Title:

Administration of tetrodotoxin protects artificially-raised juvenile tiger puffer *Takifugu rubripes* from predators

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Abstract:

We examined the effects of tetrodotoxin (TTX) administration to the artificially-raised tiger puffer *Takifugu rubripes* juveniles on the survival after release into a mesocosm with predators in order to clarify the ecological significance of TTX. Artificial pellets containing 3 different concentrations of TTX (0 as control, 7, 14 MU/g·diet) were fed to non-toxic artificially-raised *T. rubripes* juveniles for 10 days. TTX accumulation in the various tissues of fish was detected except for control diet group, and TTX administration did not affect survival or growth of the fish. Then, a hundred fish from each diet group were released together into a salt-pond mesocosm (2,650 m^2) with predators (*Lateolabrax* sp.) for 5 days. Survival after release was significantly higher in the fish fed with TTX both 7 MU/g·diet (62 %) and 14 MU/g·diet (74 %) than the control fish (32 %).

Keywords:

tetrodotoxin · puffer · predation defense · mesocosm.

1. Introduction

Tetrodotoxin (TTX) is one of the most potent nonproteinaceous toxins known, responsible for numerous fish poisonings [1], and is especially known in pufferfishes in the order Tetradontiformes. Since the food poisoning caused by pufferfish is a serious hazard to public health in Japan, tremendous attentions have been paid to the epidemiological studies (see reviews [2-4]). On the other hand, ecological significance of possessing tetrodotoxin by pufferfish has not been clearly revealed. It is widely accepted that pufferfish accumulates TTX via the food chain [5,6] which is originally produced by bacteria of the genera *Vibrio* and *Shewanella* [7-10]. Pufferfish accumulates TTX throughout the life stage in the wild and part of the accumulated TTX are transferred into ovary and eggs when they matured [11,12]. Recently, Itoi et al [13] revealed that maternal TTX of pufferfish (tiger puffer *Takifugu rubripes* and grass puffer *T. niphobles*) is primarily localized in the body surface of the larval pufferfish, and observed that various predatory fishes ingested pufferfish larvae but spat them out promptly. Their study demonstrated that miniscule amounts of TTX in pufferfish larvae can be detected by the predatory fishes and TTX has an apparent function for protection from predators in the early life stage of pufferfish.

Tiger puffer *T. rubripes* is a commercially important species in Japan, and the stock enhancement programs have been being practiced due to the decline of natural stocks [14]. It is reported that the major cause of mortality in artificially-raised *T. rubripes* juveniles is predation after release [15,16]. Shimizu et al [17,18] elucidated that there are behavioral deficits of anti-predator response in the artificially-raised tiger puffer juveniles, and that artificially-raised tiger puffer does not possess TTX while all wild juveniles are toxic. It is known that artificially-raised *T. rubripes* becomes non-toxic when fed with non-toxic diets in an environment where the invasion of TTX-bearing organisms was eliminated [19,20]. Such non-toxic *T. rubripes* juveniles are attracted to TTX by olfactory [21] and accumulate TTX when they are fed TTX-containing diet [22]. Furthermore, TTX was detected not only in liver but also basal cell of skin both in the wild juveniles and artificially-raised juveniles to which TTX were orally administrated [22].

Thus, we hypothesized that bearing of TTX in the skin of *T. rubripes* juveniles may be functional as predator defense same as in the larval stage of pufferfish [13]. To test this hypothesis, we fed diets containing different amount of TTX to non-toxic artificially-raised *T. rubripes* juveniles. Then, we conducted a release experiment in a salt pond mesocosm with predators and determined whether the survival of *T. rubripes* juveniles is affected by TTX accumulation.

2. Materials and methods

2.1 Experimental fish

Tiger puffer *T. rubripes* juveniles were purchased from a private fish farmer (Tawaki-Suisan Co., Kumamoto, Japan). They were cultured in an indoor tank from hatching on 22 May 2008 and were transferred to Research Center for Marine Invertebrates, National Research Institute of Fisheries and Environment of Inland Sea, Japan Fisheries Research and Education Agency (FRA), Japan on 3 July 2008 (42 days after hatching). Fish were kept as a

stock in a net cage held in a 40 kl concrete tank with flow-through system and were fed with commercial diet (Otohime S2, Marubeni Nisshin Feed Co., Ltd., Japan) until satiation 6 times daily.

