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Tetrodotoxin content of Rough-skinned Newts, *Taricha granulosa* (Salamandridae), from their northern distribution range, British Columbia, Canada, and Southeast-Alaska, USA

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Abstract. Rough-skinned Newts, *Taricha granulosa*, occur along the west coast of North America, reaching their most northern range limits in British Columbia (BC), Canada, and Southeast-Alaska (AK), USA. They contain one of the most potent neurotoxins, tetrodotoxin (TTX), which varies considerably in concentration between individuals and populations. Analysis of specimens from a coastal area of Prince Rupert (BC) were found to contain low TTX-levels and traces only of its analogue 6-*epi*TTX; the toxin was not detected in juveniles from the eastern ranges, Terrace (BC). Likewise, low values were detected in specimens from Revillagigedo Island (AK), whereas newts from a pond near Juneau (AK) exhibited about ten-times higher TTX-concentrations. While *T. granulosa* colonised the archipelago of Southeast-Alaska after the great ice age 10,000 years ago, newts have been introduced in certain areas, such as to Juneau from opposite Shelter Island in the 1960s. The high variation in toxicity raises the question of the toxin's biogenetic origin, whether it is endogenously synthesised or sequestered from unknown external sources. Further studies on toxin levels in distant populations may provide some hints which local conditions may favour the occurrence of TTX in *T. granulosa*.

Key words. Amphibia, Urodela, *Taricha granulosa*, tetrodotoxin, British Columbia, Canada, Alaska, USA.

Introduction

Rough-skinned Newts, *Taricha granulosa*, are widely distributed along the west coast of the United States and Canada, ranging from the San Francisco Bay, California, to southeastern Alaska, including coastal and various island areas of British Columbia (BC), Canada, and of Southeast-Alaska, USA (Nussbaum et al. 1983, Petranka 1998, Matsuda et al. 2006). Like all members of the genus *Taricha*, they contain one of the most potent neurotoxins, tetrodotoxin (TTX), which is considered to play a major role in the newt's defence (Mosher et al. 1964, Brodie 1968, Hanifin 2010). TTX specifically blocks voltage-gated sodium channels of excitable membranes (Kao 1966, Narahashi et al. 1967, Moczydlowski 2013). High concentrations of the toxin are found in the skin, ovaries and eggs of the newts, and minor amounts in muscle tissue and blood (Wakely et al. 1966, Hanifin et al. 1999). However, TTX levels vary considerably between individuals and populations across broad geographical scales, ranging from zero to mg-amounts in the skin (Hanifin et al. 1999, 2008, Brodie et al. 2002).

The peripheral neuromuscular system of *T. granulosa* is highly resistant to TTX, which is the result of three amino acid substitutions at the TTX-binding site in the outer pore of the voltage-gated sodium channel, Na_v 1.4 (Hanifin & Gilly 2015). Likewise, garter snakes, *Thamnophis* spp., which predate on the newts (Brodie & Brodie 1990, Brodie et al. 2005), have developed resistance to TTX (Feldman et al. 2009). Besides Na_v 1.4, two additional Na_v paralogues, Na_v 1.6 and 1.7, which are expressed in the peripheral nervous system of the snake, contain at least one amino acid substitution identical in all three paralogues leading to toxin-resistance (Geffeney et al. 2005, Feldman et al. 2010, 2012, McGlothlin et al. 2014). Predator-prey interaction of snake and newt seems to fuel an "arms-race" based on snake resistance and newt toxicity, which are closely related in areas where they co-occur (Brodie 2005, Hanifin et al. 2008). Outside the range of the newts, *T. sirtalis* populations tend to lack express insensibility to TTX. Conversely, newts living in sympatry with TTX-resistant snakes may exhibit high TTX-levels (Brodie et al. 2002, Hanifin et al. 2008). Reciprocal selection has been suggested to be the driving force of the newt's variability in TTX-content. Although toxin levels in newts were found to be low in areas where garter snakes are not present, such as in Southeast-Alaska (Mebs et al. 2016, Hague et al.

2016), a high phenotypic variation of toxin concentrations is significant. It is still unknown, which factors, internal or external, influence toxin variability.

