



Title	Administration of tetrodotoxin protects artificially raised juvenile tiger puffer Takifugu rubripes from predators
Author(s)	Sakakura, Yoshitaka; Takatani, Tomohiro; Nakayasu, Junichi; Yamazaki, Hideki; Sakiyama, Kazutaka
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1 **Title:**

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

4  
5 **Author:**

6 Yoshitaka Sakakura<sup>1</sup> · Tomohiro Takatani<sup>1</sup> · Junichi Nakayasu<sup>1</sup> · Hideki Yamazaki<sup>2</sup> ·  
7 Kazutaka Sakiyama<sup>3</sup>

8  
9 **Affiliations:**

10 <sup>1</sup> Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Bunkyo 1-  
11 14, Nagasaki 852-8521, Japan

12 <sup>2</sup> Research Center for Marine Invertebrates, National Research Institute of Fisheries and  
13 Environment of Inland Sea, Japan Fisheries Research and Education Agency, Onomichi,  
14 Hiroshima 722-0061, Japan

15 <sup>3</sup> Japan Sea National Research Institute of Fisheries, Japan Fisheries Research and Education  
16 Agency, Miyazu, Kyoto 626-0052, Japan

17  
18 **Corresponding author:**

19 Yoshitaka Sakakura

20 Tel/fax: +81-95-819-2823

21 e-mail: [sakakura@nagasaki-u.ac.jp](mailto:sakakura@nagasaki-u.ac.jp)

22  
23 **Abstract:**

24 We examined the effects of tetrodotoxin (TTX) administration to the artificially-raised tiger  
25 puffer *Takifugu rubripes* juveniles on the survival after release into a mesocosm with  
26 predators in order to clarify the ecological significance of TTX. Artificial pellets containing 3  
27 different concentrations of TTX (0 as control, 7, 14 MU/g-diet) were fed to non-toxic  
28 artificially-raised *T. rubripes* juveniles for 10 days. TTX accumulation in the various tissues  
29 of fish was detected except for control diet group, and TTX administration did not affect  
30 survival or growth of the fish. Then, a hundred fish from each diet group were released  
31 together into a salt-pond mesocosm (2,650 m<sup>2</sup>) with predators (*Lateolabrax* sp.) for 5 days.  
32 Survival after release was significantly higher in the fish fed with TTX both 7 MU/g-diet  
33 (62 %) and 14 MU/g-diet (74 %) than the control fish (32 %).

34  
35 **Keywords:**

36 tetrodotoxin · puffer · predation defense · mesocosm.

## 38 **1. Introduction**

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

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

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

72

## 73 **2. Materials and methods**

### 74 *2.1 Experimental fish*

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

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

83

## 84 *2.2 Experimental diet*

85 TTX was purified from the ovaries (1.4 kg) of wild-caught 3 adult *T. rubripes*  
86 according to the method of Ikeda et al [23] with a slight modification. The extract was  
87 partially purified with Bio-Gel P-2 column (Bio-Rad Laboratories Inc., Hercules, CA, USA)  
88 and the absorbed TTX by the gel was eluted with 0.05 M AcOH. TTX fraction was analyzed  
89 by LC/MS analysis on an Alliance LC/MS system equipped with a ZSpray MS detector  
90 (Waters, Milford, MA, USA) following Nakashima et al [24]. The amount of TTX (in ng)  
91 determined by LC/MS was converted to MU (mouse unit) based on the specific toxicity of  
92 TTX (220 ng/MU). Purified TTX was dissolved in distilled water at the toxicity of 1,678  
93 MU/ml. TTX solution (24 ml), distilled water (2 ml) and 6 g of soy lecithin (Nacalai Tesque  
94 Inc., Japan) were homogenized in an ice bath for 3 min at 14,000 rpm. Then, TTX containing  
95 emulsion was made by adding 8 ml of cod liver oil (Riken Feed Oil Omega, RIKEN Vitamin  
96 Co., Ltd, Japan) and homogenizing TTX solution and feed oil in an ice bath for 3 min at  
97 14,000 rpm. Control emulsion was also prepared in the same manner of TTX containing  
98 emulsion replacing same amount of TTX solution with distilled water. Three different  
99 combinations of emulsion were prepared; control (40 ml), 25 MU (30 ml control and 10 ml of  
100 TTX containing emulsion), 50 MU (20 ml control and 20 ml of TTX containing emulsion).  
101 Each emulsion was sprayed onto the 360 g of diet (Otohime EP1) adjusting the concentration  
102 of TTX with 0, 25 and 50 MU/g·diet, respectively. A part of these diets were subjected to the  
103 measurement of concentrations of adsorbed TTX in diet as described above. The effective  
104 concentrations of TTX in 3 diets were, 0, 7 and 14 MU/g·diet, respectively.