2.2 Experimental diet

TTX was purified from the ovaries (1.4 kg) of wild-caught 3 adult *T. rubripes* according to the method of Ikeda et al [23] with a slight modification. The extract was partially purified with Bio-Gel P-2 column (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the absorbed TTX by the gel was eluted with 0.05 M AcOH. TTX fraction was analyzed by LC/MS analysis on an Alliance LC/MS system equipped with a ZSpray MS detector (Waters, Milford, MA, USA) following Nakashima et al [24]. The amount of TTX (in ng) determined by LC/MS was converted to MU (mouse unit) based on the specific toxicity of TTX (220 ng/MU). Purified TTX was dissolved in distilled water at the toxicity of 1,678 MU/ml. TTX solution (24 ml), distilled water (2 ml) and 6 g of soy lecithin (Nacalai Tesque Inc., Japan) were homogenized in an ice bath for 3 min at 14,000 rpm. Then, TTX containing emulsion was made by adding 8 ml of cod liver oil (Riken Feed Oil Omega, RIKEN Vitamin Co., Ltd, Japan) and homogenizing TTX solution and feed oil in an ice bath for 3 min at 14,000 rpm. Control emulsion was also prepared in the same manner of TTX containing emulsion replacing same amount of TTX solution with distilled water. Three different combinations of emulsion were prepared; control (40 ml), 25 MU (30 ml control and 10 ml of TTX containing emulsion), 50 MU (20 ml control and 20 ml of TTX containing emulsion). Each emulsion was sprayed onto the 360 g of diet (Otohime EP1) adjusting the concentration of TTX with 0, 25 and 50 MU/g·diet, respectively. A part of these diets were subjected to the measurement of concentrations of adsorbed TTX in diet as described above. The effective concentrations of TTX in 3 diets were, 0, 7 and 14 MU/g·diet, respectively.

2.3 TTX administration

The toxin administration was carried out for 10 days from 12 July 2008. A total of 600 cultured juveniles were taken from the stock cage and were randomly divided into 3 groups. All fish were marked individually using visible implant elastomer tags (VIE, Northwest Marine Technology, Inc., USA) to discriminate the following 3 diet groups. Fish in each diet group were fed with 3 different TTX-containing diets (0, 7 and 14 MU/g·diet). Fish were kept in 2 kl tank for each diet group with flow through system (2 kl/hour) and were fed 6 times a day with 3-7 % body weight (BW) on each diet group. Experimental tanks were located outdoor and water temperature ranged 25.1-30.5 °C during the trial.

At the initial day of feeding trial, 60 fish were sampled from the stock cage prior to assigning the fish for TTX administration (standard length, SL, 4.1±0.4 cm; BW, 2.3±0.7 g; average±standard deviations, n=60). Then, 20 fish per diet group were randomly collected at 5 days after toxin administration, and all survived fish after 10 days TTX administration were counted and measured and 9-18 fish from each diet group were sampled. All sampled fish were stored at -20 °C until TTX analysis. We measured SL and total length of each fish by a digital caliper (CD20-GM, Mitsutoyo Corp., Japan) and BW by an electric balance (PB153-S,

Mettler-Toledo Inc., USA) up to 2 decimal digits. Degree of loss of caudal fin (DLCF) was calculated with following equation (1) [25]

$$DLCF(\%) = \left(1 - \frac{Lth - Lsh}{Ltw - Lsh}\right) \times 100, \quad (1)$$

where, *Lth* and *Lsh* indicate the TL and SL of a measured fish, and *Ltw* is an estimated TL from the wild fish of the same SL which has no loss of caudal fin from the following equation (2).

$$Ltw = 1.1806 \times Lsh + 6.0142 (n = 4{,}019, R^2 = 0.991), \quad (2)$$

DLCF is used as an indicator of degree of agonistic interactions in tiger puffer where high DLCF shows higher loss of caudal fin of a fish by being nipped from other individuals.

