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Tetrodotoxin Retention in the Toxic Goby *Yongeichthys criniger*

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Abstract: To investigate tetrodotoxin (TTX) retention by the toxic goby *Yongeichthys criniger*, rearing experiments feeding nontoxic diets were conducted using 12 (Group I) and 17 (Group II) specimens collected from a natural environment. The specimens were reared in an aquarium with aeration and fed a diet lacking TTX for 60 days. Specimens were removed at 0, 20, 40, and 60 days (Group I) or 0, 30, and 60 days (Group II) after initiation of rearing. Liquid chromatography/mass spectrometry and liquid chromatography-tandem mass spectrometry revealed that whole-body concentrations and amounts of TTX decreased with increasing rearing duration in Group I. There were similar decreases in Group II, but the trend differed among tissues; the concentrations and amounts of TTX in the skin exhibited the greatest decreases. The results imply that *Y. criniger* has low TTX retention ability.

Keywords: *Yongeichthys criniger*; tetrodotoxin; rearing experiment

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1. Introduction

Tetrodotoxin (TTX) is a potent neurotoxin long thought to be unique to pufferfish. However, it is present in diverse marine phyla; among fish, the goby *Yongeichthys criniger* and pufferfish have TTX [1]. In Japan, *Y. criniger* inhabits estuarine areas of the Nansei Islands, which have mature mangrove forests [2,3]. In the Okinawa/Amami region, the goby has long been known to be toxic [4], and its toxin was confirmed to be TTX by Noguchi and Hashimoto [5]. Like the marine pufferfish, the goby takes up TTX by ingesting benthic TTX-bearing organisms and accumulates it in certain tissues [1], but it is unclear whether the goby accumulates and retains or eliminates TTX by the same or a different mechanism as the pufferfish. Therefore, the absorption, transportation, accumulation, retention, and elimination of TTX in *Y. criniger* and pufferfish warrant investigation.

Like marine pufferfish of the genus *Takifugu*, *Y. criniger* accumulates a large amount of TTX in the skin and ovary, the latter increasing with maturation [6–9]. *Y. criniger* accumulates TTX in muscle and testis, where it is rarely detected in *Takifugu* [6–10]. This is similar to the brackish water pufferfish, *Chelonodon patoca* [11]. Elimination of TTX in *Y. criniger* has been reported via the skin and ovaries. A toxicity equivalent to 3.1–187 μg of TTX was detected in gauze used to wipe the skin of *Y. criniger* [8,9,12]. Marine pufferfish also eliminate TTX from their skin [13,14]. Toxicity has been detected in the ovulated eggs of *Y. criniger* [15], indicating that TTX in ovary is retained in eggs and eliminated by ovulation. This is the case in pufferfish [16,17].

TTX retention has been examined using nontoxic *Takifugu alboplumbeus* and *T. rubripes* cultured from hatching with nontoxic feeding. That is, in these studies nontoxic individuals of both species were fed a toxic diet to accumulate TTX and switched to a nontoxic diet to investigate TTX retention. The two species retained toxin for 170–210 and 45 days after switching to a nontoxic diet, respectively [18–20]. However, no study has evaluated TTX retention in *Y. criniger*.

We investigated TTX retention by *Y. criniger* by rearing individuals collected from a natural environment on a nontoxic diet as part of a study to clarify differences in TTX absorption, transportation, accumulation, and retention/elimination between *Y. criniger* and pufferfish.

2. Materials and Methods

2.1. Goby Specimens

In June 2010 (Group I) and 2015 (Group II), wild specimens of *Y. criniger* were collected from the Fukido River, Okinawa Prefecture, Japan, and transported live to Nagasaki University. The body sizes of Groups I and II are shown in Tables 1 and 2, respectively. As a control, 3 of 12 specimens in Group I and 6 of 17 specimens in Group II were used for TTX quantification without rearing. The remaining specimens were subjected to the rearing experiments. Because the gonads were not of sufficient size (≤0.06 g), the specimens were used without distinguishing between females and males.

Table 1. Body size of the *Y. criniger* specimens of Group I.

Rearing Day	No.	Body Length (mm)	Body Weight (g)
0	1	34	0.73
	2	35	0.85
	3	34	0.68
20	4	38	0.85
	5	35	0.77
	6	40	1.14
40	7	42	1.15
	8	42	1.28
	9	39	0.98
60	10	44	1.51
	11	36	0.73
	12	34	0.59

Table 2. Body size of the *Y. criniger* specimens of Group II.