105

## 106 *2.3 TTX administration*

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

115 At the initial day of feeding trial, 60 fish were sampled from the stock cage prior to  
116 assigning the fish for TTX administration (standard length, SL, 4.1±0.4 cm; BW, 2.3±0.7 g;  
117 average±standard deviations, n=60). Then, 20 fish per diet group were randomly collected at  
118 5 days after toxin administration, and all survived fish after 10 days TTX administration were  
119 counted and measured and 9-18 fish from each diet group were sampled. All sampled fish  
120 were stored at -20 °C until TTX analysis. We measured SL and total length of each fish by a  
121 digital caliper (CD20-GM, Mitsutoyo Corp., Japan) and BW by an electric balance (PB153-S,

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

$$124 \quad DLCF(\%) = \left(1 - \frac{Lth - Lsh}{Ltw - Lsh}\right) \times 100, \quad (1)$$

125 where, *Lth* and *Lsh* indicate the TL and SL of a measured fish, and *Ltw* is an estimated TL  
126 from the wild fish of the same SL which has no loss of caudal fin from the following equation  
127 (2).

$$128 \quad Ltw = 1.1806 \times Lsh + 6.0142 (n = 4,019, R^2 = 0.991), \quad (2)$$

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

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

144

#### 145 2.4 Release experiment in a mesocosm

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

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

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

164

### 165 *2.5 Statistical analysis*

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

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

177

## 178 **3. Results**

### 179 *3.1 TTX administration*

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

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

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

199

### 200 *3.2 Release trial in a mesocosm*

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

203 was significantly different with TTX administration, where survival of TTX administered fish  
204 (62 and 74 %) were about 2 times higher than that of control diet ( $\chi^2$ -test,  $df=2$ ,  $\chi^2=37.987$ ,  
205  $P<0.001$ ; Tukey's wholly significant difference analysis,  $P<0.05$ ). No significant difference  
206 was detected both in SL and BW during the release period in all diet groups. DLCF decreased  
207 during the release trial (2-way ANOVA,  $df=1$ ,  $F=76.504$ ,  $P<0.001$ ) and was significantly  
208 higher in the fish from control diet than those of the fish fed with TTX containing diet (2-way  
209 ANOVA,  $df=2$ ,  $F=6.309$ ,  $P<0.001$ ; Tukey-Kramer HSD test,  $P=0.002$ ). Gut contents analysis  
210 of tiger puffer juveniles at the end of the release trial revealed that 95-100 % of observed fish  
211 from each diet group ( $n=20$ ) fed on the zooplanktons such as mysids, zoea of crustaceans,  
212 Myodocopa and copepods. There was no mortality in the sea bass during the trial and a total  
213 of 25 fish was recaptured at the end of the release trial. We found a total of 6 VIEs from the  
214 gut contents of sea bass throughout the release trial; 2 from control diet (day 1 and 4), 1 from  
215 7 MU/g·diet (day 5), and 3 from 14 MU/g·diet group (day 0, 3 and 5), respectively.