We quantified TTX concentrations from the fish at initial, 5 and 10 days after TTX administration. Fish from each diet group at the same sampling date (*n*=9-20) were dissected into different anatomic tissues (liver, skin, muscle, brain and others) and weighed by an electric balance. These tissues were pooled with 2-3 individuals and were extracted with 0.1% acetic acid [26]. Each extract was filtered through a 0.45 μm cellulose acetate membrane (DISMIC-13CP, ADVANTEC, Tokyo, Japan) and subjected to LC/MS analysis [24]. Toxicity of each tissue (MU/g·tissue) was converted into an amount of 1 fish with average BW of the pooled individuals. We also collected wild *T. rubripes* juveniles (SL 8.6±0.6 cm, BW 9.9±1.5 g, *n*=10) as a reference from a set net at off Kasaoka city, Okayama prefecture, Japan on 3 August 2009. Wild juveniles were dissected and TTX were quantified in the same manner as described above. Because of the small sample size and the uncertainty of TTX amount, tissues of 10 fish were pooled and measured and then converted into an average value of 10 fish.

2.4 Release experiment in a mesocosm

The mesocosm used in this study is an artificial outdoor pond (2,650 m^2) that is a revamped saltpan at FRA [16]. The pond experiences tidal seawater exchange through an inlet with pond water volumes of 3,250–4,500 m^3; its average depth is 1.8 m. Screens (5 mm mesh) installed at the inlet and drain outlet prevent movement of larger animals in and out of the pond, while allowing the inflow of zooplankton into the pond. Animals that dominantly appeared in the pond water were copepoda and mysidacea [27], and amphipoda and polychaeta dominated the benthos [28], producing an environment resembling that of natural tidal flat. Fifty sea bass *Lateolabrax* sp. (TL 39.7±1.7 cm), which were artificially-raised by a local hatchery (Kaneto Suisan Co., Fukuyama, Japan), were introduced into the mesocosm 3 days before the release of tiger puffer juveniles.

A total of 300 tiger puffer juveniles (100 fish from each diet group) were released into the mesocosm for 5 days from 23 July 2008. Five of the sea bass were captured 4 hours after release of tiger puffer (day 0) and each day using a gill net throughout the trial period to check their stomach contents. At the end of the trial, all pond seawater was drained and then all surviving released fishes were collected. All surviving tiger puffer juveniles were individually

discriminated by VIE to check the diet group and the survival rate was calculated for each diet group. Then, TL, SL and BW were measured and DLCF were calculated. Twenty fish of each diet group were subjected to gut contents analysis.

2.5 Statistical analysis

Survival rate among 3 diet groups during TTX administration period and at the end of the release trial were compared using Chi-square test followed by Tukey's wholly significant difference analysis. Differences in mean values of growth parameters (SL, BW and DLCF) and TTX accumulation among diet groups during the TTX administration period were compared using 2-way ANOVA followed by Tukey-Kramer HSD test. Differences in mean values of growth parameters (SL, BW and DLCF) among diet groups during the release trial were compared using 2-way ANOVA followed by Tukey-Kramer HSD test.

Statistical analysis was carried out using R. version 2.15.3 (R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/ "Accessed 20 June 2015") and p-values < 0.05 were considered significant in all analyses.

3. Results

3.1 TTX administration

Survival and growth of tiger puffer juveniles during TTX administration are shown in Table 1. Survival (60.6-65.5 %), SL (4.8-5.0 cm) and BW (3.6-3.8 g) were not different among diet groups. DLCFs were also not different among diet groups, whereas average DLCFs in TTX containing diet groups (67.5-73.1 %) showed lower trend than the control diet group (79.3 %).

Fish fed with TTX containing diets accumulated TTX in various tissues, such as liver, muscle, skin and brain, and TTX was mostly detected from skin and muscle (Fig.1). TTX was not detected in all the fish fed with the control diet throughout the administration period. When fish were fed with TTX containing diets, toxicity of whole body significantly increased according to the administration period (2-way ANOVA, df=2, F=19.337, P<0.001) and TTX concentration in the diet (2-way ANOVA, df=2, F=27.143, P<0.001). Interaction effects on the TTX accumulation were detected between administration period and TTX concentration in the diet (2-way ANOVA, df=4, F=7.179, P=0.0012), and fish fed with TTX at 14 MU/g·diet showed the highest toxicity (2.2±0.7 MU/g·fish) at the end of the administration trial. Average total TTX amount per fish at the end of the administration period reached 4.5 MU/fish for 7 MU/g·diet group and 8.7 MU/fish for 14.0 MU/g·diet group, respectively.

