

Occurrence of paralytic shellfish toxins in Cambodian Mekong pufferfish *Tetraodon*
turgidus: selective toxin accumulation in the skin

Laymithuna Ngy^a, Kenji Tada^b, Chun-Fai Yu^c, Tomohiro Takatani^b,
Osamu Arakawa^{b,*}

^a*Graduate School of Science and Technology, Nagasaki University, Nagasaki 852-8521, Japan*

^b*Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan*

^c*Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University,
Hung Hom, Kowloon, Hong Kong, China*

*Corresponding author. Tel./fax: +81 95 819 2844.

E-mail address: arakawa@nagasaki-u.ac.jp (O. Arakawa)

Abstract

The toxicity of two species of wild Cambodian freshwater pufferfish of the genus *Tetraodon*, *T. turgidus* and *Tetraodon sp.*, was investigated. *Tetraodon sp.* was non-toxic. The toxicity of *T. turgidus* localized mainly in the skin and ovary. Paralytic shellfish toxins (PSTs), comprising saxitoxin (STX) and decarbamoylsaxitoxin (dcSTX) account for $\approx 85\%$ of the total toxicity. Artificially-reared specimens of the same species were non-toxic. When PST (dcSTX, 50 MU/individual) was administered intramuscularly into cultured specimens, toxins transferred via the blood from the muscle into other body tissues, especially the skin. The majority (92.8%) of the toxin remaining in the body accumulated in the skin within 48 h. When the same dosage of TTX was similarly administered, all specimens died within 3 to 4 h, suggesting that this species is not resistant to TTX. Toxin analysis in the dead specimens revealed that more than half of the administered TTX remained in the muscle and a small amount was transferred into the skin. The presence of both toxic and non-toxic wild specimens in the same species indicates that PSTs of *T. turgidus* are derived from an exogenous origin, and are selectively transferred via the blood into the skin, where the toxins accumulate.

Keywords: Paralytic shellfish toxins; Saxitoxin; Tetrodotoxin; Mekong pufferfish; *Tetraodon turgidus*; Cambodia; Intramuscular administration

1. Introduction

Marine pufferfish of the family Tetraodontidae possess tetrodotoxin (TTX). Toxicity is generally high in the liver and ovary in these fish and human ingestion of these organs often causes food poisoning, especially in Japan (Noguchi and Ebesu, 2001). TTX and paralytic shellfish toxins (PSTs) are potent neurotoxins of low molecular weight (Fig. 1) that inhibit nerve and muscle conduction by selectively blocking sodium channels (Narahashi, 2001). TTX also detected in other organisms, including newts, gobies, some species of frogs, blue-ringed octopuses, carnivorous gastropods, starfish, toxic crabs, flat worms, and ribbon worms (Noguchi et al., 1997; Miyazawa and Noguchi, 2001). These TTX-bearing animals are thought to accumulate TTX through the food chain, starting from the marine bacteria that produce TTX (Noguchi et al., 2006).

Small-sized pufferfish from brackish water or freshwater also possess paralytic toxins, mainly in their skin, and occasionally cause food poisoning and fatalities in humans in Asian-Pacific countries, such as Thailand and Bangladesh (Laobhripatr et al., 1990; Mahmud et al., 2000; Panichpisal et al., 2003). The toxic principles are different depending on the species and/or their habitats. For example, *Tetraodon nigroviridis*, *T. steindachneri*, and *T. ocellatus* collected from Thailand or Taiwan possess TTX (Mahmud et al., 1999a, b; Lin et al., 2002), whereas the main toxins of *T. leiurus*, *T. suvatii* from Thailand, *T. cutcutia*, *Chelonodon patoca* from Bangladesh, and *Colomesus asellus* from Brazil are PSTs (Kungsuwan et al., 1997; Zaman et al., 1997, 1998; Oliveira et al., 2006). The presence of both TTX and PSTs within the same species is also documented for the Thailand freshwater pufferfish *T. fangi* (Saitanu et al., 1991;

Sato et al., 1997), whereas palytoxin-like substance(s) in addition to PSTs are detected in the Bangladeshi specimens of *Tetraodon sp.* (Taniyama et al., 2001). Similarly, some marine pufferfishes possess PSTs as their main toxin (Nakashima et al., 2004; Landberg et al., 2006). Although the origin of PSTs in freshwater pufferfish is unclear, they possibly derive from the food chain, starting from PST-producing cyanobacteria (Lagos et al., 1999; Pereira et al., 2000).

