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ARTICLE

Functional and physiological resistance of crayfish to amphibian toxins: tetrodotoxin resistance in the white river crayfish (*Procambarus acutus*)

N.J. Wilson, A.N. Stokes, G.R. Hopkins, E.D. Brodie, Jr., and C.R. Williams

Abstract: Freshwater crayfish are reported to consume early life-history stages of a number of toxic amphibians. Although previous research indicates toxic amphibians are palatable to crayfish, the potential toxicity associated with consumption of toxic prey has been poorly described. We sought to characterise the supposed tetrodotoxin (TTX) resistance of freshwater crayfish, which have been observed to eat the eggs and larvae of toxic *Taricha* Gray, 1850 newts. White river crayfish (*Procambarus acutus* (Girard, 1852)) consumed 7.7 ± 4.0 Rough-skinned Newt (*Taricha granulosa* (Skilton, 1849)) eggs (mean ± SD) when offered 10 eggs in controlled feeding trials. Eggs were determined to contain a concentration of 1239 ± 571 ng (mean ± SD) of TTX. A dose-response assay was then performed to compare ingested doses with physiological TTX resistance. Crayfish were highly susceptible to TTX when administered as an intramuscular injection; TTX doses of 0.1 mass-adjusted mouse units were lethal to 100% of *P. acutus* crayfish. We established that while crayfish were capable consumers of highly toxic newt eggs, these decapods did not demonstrate physiological resistance to TTX. These findings suggest that crayfish have some functional resistance that renders them capable of consuming TTX-bearing prey despite a lack of physiological resistance to TTX.

Key words: Rough-skinned Newt, *Taricha granulosa*, white river crayfish, *Procambarus acutus*, predation, tetrodotoxin, toxicity, resistance.

Résumé : Des observations indiquent que les écrevisses d'eau douce consommeraient les premiers stades du cycle de vie de divers amphibiens toxiques. Si des travaux antérieurs indiquent que des écrevisses affectionnent certains amphibiens toxiques, la toxicité potentielle associée à la consommation de proies toxiques n'a pas été décrite en détail. Nous avons tenté de caractériser la résistance présumée à la tétrodotoxine (TTX) des écrevisses d'eau douce, dont la consommation d'œufs et de larves de tritons toxiques du genre *Taricha* Gray, 1850 a été observée. Des écrevisses blanches de rivière (*Procambarus acutus* (Girard, 1852)) auxquelles étaient offerts 10 œufs de triton rugueux (*Taricha granulosa* (Skilton, 1849)) dans le cadre d'essais d'alimentation contrôlés ont consommé 7,7 ± 4,0 œufs (moyenne ± ÉT). Il a été déterminé que les œufs avaient des concentrations de TTX de 1239 ± 571 ng (moyenne ± ÉT). Un essai dose-réponse a ensuite été mené pour comparer les doses ingérées à la résistance physiologique au TTX. Les écrevisses étaient très susceptibles au TTX administré par injection intramusculaire; des doses de 0,1 unité-souris ajustée pour la masse étaient létales pour 100 % des écrevisses *P. acutus*. Nous avons établi que les écrevisses ne présentaient pas une résistance physiologique au TTX, même si ces décapodes pouvaient très bien consommer des œufs de triton très toxiques. Ces constatations donnent à penser que les écrevisses ont une forme de résistance fonctionnelle qui les rend aptes à consommer des proies contenant du TTX malgré l'absence de résistance physiologique à ce composé. [Traduit par la Rédaction]

Mots-clés : triton rugueux, *Taricha granulosa*, écrevisse blanche de rivière, *Procambarus acutus*, prédation, tétrodotoxine, toxicité, résistance.

Introduction

Crayfish are capable consumers of a range of toxic amphibians, particularly the egg and larval stages (Wilson and Williams 2014). In many instances, crayfish appear unharmed by consumption of these amphibian toxins (Wilson and Williams 2014). Palatability of toxic amphibians has been reported for several crayfish species, but few studies describe adverse effects of consuming toxic prey. Lethal and sublethal effects of amphibian toxins on crayfish remain largely undescribed, with the limits of ingestion of toxic material unknown. Furthermore, the extent of physiological resistance to amphibian toxins in freshwater crayfish remains unclear.