The TTX content of each tissue in the wild specimens was 1.0 MU/g·skin, 0.7 MU/g·muscle, 1.6 MU/g·liver and 0.5 MU/g·brain, respectively. Total TTX amount of a wild juvenile was estimated as 6.0 MU/fish.

3.2 Release trial in a mesocosm

Survival and growth of tiger puffer juveniles during the release trial were summarized in Table 1 and Fig.2. Survival at 5 days' post-release of hatchery reared tiger puffer juveniles

was significantly different with TTX administration, where survival of TTX administered fish (62 and 74 %) were about 2 times higher than that of control diet (χ^2-test, df=2, χ^2=37.987, P<0.001; Tukey's wholly significant difference analysis, P<0.05). No significant difference was detected both in SL and BW during the release period in all diet groups. DLCF decreased during the release trial (2-way ANOVA, df=1, F=76.504, P<0.001) and was significantly higher in the fish from control diet than those of the fish fed with TTX containing diet (2-way ANOVA, df=2, F=6.309, P<0.001; Tukey-Kramer HSD test, P=0.002). Gut contents analysis of tiger puffer juveniles at the end of the release trial revealed that 95-100 % of observed fish from each diet group (n=20) fed on the zooplanktons such as mysids, zoea of crustaceans, Myodocopa and copepods. There was no mortality in the sea bass during the trial and a total of 25 fish was recaptured at the end of the release trial. We found a total of 6 VIEs from the gut contents of sea bass throughout the release trial; 2 from control diet (day 1 and 4), 1 from 7 MU/g·diet (day 5), and 3 from 14 MU/g·diet group (day 0, 3 and 5), respectively.

4. Discussion

Non-toxic hatchery-reared tiger puffer *T. rubripes* juveniles accumulated TTX by oral administration of TTX and the localization of TTX in tissues was similar to that of wild juveniles. Therefore, TTX accumulation patterns in the artificially-raised juveniles in this study are considered reasonable. In the adult *T. rubripes*, TTX is generally detected in liver and ovary but not from skin and muscle [3]. However, most of TTX was detected in skin and muscle in case of juveniles (Fig.1). Okita et al [22] also detected TTX from hepatic tissue, basal cell of skin and olfactory, olfactory epithelium, optic nerve and brain in wild-caught *T. rubripes* juveniles (SL 4.7-9.4 cm), by immunohistochemical technique with anti-TTX monoclonal antibody. They also confirmed the same TTX localization in non-toxic artificially-raised juveniles after 5-days TTX administration with similar method of this study. However, Ikeda et al [23] reported that intramuscularly administered TTX in *T. rubripes* juveniles decreased rapidly from muscle and TTX were transferred to liver and skin. The duration of TTX administration is different between this study and Ikeda et al [23]; the former fed TTX continuously throughout the experimental period but the latter administered once at the beginning of the experiment. We assume that the TTX accumulation in skin and muscle of *T. rubripes* is juvenile stage-dependent phenomenon, and that the muscle of juveniles has low capacity for TTX and TTX in muscle immediately transferred to skin and liver when the supply of exogenous TTX was eliminated. Recently, Itoi et al [29] reported that TTX was detected from skin but from muscle of juvenile *T. rubripes* after artificially-raised juveniles were fed with toxic eggs of their adult. The difference in TTX accumulation in muscle of juvenile *T. rubripes* between this study and Itoi et al [29] may be due to the difference in the molecular conditions of TTX which were used for administration to fish. We used purified TTX (free form) for administration but they administered TTX by the TTX-containing eggs where TTX may be bound with organic compounds. Further study is needed to investigate whether the transfer of TTX is different between free- and organic-form of TTX in the pufferfishes.