In Cambodia, poisoning incidents from the consumption of freshwater pufferfish collected from lakes and rivers are common and sometimes result in human fatalities. Because no toxicologic information on these fish is currently available, we examined the toxicity and toxin profiles of two Cambodian indigenous freshwater pufferfish species, *T. turgidus* and *Tetraodon sp.* In addition, to elucidate the mechanisms of toxin accumulation, metabolism, and elimination in pufferfish, both PST and TTX were administered intramuscularly into artificially reared specimens of *T. turgidus* (non-toxic as compared with their wild counterparts), and the subsequent changes in the distribution of toxins inside their bodies were investigated.

2. Materials and methods

2.1. Toxicity assay and toxin identification of wild pufferfish specimens

2.1.1. Pufferfish specimens

Wild specimens of the Mekong pufferfish *T. turgidus* and *Tetraodon sp.* were collected from several lakes in Kandal and Phnom Penh, Cambodia, respectively, within two successive months during rainy (April–May, 2005) and dry (December 2005–January 2006) seasons. These specimens were immediately frozen, transported by

air to our laboratory in Nagasaki University, and stored below -20°C until assay.

2.1.2. Toxicity assay

After thawing, the specimens were dissected into different anatomic tissues: skin, muscle, liver, intestine, and gonads (testis/ovary). Each tissue was examined for its toxicity by mouse bioassay according to the methods of the Association of Official Analytical Chemists (AOAC, 2003). Lethal potency was expressed in mouse units (MU), where one MU is defined as the amount of toxin required to kill a 20-g male mouse of ddY strain within 15 min after intraperitoneal administration.

2.1.3. Toxin identification

After mouse bioassay, a part of each tissue extract was filtered through a USY-1 membrane (0.45 μm ; Toyo Roshi Co., Ltd, Japan), and toxin identification was performed by high performance liquid chromatography with post-column fluorescence derivatization (HPLC-FLD) for PST and liquid chromatography/mass spectrometry (LC/MS) for TTX.

HPLC-FLD was performed on a Hitachi L-7100 HPLC system according to the method previously reported (Arakawa et al., 1995; Oshima, 1995; Samsur et al., 2006). Gonyautoxins 1–4 (GTX1–4), decarbamoylgonyautoxins 2, 3 (dcGTX2, 3), and neosaxitoxin (neoSTX), which were provided by the Fisheries Agency, Ministry of Agriculture, Forestry and Fisheries of Japan, as well as saxitoxin (STX) and decarbamoylsaxitoxin (dcSTX), prepared as reported previously (Arakawa et al., 1994), were used as standards to identify and quantify each individual analogue.

LC/MS was conducted on an Alliance separation module (Waters Corporation)

equipped with a ZSprayTM MS 2000 detector (Micromass Limited) as reported previously (Nakashima et al., 2004; Ngy et al., 2007). TTX standard was purchased from Wako Pure Chemical Industries, Ltd., Japan.

2.2. Investigation of toxin transfer inside the bodies of artificially reared pufferfish specimens

2.2.1. Pufferfish specimens

Artificially reared specimens of *T. turgidus* (body weight, 15.0 ± 2.6 g; body length, 6.0 ± 0.5 cm; $n = 33$) were provided by Kaikyokan (Shimonoseki City Aquarium), Japan. These specimens, hatched from artificially fertilized eggs, were reared in an aquarium with non-toxic foods for approximately 7 months. Toxins were quantified in 3 of the 33 specimens as described below for the non-administration (NA) group. The remaining specimens (30) were acclimatized in aerated tanks for 4 days before subsequent toxin administration experiments.