Among the list of toxic amphibians susceptible to crayfish predation are the tetrodotoxin (TTX) possessing *Taricha* Gray, 1850 newts, which vary in toxicity between species and across populations (Gamradt and Kats 1996; Gamradt et al. 1997; Hanifin 2010). Tetrodotoxin is one of the most potent neurotoxins found in nature and occurs in a wide variety of taxa from flatworms (class Turbellaria) to blue-ringed octopus (genus *Hapalochlaena* Robson, 1929) and pufferfish (family Tetraodontidae) (Miyazawa and Noguchi 2001; Hanifin 2010). Tetrodotoxin interferes with the propagation of action potentials by inhibiting voltage-gated sodium channels and can cause death by respiratory paralysis (Narahashi et al. 1964; Takata et al. 1966). The toxicity of TTX is often expressed in mouse units (MU; i.e., the amount required to

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kill a 20 g white mouse (*Mus musculus* L., 1758) in 10 min); 1 MU is equivalent to between 160 and 220 ng of TTX (Daly 2004). Newts, including embryos and efts, can be extremely toxic and a single Rough-skinned Newt (*Taricha granulosa* (Skilton, 1849)) egg can contain between 672 and 2767 ng of TTX (Hanifin et al. 2003). That is, at the upper boundary, a single *T. granulosa* egg can contain sufficient TTX to kill upwards of 12 white mice.

Unequivocally, the best documented predators of *Taricha* newts are garter snakes of the genus *Thamnophis* Fitzinger, 1843 (Hanifin 2010). Support of a coevolutionary arms race between *Thamnophis* and *Taricha* is strong; highly toxic newt populations have been repeatedly demonstrated to co-occur with highly TTX-resistant garter snakes (Brodie et al. 2002; Hanifin et al. 2008). Populations of TTX-resistant snakes have been shown to express genetically modified voltage-gated sodium channels that have reduced binding affinity for TTX, affording resistance (Geffeney et al. 2002).

Interestingly, these newts have few other known predators. Caddisflies (order Trichoptera) are reported to consume *Taricha* eggs and mosquitofish (*Gambusia affinis* (Baird and Girard, 1853)) have been shown to prey on newt larvae (Gamradt and Kats 1996; Lehman 2006; Gall et al. 2011). One species of crayfish has previously been documented to prey upon *Taricha*. The red swamp crayfish (*Procambarus clarkii* (Girard, 1852)) consumes newt eggs and larvae and has also been reported to attack adult *Taricha* newts (Gamradt and Kats 1996; Gamradt et al. 1997). The predation pressure posed by *P. clarkii* is such that declines in newt abundance are recorded for field sites inhabited by this invasive crayfish (Gamradt et al. 1997). The resistance demonstrated in garter snakes sets a precedent that other animals may also be resistant, and given the reports of caddisflies and crayfish as predators of immature life stages, this precedence extends to freshwater invertebrates. The scope of TTX resistance appears to extend beyond predators of newts. For example, some reef fishes have been witnessed to prey upon TTX-possessing polyclad flatworms (Ritson-Williams et al. 2006).

Building on the reports of *P. clarkii* as predators of *Taricha* newts, we studied feeding ability for another common and widespread species, the white river crayfish (*Procambarus acutus* (Girard, 1852)). It is not presently known whether crayfish are resistant to TTX in the same manner and magnitude as garter snakes. If crayfish do indeed show a high resistance to such a powerful toxic compound, then this would be a significant finding to help understand selective pressures on chemical defences in newts and predator–prey relationships more broadly. To explore this, we investigated the ability of *P. acutus* to consume *T. granulosa* eggs from a population of high toxicity in a controlled environment. Furthermore, we sought to compare this feeding ability to the physiological ability of these crayfish to withstand TTX delivered intramuscularly, bypassing the digestive tract.

Materials and methods

Study animals

Adult *P. acutus* were obtained from Carolina Biological Supply (Burlington, North Carolina, USA). *Procambarus acutus* was considered a suitable model for study because it is widely distributed across the United States; natural populations are known for 28 states and introduced populations are reported for a further 5 states (Taylor et al. 2007). Crayfish were group-housed in the laboratory in glass aquaria with gravel substrate, polyvinyl chloride (PVC) hides, and fitted with air stones to oxygenate the water. Aquaria were filled with filtered tap water and 50% water replacements were conducted weekly to maintain water quality. Crayfish were fed frozen beef-heart cubes twice weekly while housed in the laboratory.

All *T. granulosa* eggs used in this study were the offspring of adult newts collected from Hunter Creek; a small estuarine stream in Curry County, Oregon (42°23′19.60″N, 124°25′21.54″W). Invasive *Procambarus* and native *Pacifastacus* Bott, 1950 crayfish are commonly found throughout the range of *Taricha* newts, and crayfish were found cohabiting with breeding newts in Hunter Creek (G.R. Hopkins, personal observation). Gravid females were transported back to the laboratory at Utah State University, where they were housed individually in plastic containers (35 cm × 20 cm × 13 cm) with 2.0 L of chilled, filtered tap water, in a temperature-controlled room at 14 °C. Each newt was injected with 10 μL luteinizing hormone releasing hormone ([des-Gly^{10}, D-His(Bzl)^{6}]-LH-RH ethylamide; Sigma No. L2761; Sigma Aldrich, St. Louis, Missouri, USA) to stimulate oviposition and given submerged polyester filling as a substrate for egg deposition. Newt eggs were collected from 27 individual females within 24 h of deposition, pooled as a single batch, and subsequently allocated to trials at random. Animal use and care was in accordance with the U.S. Department of Agriculture Animal Welfare Act and the Policy for the Humane Care and Use of Animals. Animals were cared for in accordance with a protocol that had been reviewed and approved by the Utah State University Institutional Animal Care and Use Committee.