In the present study, analyses of TTX were performed using specimens of *T. granulosa* collected from the most northern parts of their distribution in British Columbia (Prince Rupert and Terrace/Kitimat area) and Southeast-Alaska, Revillagigedo Island and Juneau.

Materials and methods

Sampling

Specimens of *T. granulosa* were collected in August 2016 in forests near the shore of Lakelse and Onion Lakes, south of Terrace, BC, by checking wooden cover boards (placed by the Northern Amphibians Naturalists Society under the NWBC Reptile and Amphibian Monitoring Program) and in April 2017 by dip-netting ditches and ponds near Diana Lake, east of Prince Rupert (Fig. 2). Likewise, newts were obtained by means of dip-netting from a pond on Revillagigedo Island, AK, in June 2013 and May 2018, and from a pond near Juneau, AK (Fig. 2), in May 2018 (for geographical data s. Table 1 and Fig. 1, 2). For TTX-analysis, specimens were sacrificed by freezing, weighed and placed separately into 50-ml tubes containing 80% methanol and 0.1% acetic acid. Extraction of TTX was continued for three weeks.

Tetrodotoxin analysis

A semi-purified TTX sample containing TTX-analogues was prepared from the ovary of puffer fish, *Takifugu flavipterus*, applying a charcoal column and was used as reference in TTX-analysis (Yotsu-Yamashita et al. 2013). TTX in this sample was quantified using post-column liquid chromatography-fluorescence detection (LC-FLD) for TTX (see below for details) based on the standard curve drawn for pure TTX purchased from Sigma-Aldrich Co. (St. Louis, MD). The methanolic extracts of newt samples were evaporated to dryness at 25°C in a stream of compressed air. Each dry residue was dissolved in 0.05 M acetic acid (2.0 ml/g newt), centrifuged, and a part of the supernatant (1 ml) was applied to LC-FLD for the analysis of TTX and its analogues, which was basically performed according to the method of Yasumoto & Michishita (1985) and Shoji et al. (2001) except some technical modifications. LC-pumps Hitachi L-6000 and L-7000 (Hitachi, Tokyo, Japan) were used for the mobile phase and 4 N NaOH, respectively, a fluoromonitor (Jasco FD-2025, Hachioji, Japan), an oil bath (EYELA PS-1000, Tokyo, Japan), and a Hitachi D-2500 Chromato-Integrator were used for this system. TTX and its analogues were separated using a column (Develosil C30 UG-5, 4.6 i.d. × 250 mm; Nomura Chemical, Seto, Japan) and a mobile phase (an aqueous solution containing 1 vol% acetonitrile, 20 mM ammonium heptafluorobutyrate, and 10 mM ammonium formate buffer (pH 5.0)) at 20°C at a flow rate of 0.4 ml/min. The eluted compounds were heated with 4 N NaOH to 105°C in a stainless tube (0.46 mm i.d. × 5 m) set in a silicon oil bath. Next, the reaction products were cooled by letting them flow through the stainless tube (0.46 mm i.d. × 0.2 m) in a cooling jacket filled with cold water and detected by the fluoromonitor with set excitation at 365 nm and emission at 510 nm. Another stainless tube (0.46 mm i.d. × 5 m) was connected to the outlet of the fluoromonitor to avoid bubbling in the cell. The limits of detection of TTX and 6-*epi*TTX were 0.1 and 0.01 µg/g (s/n = 3), respectively.