2.2.2. Preparation of PST and TTX solutions

Both dcSTX (purity more than 70%), prepared from the xanthid crab *Zosimus aeneus* according to the method previously reported (Arakawa et al., 1994), and TTX, purchased from Wako (purity more than 90%), were dissolved individually in a physiologic saline solution containing 1.35% NaCl, 0.06% KCl, 0.025% CaCl₂, 0.035% MgCl₂ and 0.02% NaHCO₃ at a concentration of 500 MU/ml and used in the following toxin administration experiments.

2.2.3. Toxin administration experiments

The acclimatized pufferfish specimens were divided into two groups of 15 individuals, one group was administered PST (PST group) and the other was administered TTX (TTX group), and then maintained separately in two aerated 90-L tanks. Each specimen was intramuscularly administered 0.1 ml (equivalent to 50 MU) of either PST or TTX solution and returned to the tank immediately (total handling time <30 s/individual to minimize stress to the fish). Subsequently, five specimens from the PST group were randomly collected at 12, 24, and 48 h after toxin administration and toxin quantification was performed as described below. The specimens of the TTX group all died unexpectedly within 4 h of administration and also underwent toxin quantification shortly after death.

2.2.4. Toxin quantification

Using a syringe precoated with sodium heparin, all the blood was withdrawn from the portal vein of each specimen in the PST group and centrifuged at 4200 *g* for 10 min. The supernatant (blood plasma) obtained was ultrafiltered through an Ultrafree-MC 5000 NMWL (Millipore Corp., Bedford, MA) and submitted to HPLC-FLD analysis for PSTs. After blood collection, all specimens were dissected into different anatomic tissues and extracted with 0.1 N HCl (AOAC, 2003). The tissue specimens in the NA and TTX groups were dissected and extracted similarly without blood collection. Each tissue extract from all groups was filtered through a USY-1 membrane (0.45 μm ; Toyo Roshi Co., Ltd, Japan) and submitted to HPLC-FLD analysis for PSTs (NA and PST groups) and/or enzyme-linked immunosorbent assay (ELISA) for TTX (NA and TTX groups). ELISA was performed according to the following documented method (Kawatsu et al., 1997) with some modifications as follows:

Antigen coating: TTX conjugated with bovine serum albumin (Sigma, Chemical Co., St. Louis, MO) was dissolved in 0.05 M sodium bicarbonate buffer at a concentration of 1 µg/ml, and 100 µl of the solution was added to each well of a 96-well microtiter plate (Sumitomo Bakelite Co., Ltd, Japan). The plate was incubated overnight at 4°C, and the wells were washed three times with Dulbecco's phosphate buffered saline containing 0.05% Tween 20 (PBS-T). The residual protein-binding sites were blocked with 100 µl/well of 10% horse serum in PBS-T (HS-PBS-T) at room temperature for 1 h, and the wells were washed four times with PBS-T.

Reaction with antibodies: To each well of the antigen-coated plate was added 100 µl of each tissue extract, its dilution, or TTX standards (2–200 ng/ml), followed by 50 µl of HS-PBS-T containing 0.2 µg/ml of anti-TTX antibody. After 1 h of incubation at room temperature, the wells were washed four times with PBS-T, and then 100 µl of goat anti-mouse immunoglobulin-peroxidase conjugate (Sigma, Chemical Co., USA) diluted with HS-PBS-T by 1:1000 was added to each well. After 1 h of incubation at room temperature, the wells were washed four times with PBS-T.

Reaction with substrate solution: One hundred microliters of a substrate solution, which was prepared just before use by mixing 100 µl of a 3,3',5,5'-tetramethylbenzidine (Dojin, Japan) solution (10 mg 3,3',5,5'-tetramethylbenzidine in 1 ml of dimethylformamide), 150 µl of 0.3% hydrogen peroxide, and 10 µl of 0.05 M citrate buffer (pH 5.5), was added to each well and the plates were incubated for 10 min at room temperature in darkness. The reaction was terminated with 100 µl of 1 M H₂SO₄, and the absorbance of each well was measured at 450 nm with a microplate-auto reader (Model 550, Shimazu, Co., Japan).