Average TTX quantity in one individual at the end of TTX administration (4.5 MU/fish for 7 MU/g·diet group and 8.7 MU/fish for 14.0 MU/g·diet group) was comparable to that of a wild (6.0 MU/fish) in this study. Shimizu et al [17,18] measured TTX in the wild tiger puffer juveniles (SL 4.7-6.7 cm) from the same location in this study during the year 2004 and 2005, and TTX concentration ranged between 0.1 and 0.4 MU/g·fish, which is about one-tenth concentration of this study. Although toxicity of tiger puffer juveniles in the wild fluctuates by year, we judge that the oral administration of TTX into the artificially-raised juveniles in our study was successful to accumulate TTX into the fish with similar conditions as the wild juveniles. The minimum lethal dose of TTX for humans is estimated to be approximately 10,000 MU [1] and four toxicity levels for food safety standards are defined in Japan as follows: non-toxic (<10 MU/g·tissue), weakly toxic (10-100 MU/g·tissue), moderately toxic (100-1000 MU/g·tissue), strongly toxic (>1000 MU/g·tissue) [3]. Based on these criteria for food hygiene, both wild and TTX-administered reared *T. rubripes* juveniles in this study are regarded as non-toxic, and it will be safe if these fish are accidentally consumed. Furthermore, if these *T. rubripes* juveniles grow to market size, the TTX accumulation and distribution patterns in tissues will change into adult phase, in which skin and muscle are non-toxic.

It is noteworthy that TTX administration to the artificially-raised *T. rubripes* juveniles resulted in significantly high survival during the release trial with their predators (Fig.2). We excluded larger animals, such as crustacean and fishes which are the potential predators of tiger puffer juveniles, from the mesocosm prior to the release trial, and we found the VIEs from the gut contents of sea bass. Shimizu et al [16-18] also conducted release trials using tiger puffer and sea bass in the same mesocosm of this study and confirmed predations on tiger puffer juveniles by sea bass. Further, it is reported that the main cause of mortality in the released puffer juveniles in the wild was seabass [15]. Therefore, the main cause of mortality of tiger puffer juveniles in the mesocosm should be predations by sea bass. Comparison of survival rate between wild and non-toxic hatchery-reared *T. rubripes* juveniles after release into a mesocosm with sea bass showed that wild fish (86 %) survived better than hatcher-reared ones (56 %) 5 days after release in the previous studies [17,18]. These survival rates coincide with the difference between TTX administered (62-74 %) and non-toxic (32 %) fish in this study, and TTX administered fish accumulated TTX in their skin same as the wild juveniles from this study and a previous study [22]. Female parents of the *Takifugu* pufferfishes vertically transfer TTX to the larvae through its accumulation in the ovaries, and subsequent localization on the body surface of the larvae and various predatory fishes appeared to promptly sense and avoid TTX on the body surface of the puffer fish larvae [13]. Synthesizing these evidences and our results, we conclude that orally administered TTX in the hatchery reared *T. rubripes* juveniles is transferred into the skin (body surface) and bearing TTX in the skin of *T. rubripes* juveniles is functional as predator defense. However, bearing TTX in the skin of juvenile *T. rubripes* cannot completely avoid the risk of predation, because predation on TTX-fed juveniles was confirmed in this study. Furthermore, the result that no mortality of sea bass was confirmed in the release trial indicates that dose of TTX in *T. rubripes* juveniles was not lethal to sea bass. Our findings also propose the use of TTX

administration to the hatchery-reared tiger puffer for stock enhancement program in order to improve the post-release survival. Since wild *T. rubripes* juveniles bear TTX, administrating TTX to the non-toxic artificially-raised juveniles prior to release in a stock enhancement program seems reasonable considering the ecological characteristics of this species. However, administration of TTX for *T. rubripes* stock enhancement will be not realistic, because there are many issues to be carefully solved regarding the safety management of TTX during handling thousands of juveniles with considerable amount of TTX at each institute.

TTX in the skin of pufferfishes is functional as a predator defense chemical in their early life stages, however, the accumulation patterns of TTX seem to be different in the developmental stages. Maternal TTX in ovary of *T. rubripes* is vertically transferred to their eggs and larvae [13], and the TTX concentrations decrease during the larval stage [13,14,30]. Then, juveniles become non-toxic when they were excluded from TTX containing diets (this study, [17,18,22]). Therefore, *T. rubripes* larvae from toxic female parents are protected from predators by maternal TTX, however, juveniles requires external TTX from food organisms for their predator defense. Further field survey and rearing experiments regarding the TTX accumulation in tiger puffer are required to determine the TTX accumulation patterns in the early life stages.