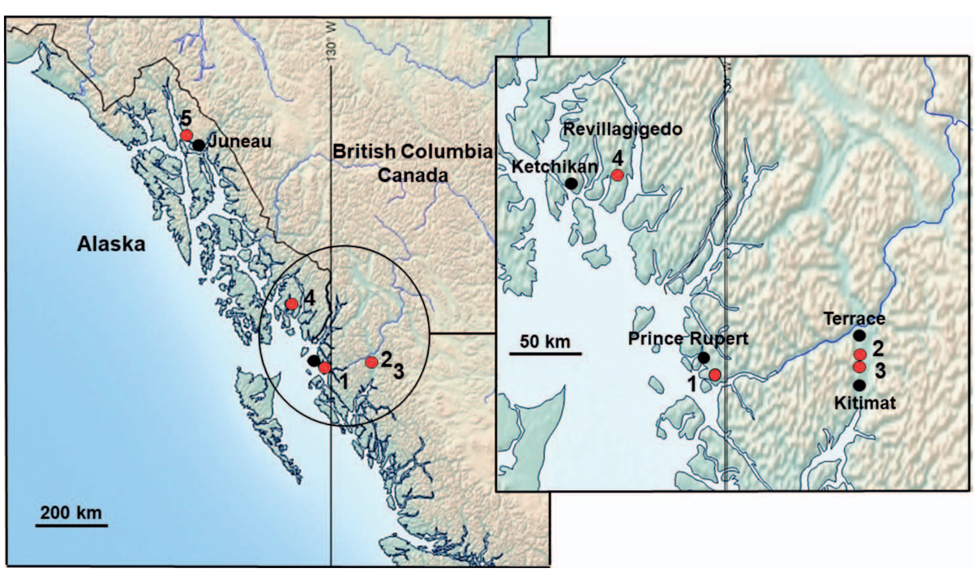

Figure 1. Collection sites of *Taricha granulosa* in northern British Columbia, Canada: 1 – Diana Lake, 2 – Lakelse Lake, 3 – Onion Lake, and in Southeast-Alaska, USA: 4 – Revillagigedo Island, 5 – Juneau. For details s. Table 1.

Table 1. Geographic details of sites where *Taricha granulosa* was sampled (British Columbia, Canada; Southeast-Alaska, USA). Altitude in meters above sea level is abbreviated as m a.s.l. *The cover boards have been placed by the Northern Amphibians Naturalists Society under the NWBC Reptile and Amphibian Monitoring Program (Project coordinator: Dr. Norma Kerby, Terrace, BC).

Location	Collection site	No. of specimens	Date
Lakelse Lake, South of Terrace BC, Canada	Forest near lake shore, under wooden cover boards* 54.2392°N, 128.3185°W, 74 m a.s.l.	5 1	August 20, 2016 May 1, 2017
Onion Lake, South of Terrace, BC, Canada	Forest near lake shore, under logs and dead wood, 54.0231°N, 128.3727°W, 80 m a.s.l.	2	August 25, 2016
Diana Lake, East of Prince Rupert, BC, Canada	Small ponds and ditches next to the lake road, 54.1364°N, 130.1006°W, 41 m a.s.l.	6	April 27/ 28, 2017
Revillagigedo Island, AK, USA	Pond, next to road to Silvis Lake 55.2418°N, 131.2948°W, 205 m a.s.l.	16	June 4, 2013 May 3, 2018
Juneau, AK, USA	Pond near Rt.7, 3rd Andreanoff Dr. 58.2418°N, 134.4509°W, 59 m a.s.l.	16	May 4/5, 2018

Figure 2. Breeding sites of *T. granulosa*. Top: Drainage ditch along the road to Diana Lake near Prince Rupert, BC, Canada (depth about 25 cm; water temperature 8°C on 27 April 2017). Bottom: Pond in Juneau area (maximum depth 50 to 60 cm, water temperature 7°C on 5 May 2018).

Results

Of the newts collected at three locations in northern BC (Fig. 1), only specimens from the Diana Lake area (Fig. 2) exhibited variable, but low concentrations of TTX not exceeding 5 µg/g and of the toxin analogue 6-*epi*TTX ranging from 0.01 to 0.17 µg/g (mean: 0.07 µg/g) (Fig. 5). In specimens from terrestrial habitats at the Lakelse and Onion Lakes (Fig. 4), not even traces of TTX were detected. However, at both locations, only juveniles with body weights of less than 2.0 g were found, suggesting 1.5 to 2 years of age. In newts from Revillagigedo Island in Southeast-Alaska, comparably low TTX-levels were estimated, but an extremely high TTX-value of 125 µg/g was estimated in one specimen (3 g body weight).

In contrast, newts from the pond in the Juneau area (Figs 2, 3, 4) exhibited about ten-times higher TTX-values (Fig. 5). In three of five juvenile specimens that had been collected by turning over logs near the pond, low TTX-concentrations were found (Fig. 5). TTX-analogues were not detected in the newts from Juneau.