TTX quantification: Percent absorbance of each well was calculated with the

assumption that the absorbance value of 0 ng/ml TTX was 100%. The TTX concentration (ng/ml) of each sample was then determined from the calibration curve that was drawn using the percent absorbance values for TTX standards. The amount of TTX (in ng) was converted to MU based on the specific toxicity of TTX (220 ng/MU).

3. Results

3.1. Toxicity and toxin profiles of wild pufferfish specimens

3.1.1. Toxicity profile

The toxicity of the wild Cambodian Mekong pufferfish *T. turgidus* is shown in Table 1. All the specimens were toxic, irrespective of the collection season. The toxicity was localized in the skin (4–37 MU/g) and ovary (15–27 MU/g), and the other tissues were all non-toxic (less than 2 MU/g). In contrast, no toxicity was detected in any tissues of the 15 specimens of *Tetraodon sp.* (data not shown).

3.1.2. Toxin profile

In HPLC-FLD analysis for PSTs, tissue extracts from both the skin and ovary of *T. turgidus* produced a main peak and a minor peak whose retention times (17.8 and 15.9 min) were consistent with those of standard STX and dcSTX, respectively (Fig. 2). The molar ratio of STX and dcSTX were calculated to be approximately 9:1. Neither other PST analogues nor TTX were detected in LC/MS (data not shown). Comparison of toxicity scores obtained from the mouse bioassay and toxicity scores calculated from the peak areas of STX and dcSTX in HPLC-FLD indicated that the main toxin in this species was PST, accounting for approximately 85% of the total toxicity detected in the

mouse bioassay (data not shown).

3.2. Toxin administration in artificially reared pufferfish specimens

3.2.1. Toxicity of artificially reared specimens

Neither PSTs nor TTX were detected in any tissues of the three artificially reared specimens of *T. turgidus* (NA group) by HPLC-FLD for PST (detection limit 0.1 MU/g) or ELISA for TTX (detection limit 0.01 MU/g).

3.2.1. Administration of PST

All 15 *T. turgidus* specimens of the PST group survived without any abnormal symptoms after administration of dcSTX at a dose of 50 MU/individual.

Changes in the toxin content (MU/g) of each pufferfish tissue during the rearing period are shown in Fig. 3-A. At 12 h after administration, the skin contained the highest amount of toxin (5.2 MU/g), followed by the intestine (4.3 MU/g) and liver (4.0 MU/g), while the muscle, which was the site of administration, retained a much lower amount (0.9 MU/g). In the blood plasma, the toxin was detected at a concentration of 1.8 MU/ml. Thereafter, the toxin content of the skin increased rapidly and reached 13.1 MU/g at 48 h, whereas those of the other tissues, including the plasma, gradually decreased to a very low level (intestine, 1.9 MU/g; other tissues, less than 1.0 MU/g or MU/ml).

Changes in the anatomic distribution of PST, demonstrated by the amount of toxin retained in each tissue (MU/individual), are shown in Fig. 3-B. The total amount of toxin remaining in the whole body (22.5–25.9 MU/individual) was nearly stable during the entire rearing period, which corresponded to approximately 45% to 50% of the

administered toxin. The amount of toxin transferred to the skin within 12 h (13.4 MU/individual) was greater than that transferred to the other tissues (≤ 3.6 MU/individual), and increased up to 24.0 MU/individual within 48 h, accounting for 92.8% of the total amount of toxin remaining in the body.

In HPLC-FLD analysis, no PST analogues other than dcSTX were detected in any tissue (data not shown).

3.2.2. TTX administration

From 5 to 15 min after TTX administration (50 MU/individual), all 15 specimens of the TTX group began to show abnormal symptoms, such as jerky swimming movements, loss of equilibrium, and lying down on the tank bottom with shallow and arrhythmic gill movements. All 15 eventually died within 3 to 4 h.