216

#### 217 **4. Discussion**

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

244 Average TTX quantity in one individual at the end of TTX administration (4.5  
245 MU/fish for 7 MU/g-diet group and 8.7 MU/fish for 14.0 MU/g-diet group) was comparable  
246 to that of a wild (6.0 MU/fish) in this study. Shimizu et al [17,18] measured TTX in the wild  
247 tiger puffer juveniles (SL 4.7-6.7 cm) from the same location in this study during the year  
248 2004 and 2005, and TTX concentration ranged between 0.1 and 0.4 MU/g·fish, which is  
249 about one-tenth concentration of this study. Although toxicity of tiger puffer juveniles in the  
250 wild fluctuates by year, we judge that the oral administration of TTX into the artificially-  
251 raised juveniles in our study was successful to accumulate TTX into the fish with similar  
252 conditions as the wild juveniles. The minimum lethal dose of TTX for humans is estimated to  
253 be approximately 10,000 MU [1] and four toxicity levels for food safety standards are defined  
254 in Japan as follows: non-toxic (<10 MU/g·tissue), weakly toxic (10-100 MU/g·tissue),  
255 moderately toxic (100-1000 MU/g·tissue), strongly toxic (>1000 MU/g·tissue) [3]. Based on  
256 these criteria for food hygiene, both wild and TTX-administered reared *T. rubripes* juveniles  
257 in this study are regarded as non-toxic, and it will be safe if these fish are accidentally  
258 consumed. Furthermore, if these *T. rubripes* juveniles grow to market size, the TTX  
259 accumulation and distribution patterns in tissues will change into adult phase, in which skin  
260 and muscle are non-toxic.

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



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

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

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

313 We detected TTX in the brain of wild and TTX administered juvenile *T. rubripes* in  
314 accordance with the previous study [22]. Okita et al [22] observed localization of TTX in a  
315 brain of TTX administered *T. rubripes* juvenile and detected high concentration of TTX at the  
316 molecular layer and purkinje cells in brain, which serve as the sole output of the cerebellar  
317 cortex of the cerebellar corpus in the cerebellum [34]. They postulated that TTX transferred to  
318 the central nervous system is physiologically functional to *T. rubripes* juveniles, because the  
319 piscine cerebellar corpus may play a role in motor learning and motor control. Fear response  
320 of non-toxic hatchery-reared *T. rubripes* juveniles is different from that of toxic wild juveniles  
321 [17,18]; when *T. rubripes* juveniles were transferred to a new environment, wild juveniles  
322 swim around the bottom and often show bottom-dwelling behavior but the hatchery reared  
323 juveniles swim in the water column around the water surface. It is pointed out that the  
324 behavioral deficits in fear response can be a major cause of mortality in the reared juveniles  
325 shortly after the release [17,18]. Thus, the reason of difference in survival rate between non-  
326 toxic and TTX-administered tiger puffer juveniles in this study may be not only because  
327 accumulated TTX in the skin of fish act as predator defense chemical, but also because TTX