Feeding trials

Feeding trials were conducted in 7 L circular aquaria, filled with 5 L of filtered tap water. Each individual aquarium was fitted with an air stone for oxygenation. Thirty crayfish were randomly assigned to one of three treatment groups; one group was given 10 newt eggs, the second group was provided with 30 newt eggs, and the final group acted as a control and was fed pellets of chicken egg yolk set in agar. Control crayfish were offered pellets so that the handling and feeding behaviour exhibited by crayfish offered toxic newt eggs could be compared with that of the handling and feeding behaviour displayed when crayfish were offered an innocuous meal. Pellets were not specifically designed to mimic newt eggs. Pellets were created by using a flat-bottom 96-well cell culture plate as a pellet mould, producing pellets that were 11.5 mm in length and 6.4 mm in diameter. Each dosed pellet contained approximately 72 μL of chicken yolk mixed into 300 μL of agar. All crayfish were fasted for 24 h prior to commencing feeding trials and allowed to acclimate in individual aquaria prior to introduction of newt eggs. Feeding trials were conducted for 24 h with a 14 h light : 10 h dark photoperiod at 24 °C.

Egg consumption, righting response, and signs of lethargy or agitation were monitored for all crayfish. The treatment group that was offered 10 newt eggs was monitored once per hour for the first 2 h and again 24 h after newt eggs were first introduced. In an effort to capture the possible effects of consuming toxic eggs, crayfish in the 30-egg treatment group were monitored with increased frequency; observations were taken once per hour for the first 5 h after eggs were introduced and then again at 24 h at the end of the feeding trial. All crayfish were held in isolation for an additional 24 h once the feeding trial had concluded (any remaining newt eggs were removed) to confirm whether crayfish completely recovered from toxin exposure. This included monitoring the survivorship of crayfish.

Behavioural assessments were based on observation periods of 2 min per crayfish at each of the aforementioned intervals. Each behavioural assessment was conducted by first observing crayfish for one uninterrupted minute for signs of agitation such as defensive posturing, erratic movements, and finally, movement or cleaning of mouthparts to suggest distaste as has been described previously (Formanowicz and Brodie 1982). Care was taken to ensure observations were carried out as unobtrusively as possible to reduce the impact of the researcher's presence on crayfish behaviour. Next, lethargy was monitored by prodding each animal anteriorly with a blunt metal probe and monitoring the burst response (also known as the "caridoid escape reaction"). Lethargy was scored binomially by comparing burst response in test animals to that observed for control animals (i.e., burst response

either unchanged or reduced compared with control). The final measurement recorded was righting response, which involved using a blunt metal probe to overturn crayfish. Classification of righting response is defined in Table 1.

Determination of dose response

Tetrodotoxin (lyophilised in citrate buffer; available from Abcam, Cambridge, Massachusetts, USA) was dissolved in Ringer's solution and administered via intramuscular injection between the abdominal tergites. Doses administered to crayfish have been reported as mass-adjusted mouse units (MAMU, which is the amount of TTX required to kill 1 g of mouse in 10 min multiplied by the mean mass of the organism tested based on the amount of TTX needed to kill a 20 g mouse in 10 min) (Brodie and Brodie 1990). Crayfish tested in dose-response assays were variable in size (22.0 ± 6.8 g, mean ± SD); presentation of doses as MAMU accounted for the size differences between individual crayfish. Given the amount of TTX that was ingested without ill effect in feeding trials, it was initially expected that crayfish would be at least as resistant to TTX as mice. To test this, an initial first dose of 1 MAMU was administered to crayfish to observe response. Crayfish were monitored for signs of agitation including erratic movement, cleaning behaviour, and defensive posturing. Righting response was also monitored; an animal that failed to right itself within 1 min was deemed unable to self-right. Observations were made across the first 5–10 min and again 2 h after the dose was administered to monitor crayfish recovery. Each crayfish received a single dose and was not subject to further testing. Doses were adjusted iteratively to detect the ranges at which righting response was unaffected, impaired, and temporarily lost. A control group received 0.3 mL of Ringer's solution (no TTX) and this was administered using the same technique. The same behavioural assessments were conducted for the control group as for crayfish that were administered TTX. The survivorship of crayfish within control and treatment groups was monitored for a minimum of 24 h after administering the intramuscular injection.