The anatomic distribution of TTX in the dead specimens is shown in Fig. 4. The majority of the toxin was detected in the muscle (25.3 MU/individual), whereas only 2.0 to 6.3 MU/individual was observed in other tissues, though their toxin contents per 1 gram of tissue were more or less similar to each other (8.7–10.6 MU/g). In marked contrast to the PST group, only a very limited amount of toxin (1.1 MU/g or 2.4 MU/individual) was transferred to the skin. The total amount of toxin remaining in the whole body was 36.0 MU/individual, which corresponded to 72.0% of the administered toxin, and the majority (70.3% of the remaining toxin) was detected in the muscle.

4. Discussion

Of the two wild Cambodian freshwater pufferfish species examined in the present

study, *T. turgidus* was toxic and *Tetraodon* sp. was non-toxic, both in the rainy and dry seasons. As in the freshwater or brackish water species previously reported (Laobhripatr et al., 1990; Mahmud et al., 1999a, b; Lin et al., 2002; Panichpisal et al., 2003), the toxin localized mainly in the skin (Table 1), and the toxicity scores (4–37 MU/g) were comparable to those of the Bangladeshi freshwater pufferfishes *T. cutcutia* (2–20 MU/g) and *C. patoca* (2–40 MU/g) (Zaman et al., 1997), and the Brazilian freshwater species *C. asellus* (19–53 MU/g) (Oliveira et al., 2006). Because the minimal lethal dose (MLD) of PST in humans is estimated to be 3000 MU (Noguchi et al., 1997), the consumption of approximately 100 g of the skin or ovary (with a toxicity of approximately 30 MU/g) can be fatal. Therefore, *T. turgidus* is considered a hazardous species unsuitable for human consumption and might be one of the causative species in the past pufferfish poisonings that have occurred in Cambodia.

The main toxin in *T. turgidus* is PST comprising STX as the main and dcSTX as the minor analogue (Fig. 2), which accounted for about 85% of the total toxicity. Neither TTX nor other PST components such as neoSTX and GTXs, which are present in some other freshwater pufferfishes (Kungsuwan et al., 1997; Oliveira et al., 2006), were detected in the present study. Zaman et al. (1997, 1998) reported the presence of carbamoyl-*N*-methyl derivatives of STX and GTXs, which were undetectable by HPLC-FLD analysis, in Bangladesh freshwater puffers. The remaining toxicity (15%) of *T. turgidus* might be explained by such ‘invisible’ derivatives, though further studies are needed to clarify this point.

In contrast, neither PST nor TTX were detected in the artificially reared specimens of *T. turgidus*. Marine puffer species such as *Takifugu niphobles* and *T. rubripes* are also non-toxic when they are artificially reared on non-toxic diets after hatching (Matsui et

al., 1982; Saito et al., 1984). Furthermore, such non-toxic pufferfish become toxic when orally administered TTX (Matsui et al., 1981; Yamamori et al., 2004; Honda et al., 2005), indicating that TTX in the pufferfish must be derived from an exogenous origin, i.e., toxic food organisms (Noguchi et al., 2006). The present result strongly suggests that PSTs in the freshwater pufferfish are also exogenous. The toxic food organism(s) ingested directly by the pufferfish, however, have not yet been identified, though some species of cyanobacteria are known to produce PST in a freshwater environment (Lagos et al., 1999; Pereira et al., 2000).

When PST (dcSTX, 50 MU/individual) was intramuscularly administered into the artificially reared specimens, the toxin was rapidly transferred from the muscle to the other tissues. The amount of toxin transferred to the skin within 12 h after administration was 13.4 MU/individual and gradually increased thereafter. The majority (24.0 MU/individual or 92.8%) of the toxin remaining in the body eventually accumulated in the skin (Fig. 3). To our knowledge, this is the first study to examine the rapid accumulation of PSTs in freshwater pufferfish skin. In contrast, only a small portion of the toxin (3.6 and 0.8 MU/individual, respectively) moved transitorily to the intestine and liver at 12 h and decreased thereafter. These results, combined with detection of the toxin in blood plasma and the finding that PSTs found in wild specimens localize mainly in the skin, suggest that freshwater pufferfish are equipped with a particular mechanism whereby PSTs taken into the body are selectively transferred and accumulated in the skin via the blood. TTX-binding proteins, which also bind to PSTs, are present in the blood plasma of marine pufferfishes, which suggests that TTX is transported via the blood in the puffer body (Matsui et al., 2000; Yotsu-Yamashita et al., 2001).