Discussion

As a common phenomenon, high variations of TTX-levels occur not only in New World newts, such as in *Taricha* (Hanifin et al. 1999, 2008) and *Notophthalmus* species (Yotsu-Yamashita et al. 2012, Bucciarelli et al. 2014), but also in newt species from Asia, e.g., of the genera *Cynops, Pachytriton, Laotriton* and *Paramesotriton* (Yotsu-Yamashita et al. 2017). As is demonstrated in the present study, this was also observed in populations of *T. granulosa* from the northernmost parts of their distribution range, the panhandle of Southeast-Alaska, USA, and Northwest-BC, Canada, confirming the results of Mebs et al. (2016) and Hague et al. (2016). Contrary to the expectation that TTX-concentrations would be low and even approach negative values in newts from these areas, those from the Juneau population were found to contain toxin levels comparable to those estimated in specimens from Texada Island in the southern part of BC (Mebs et al. 2016).

About 90,000 years ago, the northern parts of North America were under an immense ice cover, forcing the fauna to the south (Barnosky et al. 1987, Mann & Hamilton 1995). After the Pleistocene, about 10,000 years ago, the ice sheet receded and amphibians including *T. granulosa* recolonised Southeast-Alaska and BC by moving up the Pacific coast from southern refugia probably in Oregon or Washington State (Kuchta & Tan 2005, Matsuda et al. 2006).

According to data kindly provided by Dr. Norma Kerby (Terrace, BC, Canada) from the Northern Amphibians Naturalists Society, which has been maintaining monitoring locations and recording sightings of amphibians and reptiles in northern British Columbia, *T granulosa* newts are sparsely distributed in the coastal inland valleys of Northwest-BC and seldom encountered farther north than the Nass Valley and Skeena River area (N 55°.30). In contrast, numerous populations of the newts inhabit the archipelago of Southeast-Alaska (MacDonald & Cook 2007). However, only few records exist for mainland populations, i.e., from Stikine River, Checats Lake, and the boreal interior near Hyder (Carstensen et al. 2009). *T. granulosa* seems to be largely limited to coastal habitats and has most likely migrated northwards up the coast during the Holocene after the great ice age. It could have colonised the present islands when overland connections still existed after the ice retreated (Carstensen et al. 2014). It appears rather unlikely that the newts are resistant to saltwater and were able to colonise the islands unassisted in recent years.

Moreover, human introduction of amphibians, including newts, has been found to be a common practice. Often, they are moved from place to place, captured for educational purposes or kept as pets, and released later (Joshua Ream pers. comm.). Even the possibility that island populations of the newts were established intentionally or accidentally by the native Tlingit people has been contemplated (Richard Carstensen pers. comm.). It has been reported that *T. granulosa* from the Juneau area was introduced in the 1960s from opposite Shelter Island, 2.5 miles off the mainland. Therefore, the specimens collected for this study more than 50 years later are supposed to be descendants from this introduction (Carstensen 2003). Whether the newts on Shelter Island are a natural population, is still not clear, as this locality lies in close proximity to Admiralty Island (1.5 miles) on which they are widespread (MacDonald 2010).

TTX-levels appear to be generally low in populations of *T. granulosa* occurring in areas where toxin-resistant predators such as snakes of the genus *Thamnophis* are not present. This has been demonstrated for newts from Alaska and northern British Columbia, which live outside the geographic range of these snakes (Hague et al. 2016, Mebs et al. 2016). Among them, however, a small number of specimens were found to contain high TTX-concentrations,

Figure 3. Mating ball formed by mass amplexus of *T. granulosa* in the pond near Juneau (5 May 2018).

which correspond to those of newts living in sympatry with *T. sirtalis* in the south.

According to Dr. Norma Kirby (pers. comm.), *T. sirtalis* and *T. elegans* have been sighted in the Lakelse and Onion Lake area, but their distribution range ends halfway between Terrace and Prince Rupert, BC. Garter snakes have never been recorded from the Prince Rupert area, including Diana Lake. The high TTX-levels detected in newts from the Juneau population may indicate that predators other than snakes may trigger toxin-variance, but may also lend support to the assumption that exogenous factors influence toxin-levels.