Quantification of TTX in newt eggs: converting dose response to harmful ingested dose

A subsample of newt eggs was taken from the pooled batch used in feeding trials to quantify the amount of TTX present in the newt eggs used in this study. Eighteen eggs were analysed in groups of three and extracted using the methods of Hanifin et al. (2002). Performing extractions in six groups of three eggs was efficient yet ensured that a representative number of eggs were sampled, giving confidence that the results obtained reflected the mean TTX concentration of eggs as well as demonstrating the variability. Egg groups were homogenized in 800 μL of 0.1 mol/L acetic acid and boiled for 5 min. Each sample was centrifuged at 13 000 rev/min for 20 min, after which the supernatant was pipetted into centrifugal filter tubes and centrifuged again in the same manner. Samples were stored at −80 °C for quantification.

Samples were quantified using a competitive inhibition enzymatic immunoassay as in Stokes et al. (2012). Standards were prepared by diluting TTX–citrate in 1% bovine serum albumin (BSA) prepared in phosphate-buffered saline. Standards were diluted to the linear range of the standard curve (10–500 ng/mL). Each sample was also diluted 1:100 in BSA to allow them to fall within range of the curve. The mean intraplate coefficient of variation was 6.56%. We calculated the amount of TTX in each egg by dividing by the number of eggs in each group, thereby giving a mean value of TTX per newt egg.

This enabled translation of the dose that resulted in a total loss of righting response into an estimate of the number of newt eggs that would need to be consumed to be purportedly harmful to the crayfish. This calculation included a few assumptions. Tetrodotoxin has been reported to be approximately 40 times more lethal by intraperitoneal injection (LD_{50}: 8 μg/kg) than through the oral route (LD_{50}: 334 μg/kg) for white mice (Mosher et al. 1964). A similar dose-response relationship was observed with garter snakes, with intraperitoneal injection being slightly more than 40 times greater in toxicity than oral administration of TTX (Williams et al. 2002). We have assumed the same relationship between oral and injected doses for crayfish in our calculations. Additionally, as previously stated, crayfish received intramuscular injections rather than intraperitoneal injections and this may have influenced the dosage required to elicit response.

Table 1. Classification of white river crayfish (*Procambarus acutus*) righting response during feeding trials.

Score	Definition
0	Animal able to right self rapidly (<1 s)
1	Animal able to right self readily (1–5 s)
2	Animal able to right self but slow to do so (5–10 s)
3	Animal slow to right self (10+ s)

Statistical analysis

Kruskal–Wallis tests were performed to compare the mass and size of crayfish across the control and treatment groups to determine whether all groups could be considered of equal size and mass. The Kruskal–Wallis test was also used to determine if crayfish survivorship differed among the three groups. At the conclusion of feeding trials (24 h), a Wilcoxon's rank-sum test was performed to determine whether egg consumption in the 10-egg treatment was significantly different than consumption observed in the 30-egg treatment. STATA data analysis software (version 11) was used to perform data analysis for the above mentioned tests (StataCorp LP, College Station, Texas, USA). Contingency tables and the χ^2 statistic (Zar 1999) were used to test the independence of righting and egg consumption, to evaluate changes in righting response with TTX dosage (testing the hypothesis that righting was dependent on TTX dose), and finally, to examine the association between TTX dose and display of agitated behaviour. Statistical significance was set at $\alpha = 0.05$ for all tests performed.

Results

Feeding trials

Rate of newt egg consumption was quite variable; mean (±SD) egg consumption was 7.7 ± 4.0 (77%) and 13.7 ± 12.3 (41%) for the 10-egg and 30-egg treatments, respectively (Table 2). Seven crayfish in the 10-egg treatment consumed all available eggs, while maximum consumption recorded in the 30-egg trial was 28 eggs. Egg consumption between the treatment groups was not considered statistically significantly different (Table 2). Control animals always consumed the agar pellet provided. There were no significant differences in the size (carapace length) and mass of crayfish in the control and treatment groups (see Table 3). One crayfish in the 30-egg treatment began to moult during the feeding trial and appeared to become stuck midway through shedding the exoskeleton and died; this animal was excluded from the analysis.

The death of one crayfish in the 10-egg treatment was recorded during a feeding trail. At the 2 h observation point, this animal had consumed all 10 eggs, appeared very lethargic (unresponsive to prodding), and was struggling to right (classification score = 3). This was the only animal in the feeding trial to appear lethargic and it did not survive to the 24 h observation. Overall, survivorship in the treatment groups was not significantly different to that of the control group ($\chi^2 = 1.9$, $P = 0.39$).

Delayed righting response was observed for some crayfish in the 10- and 30-egg treatments and is depicted in Fig. 1. However, statistical analysis indicated that righting response was independent of the number of newt eggs offered (Table 4). Crayfish in both treatment groups (with the exception of the previously mentioned fatality) appeared to recover completely within 24 h. Right-

Table 2. Size and mass of white river crayfish (*Procambarus acutus*) within treatment groups in which the number of Rough-skinned Newt (*Taricha granulosa*) eggs consumed and (or) damaged was recorded.