Rearing Day	No.	Body Length (mm)	Body Weight (g)
0	1	66	5.17
	2	56	4.19
	3	64	5.37
	4	67	6.77
	5	59	4.84
	6	72	7.72
30	7	77	8.94
	8	77	9.17
	9	70	8.23
	10	70	6.89
	11	55	2.94
60	12	71	7.12
	13	75	9.03
	14	57	3.37
	15	68	5.46
	16	73	7.57
	17	77	8.53

2.2. Rearing Experiments

The specimens were reared in an aerated aquarium (60 L) and fed a commercial diet without TTX until the end of the experiment. In Group I, three specimens were randomly collected at 20, 40, and 60 days after initiation of rearing. In Group II, five or six specimens were randomly collected at 30 and 60 days after initiation of rearing. These specimens, together with the non-reared specimens (rearing period day 0), were subjected to TTX quantification.

2.3. TTX Quantification

The specimens in Group I were small, making it difficult to remove tissues, whereas we were able to dissect four specific tissues from Group II specimens: skin, muscle, liver, and gonads. Whole-body (Group I) or specific tissues (Group II) were homogenized and added to three to five volumes of 0.1% acetic acid. The mixtures were heated in boiling water for 10 min and centrifuged at 3000× *g* for 15 min [21]. The extracts were passed through an HLC-DISK membrane filter (0.45 μm, Kanto Chemical Co., Inc., Tokyo, Japan).

Filtrates of Group I were analyzed by liquid chromatography/mass spectrometry (LC/MS) for TTX according to Nakashima et al. [22]. Briefly, LC/MS was performed using an Alliance system equipped with a Zspray™ MS 2000 detector (Waters Alliance, Milford, MA, USA). A Mightysil RP-18 GP column (2.0 × 250 mm, Kanto Chemical Co., Inc., Tokyo, Japan) was used with a mobile phase of 30 mmol/L heptafluorobutyric acid in 1 mmol/L ammonium acetate buffer (pH 5.0). The flow rate was set at 0.2 mL/min, and the eluate was introduced into the ion source of the MS detector for electrospray ionization of TTX in positive-ion mode. The desolvation temperature, source-block temperature, and cone voltage were 350 °C, 120 °C, and 30 V, respectively. A precursor ion (*m/z* 320) was monitored using the MassLynx™ NT operating system. A TTX standard (Wako Pure Chemical Industries, Ltd. [purity > 90%], Osaka, Japan) was dissolved in distilled water at 0.05, 0.1, 0.2, and 0.4 μg/mL and used for quantification.

Filtrates of Group II were submitted to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of TTX according to Gao et al. [23]. LC was performed on an Alliance 2690 Separations Module (Waters Alliance). The column and mobile phase conditions were as for Group I. TTX was ionized using a Quattro Micro™ API Detector (Waters), and a product ion (*m/z* 162) with a collision voltage of 38 V and a precursor ion (*m/z* 320) were monitored. A TTX standard (Wako Pure Chemical Industries, Ltd. [purity > 90%]) was dissolved in distilled water at 0.01, 0.02, and 0.04 μg/mL and used for quantification.

2.4. Statistical Analysis

The Kruskal-Wallis test was used to compare the concentrations and amounts of TTX in the whole body (Group I) or skin, muscle, liver, and gonads (Group II) among the four (Group I) and three (Group II) rearing periods. Significant differences were analyzed using the Wilcoxon rank-sum test with Bonferroni correction.

3. Results

Figures 1 and 2 show the results for the whole-body Group I specimens. The TTX concentrations were highest on day 0 (5.7–21.1 μg/g), followed by days 20 (3.0–6.2 μg/g), 40 (1.2–4.1 μg/g), and 60 (0.4–2.0 μg/g). The amounts of TTX were highest on day 0 (4.2–18.0 μg/individual) and decreased thereafter. No significant differences in TTX concentrations or amounts were detected in any rearing period.

Figures 3 and 4 show the results for the specific organs in Group II. The TTX concentrations in skin were significantly ($p < 0.05$) higher on day 0 (2.4–21.9 μg/g) than on days 30 and 60. The concentrations in muscle and gonads were significantly lower on days 30 and 60, respectively, than at other rearing times. The concentrations in the liver did not decrease significantly during rearing. The amounts of TTX were significantly higher on day 0 (9.8 μg/individual) than in the other rearing periods, and skin and muscle accounted

for 80% and 18% of the levels, respectively. The amount in skin decreased over time and was ~30% on day 60.

