.6	NE	n/a
Rejected	8.9	12.82	85.5	27	n/a

Newt mass, the concentration of tetrodotoxin (TTX) in each newt's skin, snake mass, and snake resistance to TTX in mouse adjusted mass units (MAMUs; see text) are given

NE not examined, *n/a* not applicable

[a] Resistance was assessed for individual snakes ($n = 5$) or estimated by mean resistance of the snake's litter ($n = 8$) where possible

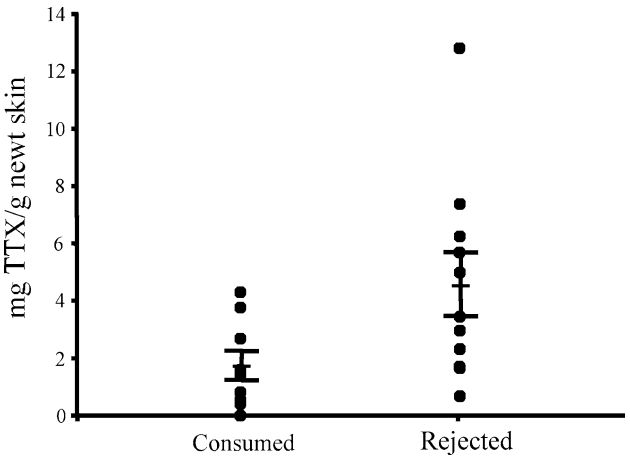

Fig. 1 Tetrodotoxin (TTX) levels of newts (*Taricha granulosa*) either rejected or consumed by garter snake predators (*Thamnophis sirtalis*). *Error bars* (SE) and mean (*horizontal bar*) are depicted

predators to prey. Once a prey item reaches a threshold maximum or minimum size (relative mass or prey diameter to gape size), the probability of consumption declines to zero (reviewed by Arnold 1993). Although newt TTX stores are marginally a function of newt size, and juveniles are much less toxic than adults, extensive individual variation in TTX levels swamps the effect of size in adults and no correlation between newt TTX levels and size emerges within populations (Brodie et al. 2005; Hanifin et al. 1999; 2004). As expected, there was also no apparent relationship between TTX concentration and newt size in this study. However, newt size presumably also interacts with newt TTX concentration by influencing deliver rate of TTX. Because TTX is water soluble and absorbed rapidly though the mucosa while swallowing newt prey (Williams et al. 2003), the rate of TTX uptake and thus intoxication should increase as swallowing time increases. Ingestion of prey near the minimum or maximum size thresholds is inefficient (e.g., Pough and Groves 1983; Jayne et al. 1988; Mori 2006; Shine 1991); i.e., relatively larger prey items take longer to consume, thus increasing a snake's exposure time and accelerating intoxication when dealing with dangerous prey.

In this study we controlled for size as well as snake resistance to directly assess the effect of concentration of TTX in newt skin to predator–prey outcomes. However, we did not quantitatively size match or assess resistance a priori for all snakes. Although there was no statistical difference between snake size and resistance between groups of snakes that either rejected or consumed a newt, which allowed us to directly assess the effect of TTX concentration, we may not have removed all variance in predator–prey outcomes due to these factors in this experiment.

Resistance (Williams et al. 2003) and TTX concentrations (this study) will not solely determine the outcome of

individual predator–prey interactions. Clearly there is extensive variation in the outcome of predator–prey interactions between snakes and newts such that at times snakes will reject newts of low toxicity (Fig. 1). In addition, snakes of very low resistance are still at risk of death if they fail to quickly and accurately reject newts that exceed their TTX tolerance (Williams et al. 2003). Even snakes from populations of the lowest resistance when repeatedly offered newts from populations of elevated toxicity do not appear to learn to avoid these dangerous prey items (BLW, unpubl. data). Thus, errors in snake assessment of newt toxicity can occur at either end of the spectrum—on occasion snakes will fail to reject newts with TTX levels that are too high (and suffer lethal consequences; Williams et al. 2003) and likewise, at times, reject newts that could have been palatable based strictly on TTX levels relative to resistance. Evolutionarily, the latter error should be less costly.

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