Treatment	*n*	Carapace length (mm)	Crayfish mass (g)	No. of eggs consumed	No. eggs of damaged but not consumed	Crayfish survivorship (%)
Control	10	46.7±3.0	25.6±6.9	—	—	100
10 eggs	10	47.7±3.7	26.4±6.8	7.7±4.0	0.0	90
30 eggs	9	47.6±3.0	27.2±7.3	13.7±12.3	1.4±1.8	100
				Consumption: $Z = -0.92$, $P = 0.36$		Survivorship: $\chi^2 = 1.9$, $P = 0.39$

Note: All values reported are mean ± SD, except survivorship which is expressed as a percentage value.

Table 3. Output of Kruskal–Wallis test comparing the size and mass of white river crayfish (*Procambarus acutus*) between treatment groups.

Treatment	*n*	Rank sum	χ^2	*P*
Carapace length (mm)				
Control	10	132.50	0.15	0.93
10-egg treatment	10	145.00		
30-egg treatment	7	100.50		
Mass (g)				
Control	10	138.00	0.41	0.82
10-egg treatment	10	128.50		
30-egg treatment	8	128.50		

ing response was never impaired for control animals. Besides impaired righting response, no other signs of agitation (such as defensive posturing or erratic movement) were observed following consumption of newt eggs.

Dose response

Tetrodotoxin was lethal to crayfish at small doses, with 0.1 or greater MAMU resulting in 100% mortality (Fig. 2). Loss of righting response was significantly associated with TTX dose (Fig. 3, Table 4). Lower doses of 0.01 and 0.02 MAMU still affected some crayfish, producing temporary paralysis and impairing righting response for several minutes. Paralysis was typically characterised by rigid, largely immobile legs and was often accompanied by curling of the abdomen. Fanning of the pleopods ("swimmerets") was often the only movement observed in otherwise immobile crayfish. All crayfish tested at these low doses recovered completely within 2 h (Fig. 3). Crayfish appeared agitated following injection, but this behaviour was not significantly associated with TTX dosage (Table 4). Intramuscular injection of saline did not impair righting ability, but some signs of agitation were observed (Fig. 3).

TTX concentration of eggs

The mean (±SD) amount of TTX per newt egg was 1239 ± 571 ng. Given that 1 MU is equivalent to 160–220 ng TTX, then 0.05 MU (which killed 90% of crayfish) is equivalent to 8–11 ng TTX. Presupposing that TTX is 40 times more lethal by injection than by oral route (Mosher et al. 1964; Williams et al. 2002), the lethal ingested dose would be expected to be approximately 320–440 ng of TTX. That is, the putative lethal ingested dose (320–440 ng) is less than the amount of TTX found in one single newt egg (1239 ng).

Discussion

The specific toxicity of an amphibian toxin towards freshwater crayfish has not, to our knowledge, been described previously. Our investigation suggests, somewhat confoundingly, that while freshwater crayfish readily consume TTX laden eggs, these animals are highly susceptible to injections of TTX. Egg consumption was not significantly different between the 10- and 30-egg treatment groups. Seemingly, crayfish have some functional resistance to TTX that enables this oral consumption despite a lack of physiological resistance to the toxin.

Some crayfish that consumed newt eggs exhibited slower righting ability than control animals and, on occasion, righting was delayed by more than 10 s. Delayed righting in the wild, even if slight, could be disadvantageous to the affected crayfish and could be ecologically significant. Although crayfish recovered from low doses of TTX delivered intramuscularly, righting response was still temporarily lost, sometimes for several minutes. In the wild, affected crayfish would be vulnerable to being preyed upon and (or) cannibalised.

Crayfish consuming newt eggs showed no apparent signs of agitation. In contrast, several crayfish that received TTX intramuscularly behaved in an agitated state, moving erratically and displaying defensive postures. Agitation was not related to the extent of dosing. Agitated behaviours were not observed at the higher doses of TTX because crayfish were incapacitated with paralysis. Defensive posturing was also observed for one crayfish that received a saline injection and we suspect this may be attributable to distress caused by the researcher handling the animal while administering the saline injection.

It was anticipated that TTX doses delivered intramuscularly would affect crayfish at lower quantities than would be observed with oral doses due to first-pass metabolism where there is a great reduction in toxin concentration prior to reaching the systemic circulation. However, the disparity between these delivery routes was exaggerated and exceeded benchmarks established with both susceptible (mice) and resistant (garter snakes) vertebrates (Mosher et al. 1964; Williams et al. 2002). Delivering TTX via injection was lethal for 90% of crayfish at 0.05 MAMU TTX (Fig. 2) and suggests that crayfish are 20 times more susceptible to TTX than are white mice. Following the assumption that oral delivery is approximately 40 times less toxic than intramuscular injection (Mosher et al. 1964; Williams et al. 2002), a single newt egg contains sufficient TTX to be lethal for the crayfish consumer. However, in controlled feeding trials, crayfish were observed to consume up to 28 eggs in a 24 h period.