Agonistic interactions such as nipping and cannibalism often occur in the cultured *T. rubripes* juveniles which are non-toxic [31]. TTX administration to these non-toxic juveniles enhances immunostimulation [32] and reduces agonistic interactions [33]. The intensity of agonistic interactions among juveniles can be expressed as occurrence of individuals with truncated caudal fin and quantified by DLCF. In this study, DLCF in fish fed with TTX-containing diets showed a tendency of lower DLCF during the TTX administration period, and TTX administered fish showed significantly lower DLCF than fish fed with control diet 5 days after the release trial. These results indicate that TTX administration to *T. rubripes* juveniles reduced agonistic interactions during administration period and immunopotentiating effect of TTX advanced regeneration of truncated caudal fin.

We detected TTX in the brain of wild and TTX administered juvenile *T. rubripes* in accordance with the previous study [22]. Okita et al [22] observed localization of TTX in a brain of TTX administered *T. rubripes* juvenile and detected high concentration of TTX at the molecular layer and purkinje cells in brain, which serve as the sole output of the cerebellar cortex of the cerebellar corpus in the cerebellum [34]. They postulated that TTX transferred to the central nervous system is physiologically functional to *T. rubripes* juveniles, because the piscine cerebellar corpus may play a role in motor learning and motor control. Fear response of non-toxic hatchery-reared *T. rubripes* juveniles is different from that of toxic wild juveniles [17,18]; when *T. rubripes* juveniles were transferred to a new environment, wild juveniles swim around the bottom and often show bottom-dwelling behavior but the hatchery reared juveniles swim in the water column around the water surface. It is pointed out that the behavioral deficits in fear response can be a major cause of mortality in the reared juveniles shortly after the release [17,18]. Thus, the reason of difference in survival rate between non-toxic and TTX-administered tiger puffer juveniles in this study may be not only because accumulated TTX in the skin of fish act as predator defense chemical, but also because TTX

in the brain affected the expression of fear response. Further study is needed to clarify whether TTX administration affect the fear response in the non-toxic hatchery-reared tiger puffer juveniles.