When TTX (50 MU/individual or 67 MU/20 g body weight) was similarly administered into the artificially reared specimens, all the specimens died within 3 to 4 h. Saito et al. (1985a) reported that the MLD of TTX administered intraperitoneally to toxic marine pufferfishes is 300 to 750 MU/20 g. Thus, if the MLD via both intraperitoneal and intramuscular administration is comparable, it can be estimated that the MLD of TTX to *T. turgidus* was less than 67 MU/20 g, or approximately 1/10 that of TTX-bearing marine species. In contrast, all specimens administered PST at the same dose (67 MU/20 g) survived without any abnormal symptoms. Saito et al. (1985b) reported that the MLD of PST (GTXs) administered intraperitoneally to the toxic marine pufferfish *T. niphobles* was 14 to 29 MU/20 g. Because the MLD of PST in *T. turgidus* appeared to be higher than 67 MU/20 g, it can be inferred that TTX-bearing marine pufferfishes are endowed with a high tolerance specifically against TTX, whereas PST-bearing freshwater species have a tolerance specifically against PST.

More than half (25.3 MU/individual) of the administered TTX remained in the muscle, and only a small amount (2.4 MU/individual) was transferred to the skin (Fig. 4). Because the rearing period after toxin administration (3–4 h) was much shorter than that in the PST administration experiment (12–48 h), the results of the two experiments cannot be directly compared. In other experiments, however, we observed that TTX administered intramuscularly to non-toxic cultured specimens of the marine pufferfish *T. rubripes* moved into the other tissues, including skin, very rapidly, usually within a few hours (unpublished data). These findings suggest that the above-mentioned toxin-transfer/accumulation mechanism in freshwater pufferfish is PST-specific and acts less effectively against TTX. This point, in addition to the properties of TTX/PST-binding proteins in marine and freshwater pufferfishes, remains to be

elucidated. Further studies along these lines are in progress.

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Figure Captions

- Fig. 1 Chemical structures of TTX (left) and PSTs (right).
- Fig. 2 Chromatograms of the standard toxins (neoSTX, dcSTX, and STX), and of tissue extracts from the wild specimens of *T. turgidus*.
- Fig. 3 Changes in the content (MU/g) (A), and the total amount (MU/individual) (B) of PST retained in each tissue of the artificially reared specimens of *T. turgidus* during the rearing period after toxin administration.
- *Drawn based on the calculated values, assuming that 100% of the administered toxin was retained in the muscle.
- Fig. 4 Anatomic distribution of TTX remaining in the dead specimens of *T. turgidus*.

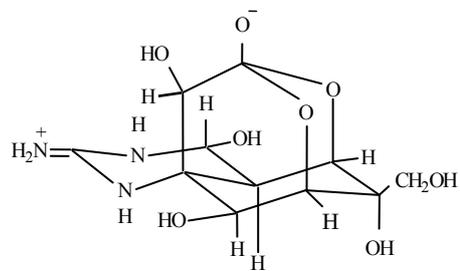
Table 1
Anatomic distribution of toxicity in the wild specimens of *T. turgidus*