Although seemingly physiologically susceptible to TTX, functionally these crayfish can consume a number of newt eggs. This apparent disparity in TTX tolerance may be attributed to other factors additional to the mode of delivery and the subsequent bioavailability of TTX. Disparity between delivery routes may be the product of a mechanism that enables crayfish to process ingested TTX that is bypassed with the injection route. Despite numerous reports of freshwater crayfish preying upon toxic amphibians, it is unknown how crayfish are able to tolerate ingestion of toxic prey (Wilson and Williams 2014). As benthic detritivores, crayfish can be exposed to a range of potentially noxious materials. This life-history evolution and consequent adaptation to tolerate toxic diets may have, in turn, afforded crayfish the ability to consume toxic amphibians. One possible mechanism is that crayfish may have symbiotic bacteria present in the gut that are capable of metabolising toxic compounds. Gut bacteria may reduce the susceptibility of host organisms to dietary toxins and have been previously suggested to enhance toxin resistance in prawns and isopods (Zachary et al. 1983; Dempsey and Kitting 1987; Harris 1993).

Another possible explanation is that crayfish may be able to either detoxify or sequester toxic compounds that are present in

Fig. 1. Righting response (RR; defined in Table 1) of white river crayfish (*Procambarus acutus*) in control (n = 10), 10-egg (n = 10), and 30-egg (n = 9) treatments following introduction of Rough-skinned Newt (*Taricha granulosa*) eggs. Note that RR was not measured for control or 10-egg treatments at 3, 4, and 5 h.

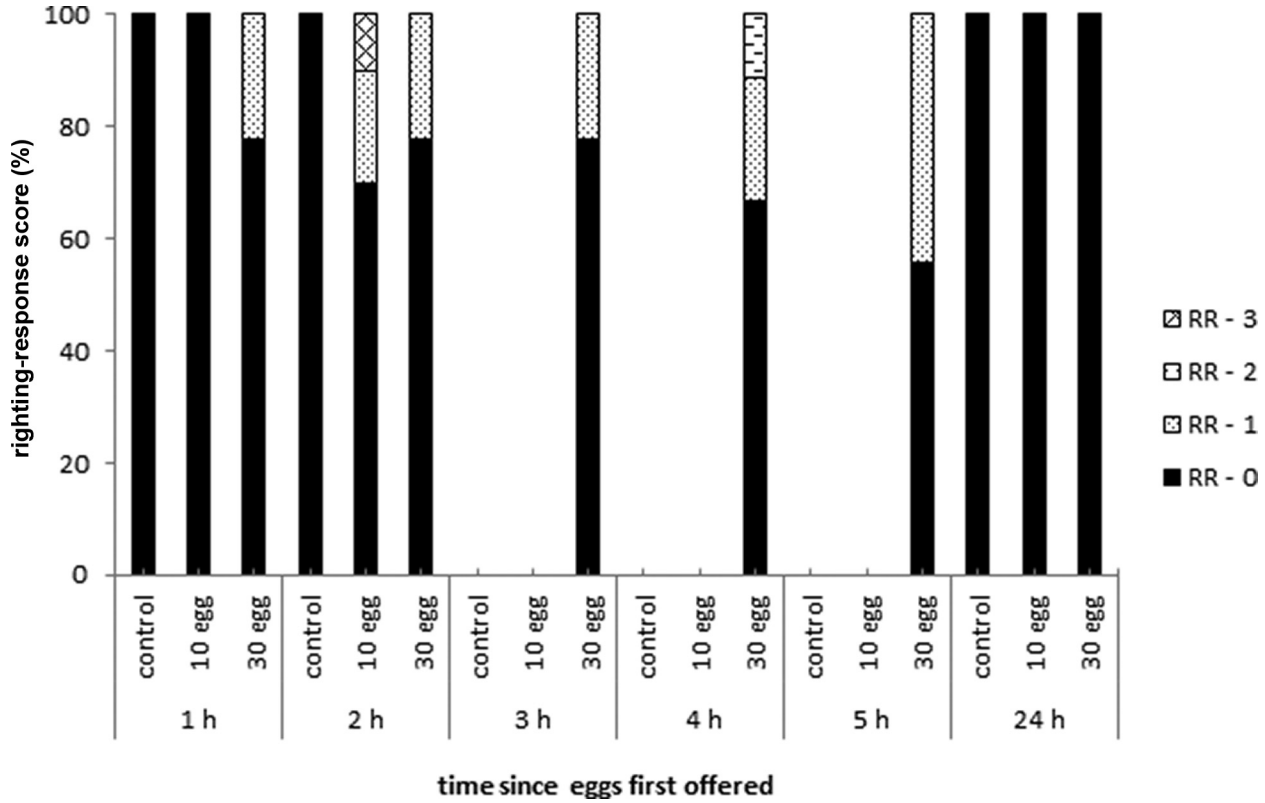


Table 4. Chi-square statistic testing the independence of righting behaviour and Rough-skinned Newt (*Taricha granulosa*) egg availability (n = 29), as well as χ^2 statistic evaluating righting (RR, n = 35) and agitation (n = 20), in response to intramuscular tetrodotoxin (TTX) dose.