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Figure legends

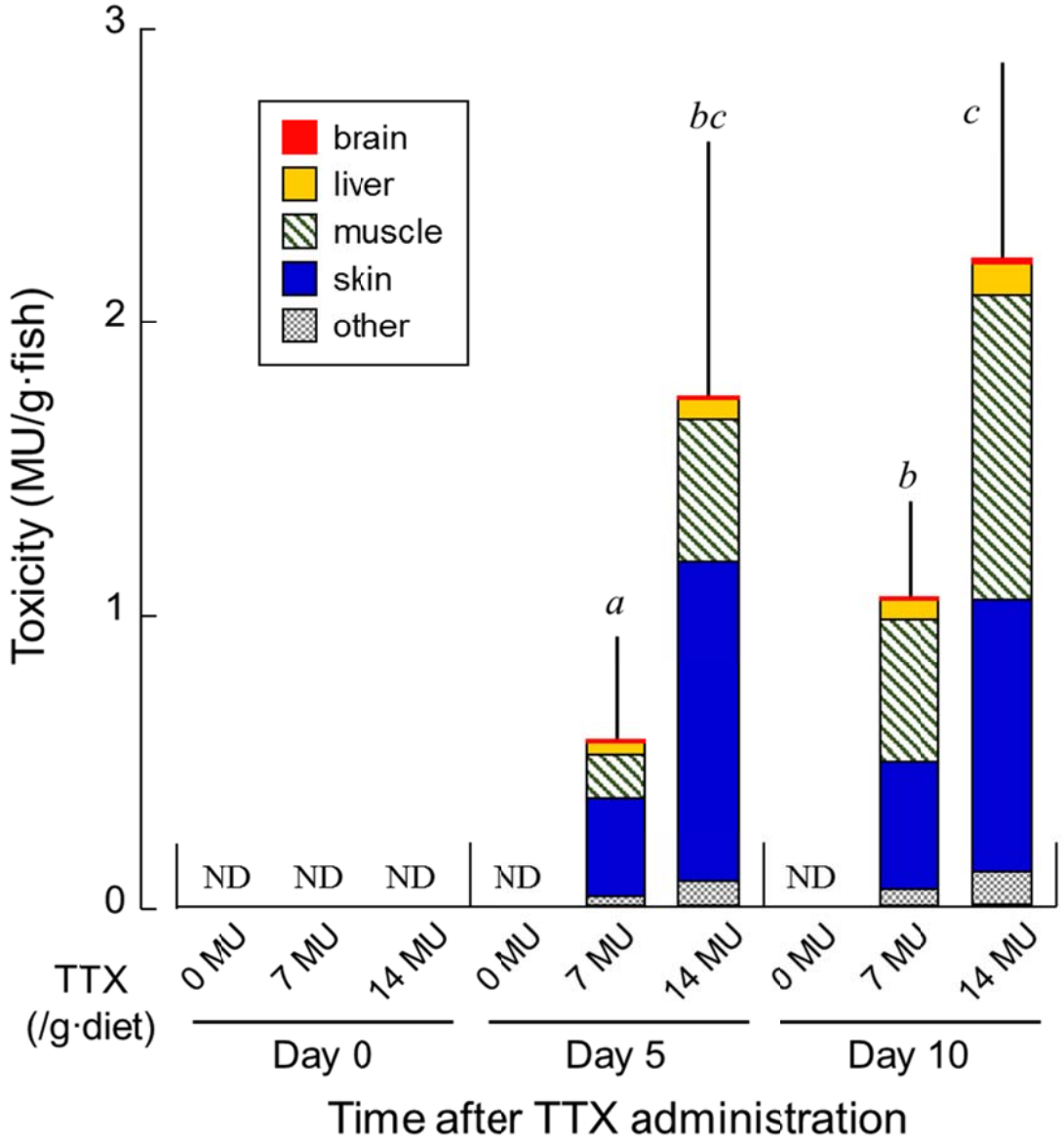

Fig. 1

Accumulation of tetrodotoxin in whole body and the tissues of *Takifugu rubripes* juveniles fed with diets containing different concentrations of tetrodotoxin for 10 days. Column represents average concentration of each tissue and bar indicates standard deviation per fish (n=3-10). Alphabetical letters on the columns denote significant differences among diet treatments in the same days for feeding (a<b<c, Tukey-Kramer HSD test, $P<0.05$).