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

331

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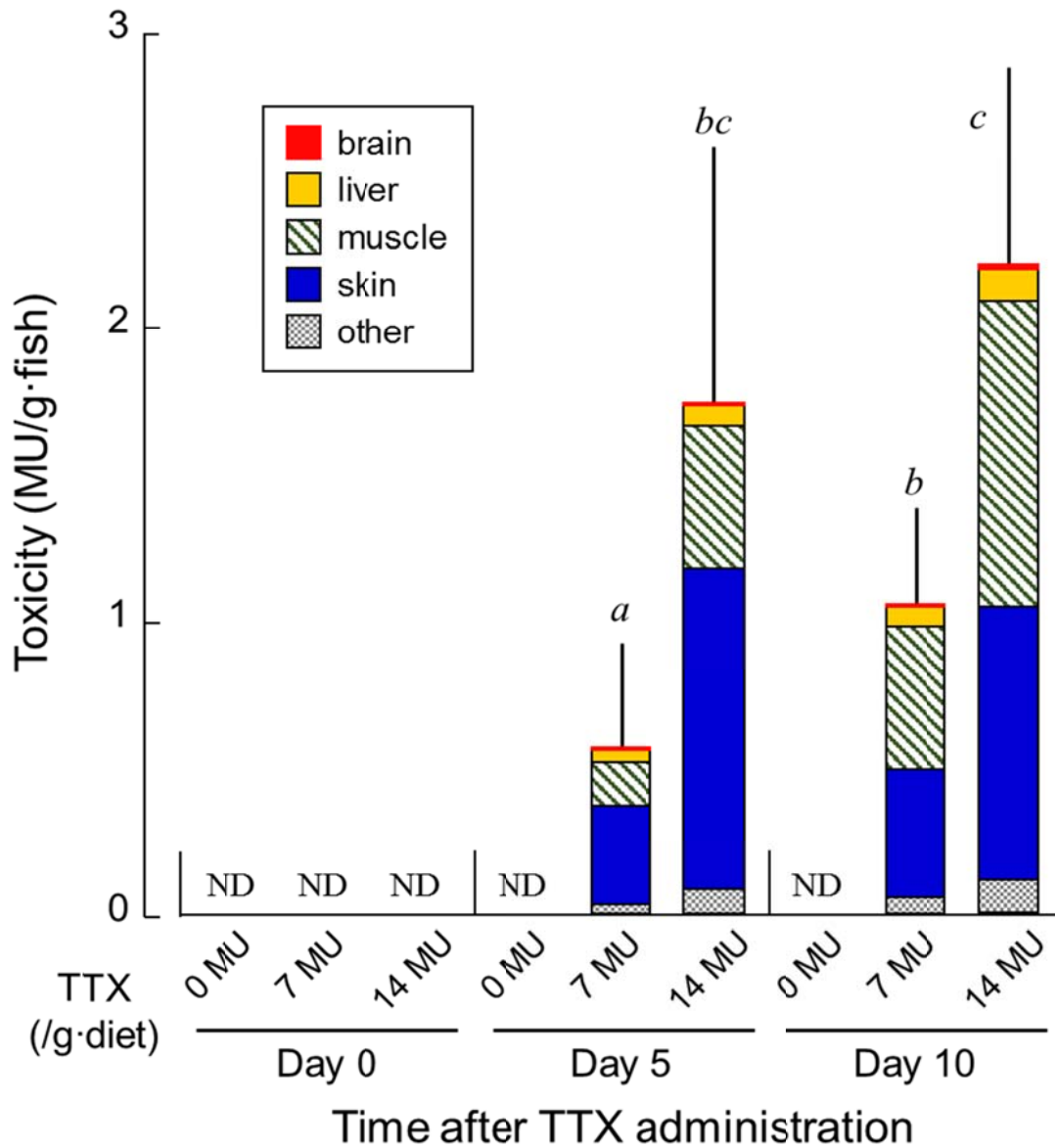
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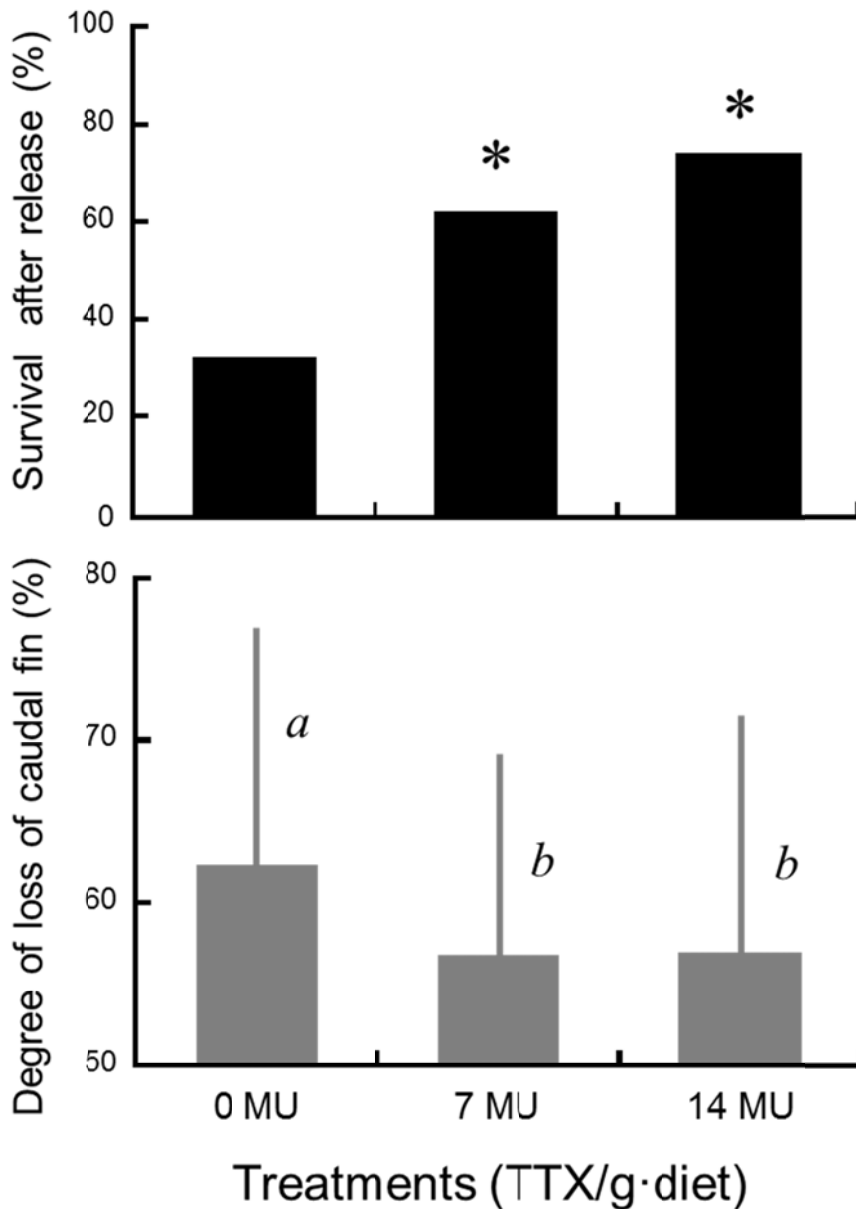
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437 **Figure legends**