Time	RR χ^2	df	P	Agitation χ^2	df	P
Rough-skinned Newt egg availability						
1 h	4.77	2	0.09	—	—	—
2 h	4.61	4	0.33	—	—	—
24 h	0.00	2	1.00	—	—	—
Intramuscular TTX injection						
1 min	21.12	4	0.0003	1.88	2	0.39
2 min	16.63	4	0.0023	2.40	2	0.30
5 min	18.48	4	0.001	3.16	2	0.21
120 min	31.25	4	0.0001	0	2	1.0

food sources. Sequestration of dietary TTX has been demonstrated in both garter snakes and caddisfly larvae (Williams et al. 2004; Gall et al. 2012). Garter snakes sequester TTX in the liver (Williams et al. 2004). Caddisfly larvae have been shown to sequester small but potentially ecologically relevant amounts of TTX, which was retained for at least 4 months when larvae were reared on a TTX-free diet (Gall et al. 2012). Previous reports indicating that cyanobacterial toxins can accumulate in the crayfish hepatopancreas without ill effect give further precedence that crayfish, like garter snakes and caddisfly larvae, may also be able to sequester TTX (Lirås et al. 1998).

The extreme toxicity via injection may be a result of bypassing gut-specific mechanisms as proposed above or could be due to the speed with which TTX was administered overwhelming clearance mechanisms. The findings of this study indicate that while TTX in eggs was not protective against crayfish predators, crayfish TTX consumptive ability is not comparable to that of the garter snake for which physiological resistance is attributable to modified sodium channels (Geffeney et al. 2002). Future research directions could examine TTX processing within the crayfish gut to determine whether TTX is metabolised, eliminated, or sequestered in the hepatopancreas or other tissues. We suspect that whatever the mechanism, this process is not specific to TTX, but rather, enables crayfish to eat a broad range of toxic compounds.

Tetrodotoxin in newt eggs was not an effective antipredator deterrent to crayfish. This study highlights the importance of examining different modes of toxin delivery. While it might appear, via one method, that crayfish are susceptible to toxins, reports that only examine toxicity in this context could underrepresent the ecological problem of invasive crayfish. Despite lacking physiological resistance to amphibian toxins, crayfish may be capable of consuming large numbers of amphibian eggs. Therefore, to understand the potential ecological impact of crayfish (or indeed other invasive predators), it is crucial to test the most ecologically realistic mode of delivery (i.e., feeding).

Our findings that *P. acutus* consume *T. granulosa* eggs are analogous with previous research describing the predation of California Newt (*Taricha torosa* (Rathke, 1833)) eggs and larvae by the highly invasive crayfish *P. clarkii* (Gamradt and Kats 1996; Gamradt et al. 1997). These findings reiterate the threat that introduced and invasive crayfish can pose to amphibian species that are otherwise largely protected from naturally occurring predators by chemical deterrents. Interestingly, the Oregon Department of Fish and Wildlife (no date) suggest that unlike the invasive *P. clarkii*, Oregon-native signal crayfish (*Pacifastacus leniusculus* (Dana, 1852)) are susceptible to newt toxins. However, this claim was not substantiated by reference to data. Introduction of *P. clarkii* to regions in Europe void of native crayfish has seen declines in amphibian

Fig. 2. Survivorship of white river crayfish (*Procambarus acutus*) following administration of tetrodotoxin via intramuscular injection. Doses are expressed in mass-adjusted mouse units (MAMU).