Collection data	Specimen no.	Sex	Body size		Toxicity determined by mouse bioassay (MU/g)				
			Weight (g)	Length (cm)	Skin	Muscle	Liver	Intestine	Testis/ovary
Rainy season (Apr–May 2005)	1	♂	24.6	9.0	8	<2	<2	<2	—
	2	♂	17.6	8.4	6	<2	<2	<2	—
	3	♂	14.7	7.5	24	<2	<2	<2	—
	4	♂	13.9	7.5	37	<2	<2	<2	—
	5	♂	12.3	7.3	15	<2	<2	<2	—
	6	♂	9.1	6.7	9	<2	<2	<2	—
	7	♂	6.4	6.6	13	<2	<2	<2	—
	8	♂	5.9	5.7	4	<2	<2	<2	—
	9	♀	23.1	7.1	14	<2	<2	<2	15
	10	♀	18.9	8.5	27	<2	<2	<2	—
	11	♀	14.2	6.2	12	<2	<2	<2	19
	12	♀	13.2	7.1	8	<2	<2	<2	—
Dry season (Dec 2005–Jan 2006)	13 ^a	♂/♀	12.6	5.8	14	<2	<2	<2	
	14 ^a	♂/♀	12.2	4.6	12	<2	<2	<2	
	15 ^a	♂/♀	12.1	5.9	11	<2	<2	<2	
	16 ^a	♂/♀	11.4	5.5	12	<2	<2	<2	27 ^b
	17 ^a	♂/♀	10.2	5.6	10	<2	<2	<2	
	18 ^a	♂/♀	9.6	5.6	14	<2	<2	<2	

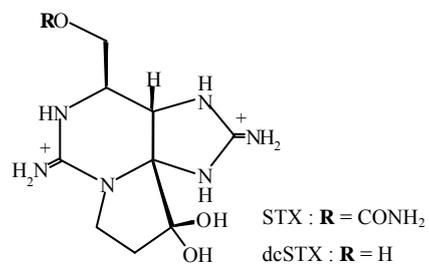
—: too small to assay.

^apooled specimen of 10 individuals.

^bpooled ovary of no. 13–18.



TTX



PSTs

Fig. 1 Ngy et al.

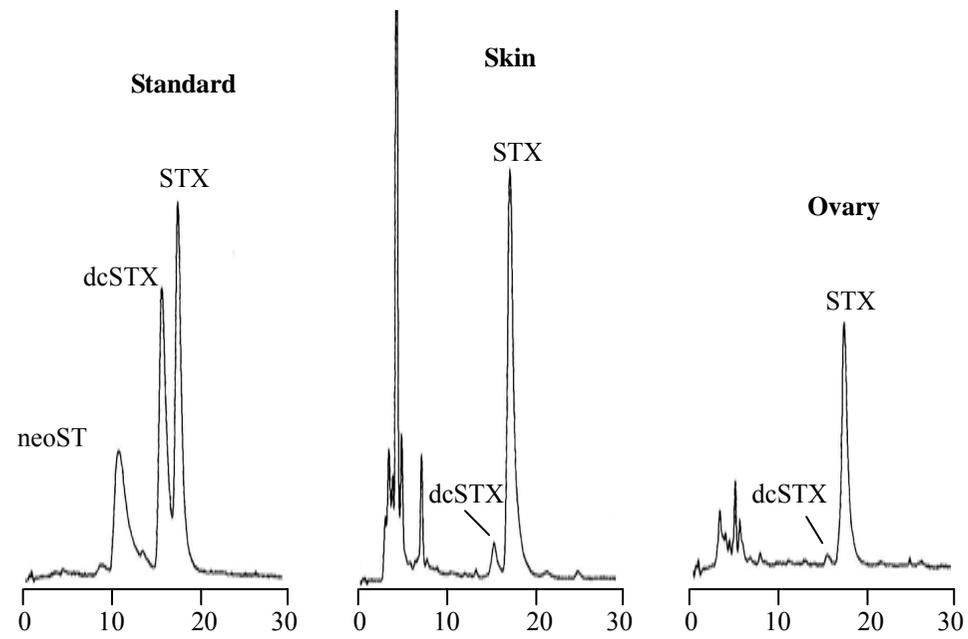


Fig. 2 Ngy et al.