438 **Fig. 1**  
 439 Accumulation of tetrodotoxin in whole body and the tissues of *Takifugu rubripes* juveniles  
 440 fed with diets containing different concentrations of tetrodotoxin for 10 days. Column  
 441 represents average concentration of each tissue and bar indicates standard deviation per fish  
 442 (n=3-10). Alphabetical letters on the columns denote significant differences among diet  
 443 treatments in the same days for feeding (a<b<c, Tukey-Kramer HSD test, P<0.05).  
 444  
 445



446  
 447 **Fig. 2**  
 448 Survival rates (upper;  $n=100$ ) and degrees of loss of caudal fin (lower; average $\pm$ SD,  $n=32-74$ )  
 449 in *Takifugu rubripes* juveniles 5 days after release into a salt-pond mesocosm. Fish were fed  
 450 with diets containing different concentrations of tetrodotoxin for 10 days prior to the release.  
 451 An asterisk indicates significant difference among treatments (Tukey's Wholly Significant  
 452 Analysis test,  $P<0.05$ ) and alphabetical letters on the columns denote significant differences  
 453 among diet treatments ( $a<b<c$ , Tukey-Kramer HSD test,  $P<0.05$ ), respectively.  
 454

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		4.3±0.3	4.8±0.4		4.8±0.3
	7 MU	4.1±0.4	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		2.6±0.6	3.6±0.8		3.6±0.6
	7 MU	2.3±0.7	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		77.1±10.4	79.3±13.0		62.4±14.5 <sup>a</sup>
	7 MU	56.6±13.6	70.0±10.8	67.5±10.2		56.8±12.3 <sup>b</sup>
	14 MU		70.4±11.4	73.1±8.6		56.9±14.6 <sup>b</sup>

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,  $P<0.05$ ).