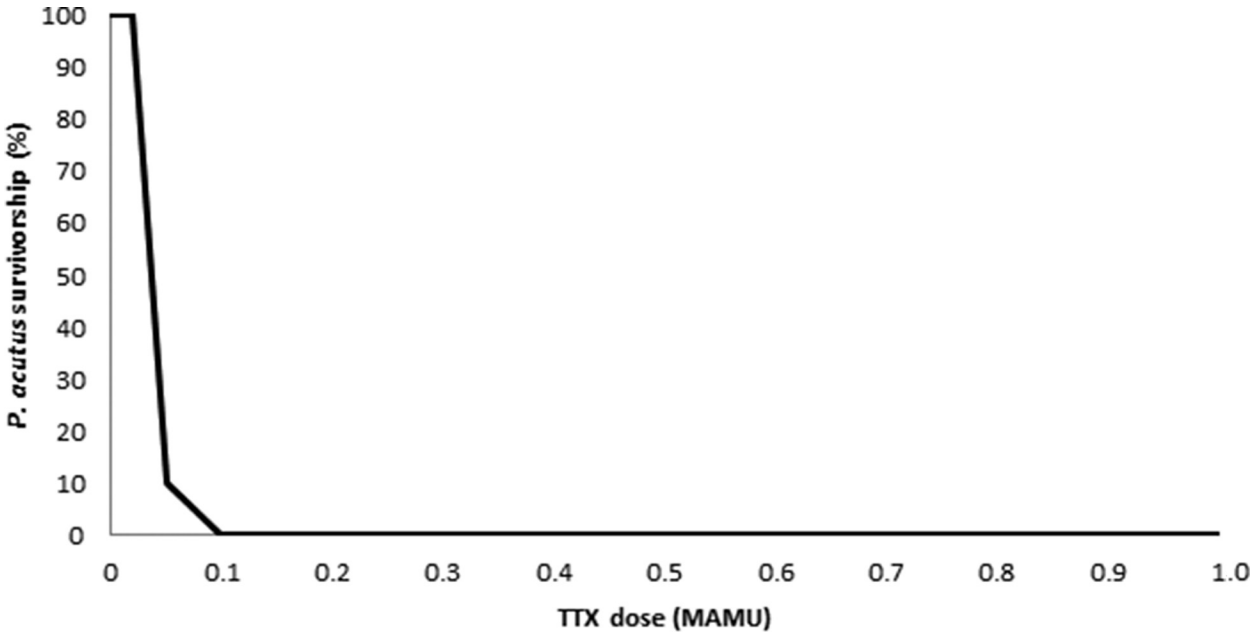
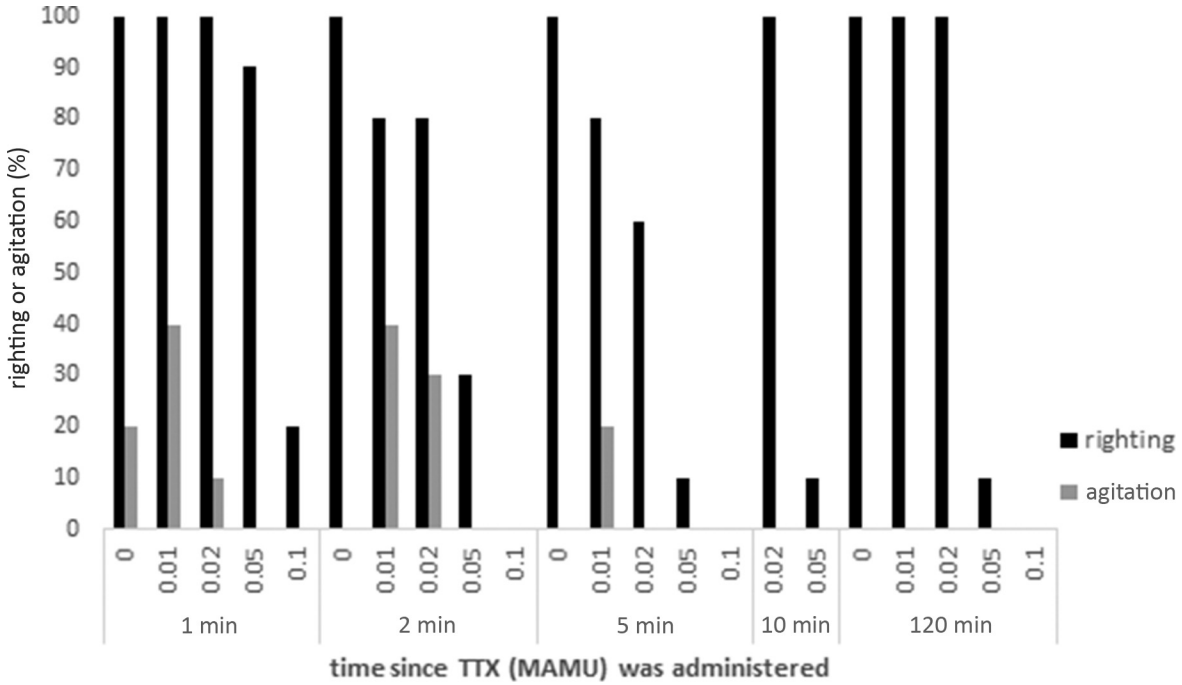


Fig. 3. Observations of righting response and agitation for white river crayfish (*Procambarus acutus*) following intramuscular injection of tetrodotoxin. Doses are expressed in mass-adjusted mouse units (MAMU). Sample size (*n*) of 5 for 0 (Ringer's solution), 0.01, and 0.1 MAMU, and *n* of 10 for 0.02 and 0.05 MAMU. No righting was recorded for doses higher than 0.1 MAMU and as such these have been omitted.



species richness at sites that crayfish were detected, further highlighting the vulnerability of amphibian fauna to invasive crayfish (Cruz et al. 2006). The invasive potential of crayfish in North America requires tighter regulation and management of aquaculture practice to reduce the risk that these predators pose to vulnerable amphibian species.

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