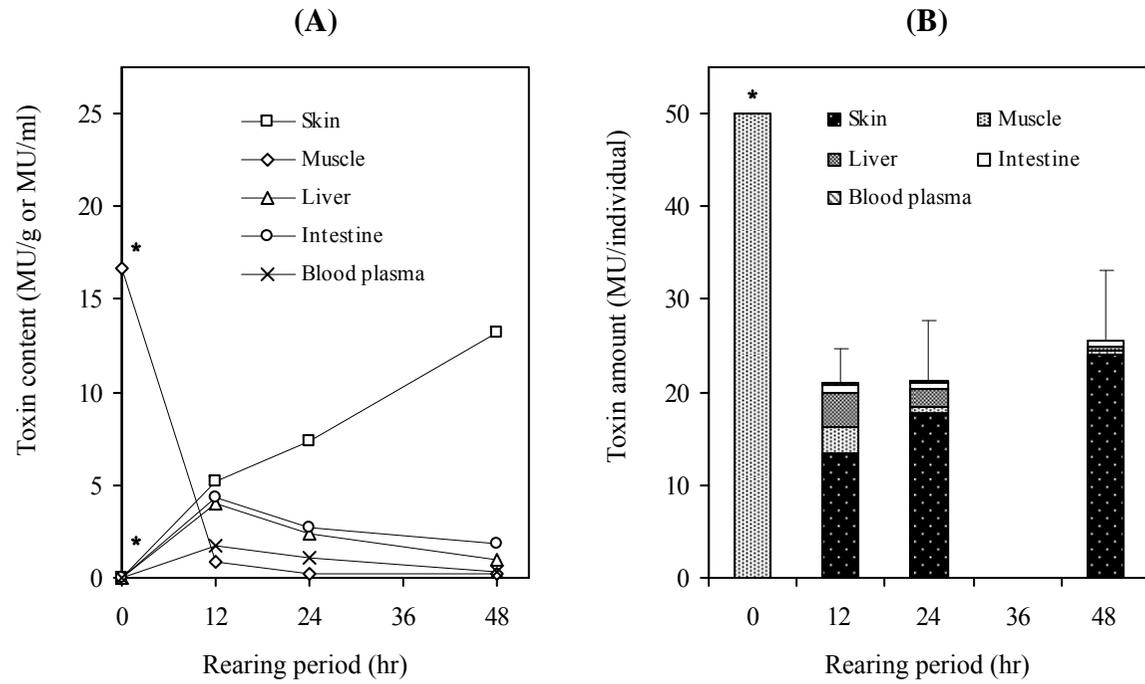


Fig. 3 Ngy et al.

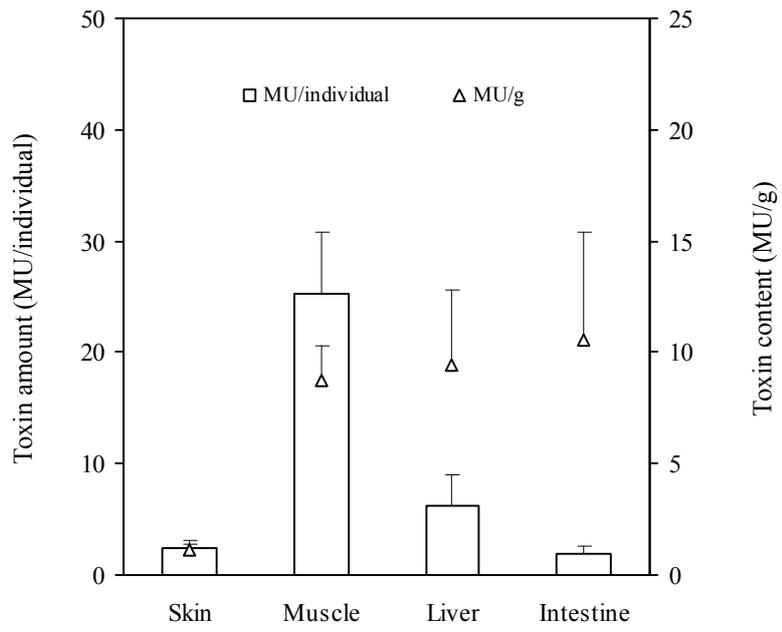


Fig. 4 Ngy et al.