Figure 4. *Taricha granulosa* from Juneau, AK, male (top), female (centre), and juvenile (below) from the Onion Lake, south of Terrace (BC).

In this respect, the still-debated question of the toxin's biogenetic origin is of interest (Hanifin et al. 2008, Hanifin 2010). In marine animals, such as in puffer fish, TTX is supposed to either be produced by bacterial endosymbionts or be sequestered through the food chain (Noguchi et al. 2006, Noguchi & Arakawa 2008, Itoi et al. 2015). However, no evidence of a bacterial source has been found in newts thus far, as has been investigated in *T. granulosa* (Cardall et al. 2004, Lehman et al. 2004). On the other hand, when raised on an artificial, toxin-free diet, long-term captiv-kept *T. granulosa* showed increased TTX-levels (Hanifin et al. 2002). Cardall et al. (2004) noted a rapid regeneration of the toxin in the skin following the electrically stimulated release of substantial amounts of TTX. These observations led to the conclusion that TTX is of endogenous origin, synthesised along still-unknown metabolic pathways.

Juveniles of *T. granulosa* from the Juneau population either contained marginal concentrations of TTX (3 of 5 specimens, Fig. 5) or the toxin was not detectable (2 specimens). This may indicate that TTX-levels slowly increase during early life stages. From experiments using adult and

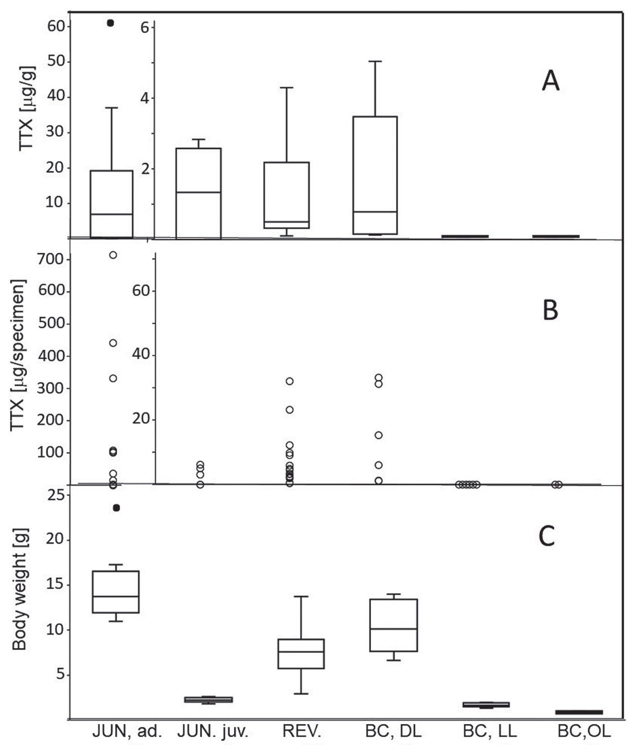

Figure 5. Concentrations of TTX in newts from different collection sites (adults from Juneau "JUN, ad." and juveniles "JUN, juv.", Revillagigedo Island "REV.", British Columbia Diana Lake "BC, DL", Lakelse Lake "BC, LL", and Onion Lake "BC, OL") expressed in µg per g body weight are given as box plots (A), and as total µg per newt (B). Note the different y-axis scale of TTX amounts in adult newts from Juneau, which were much higher than in the other samples. Body weights (g) of the newts are shown as box-plots (C).

larval Californian newts, *T. torosa*, Bucciarelli et al. (2017) concluded that individual variations in TTX-concentrations are environmentally induced. When larvae from the same egg-mass were raised under laboratory conditions or in outdoor stream-mesocosms, they responded differently to environmental circumstances. Laboratory-raised larvae built and maintained higher TTX-concentrations than stream-raised larvae. Over time, adults from the wild were reported to increase and maintain elevated TTX-levels in response to stress conditions. However, these experiments do not provide an answer to the question, whether TTX is produced by endosymbionts responding to environmental changes or whether the toxin is of external origin sequestered from TTX-bearing prey via the food chain.

No conclusive evidence exists for either the endo- or exogenous origin of the toxin. Further research is needed to explore biota of newts to identify local circumstances, which may favour the occurrence of TTX in the newts.

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