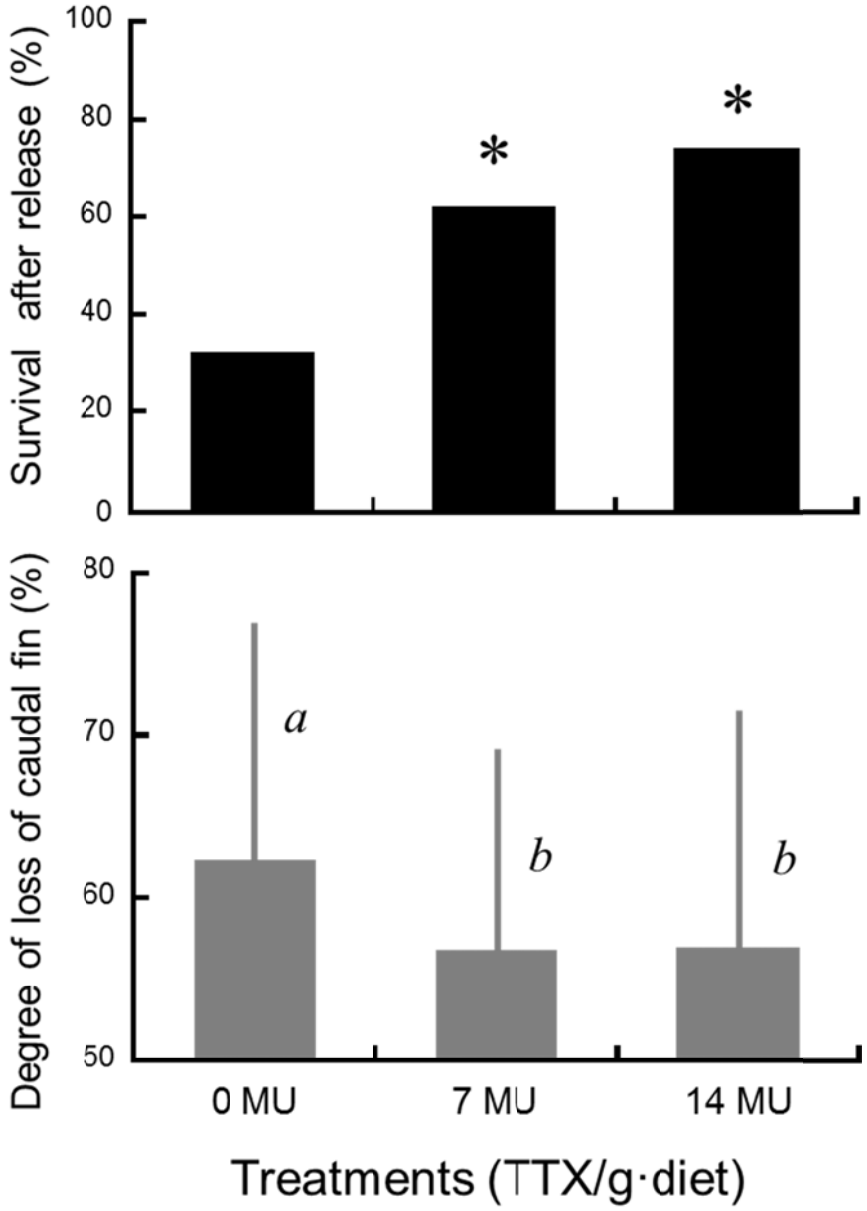

Fig. 2

Survival rates (upper; *n*=100) and degrees of loss of caudal fin (lower; average±SD, *n*=32-74) in *Takifugu rubripes* juveniles 5 days after release into a salt-pond mesocosm. Fish were fed with diets containing different concentrations of tetrodotoxin for 10 days prior to the release. An asterisk indicates significant difference among treatments (Tukey's Wholly Significant Analysis test, $P<0.05$) and alphabetical letters on the columns denote significant differences among diet treatments (a<b<c, Tukey-Kramer HSD test, $P<0.05$), respectively.

455 Table 1 Summary of TTX administration and release experiment of *Takifugu rubripes*
456 juveniles

	Treatments (TTX/g·diet)	TTX administration			Release at mesocosm	
		day 0	day 5	day 10	day 0	day 5
No. of fish (Survival %)	0 MU	200	159	118 (65.5 %)	100	32 (32.0 %)
	7 MU	200	151	116 (64.4 %)	100	62 (62.0 %)*
	14 MU	200	156	109 (60.6 %)	100	74 (74.0 %)*
Standard length (cm)	0 MU	4.1±0.4	4.3±0.3	4.8±0.4		4.8±0.3
	7 MU		4.4±0.3	5.0±0.5		5.0±0.3
	14 MU		4.5±0.4	4.8±0.4		5.0±0.4
Body weight (g)	0 MU	2.3±0.7	2.6±0.6	3.6±0.8		3.6±0.6
	7 MU		2.6±0.6	3.8±0.9		3.9±0.7
	14 MU		2.9±0.7	3.6±0.8		3.8±0.8
Degree of loss of caudal fin (%) [24]	0 MU	56.6±13.6	77.1±10.4	79.3±13.0		62.4±14.5 [a]
	7 MU		70.0±10.8	67.5±10.2		56.8±12.3 [b]
	14 MU		70.4±11.4	73.1±8.6		56.9±14.6 [b]

457 Data are indicated as average±standard deviations (n=20-100). Survival rate at 10 days after
458 TTX administration was calculated by the following equation: no. survived fish at day 10/(no.
459 initial fish (200) – no. sampled fish at day 5 (20)). Upper cases of alphabetical letters indicate
460 significant difference among the treatments (a>b, Tukey-Kramer HSD test, P<0.05). Asterisks
461 indicate significant differences (Tukey's wholly significant difference analysis, P<0.05).