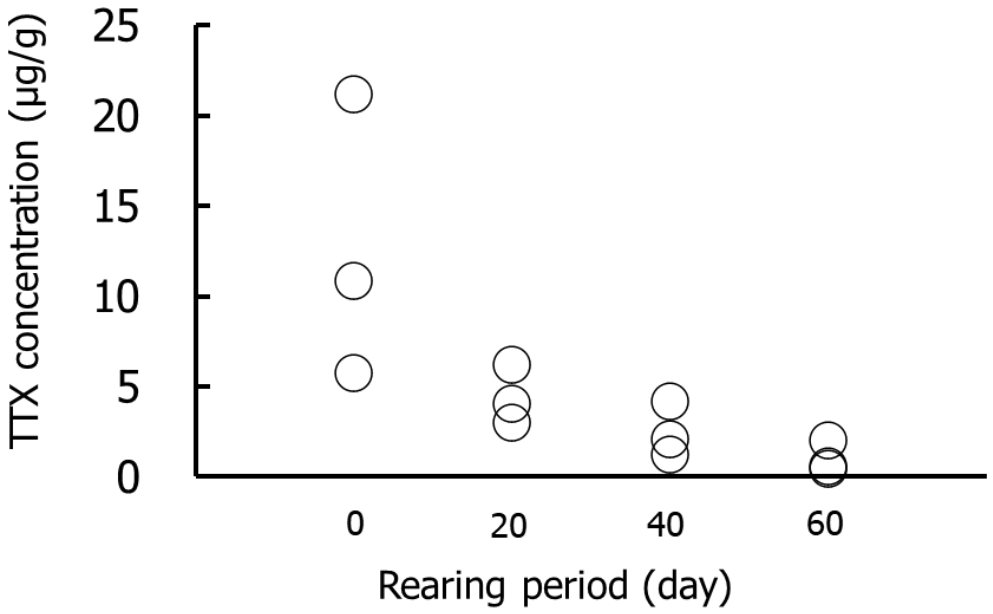

Figure 1. TTX concentrations in the whole-body during rearing in Group I. Data are individual values (circles).

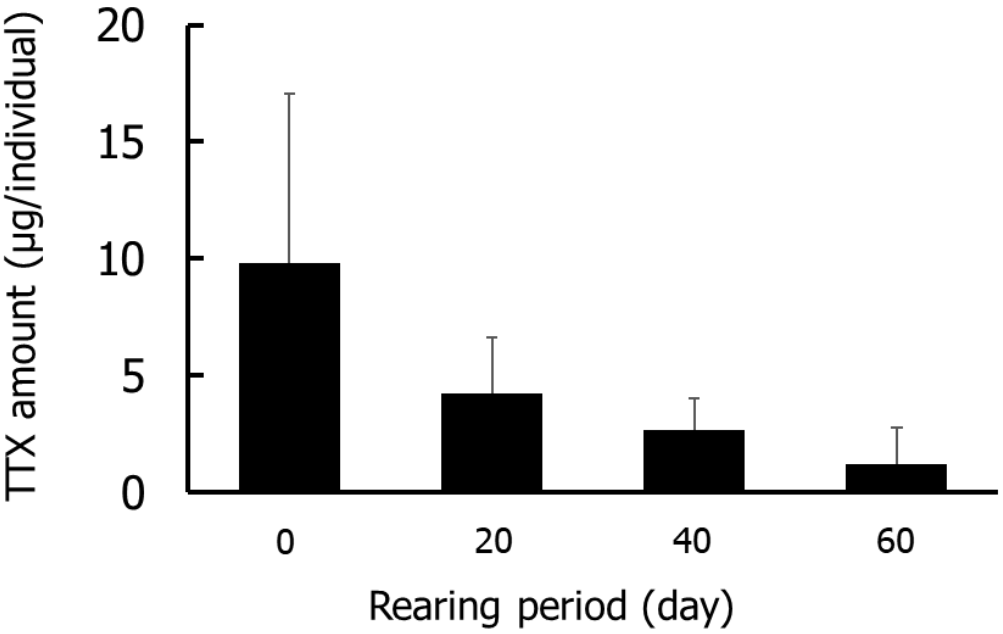

Figure 2. Amounts of TTX in the whole-body during rearing in Group I. Data are means (column) and standard deviations (error bar).

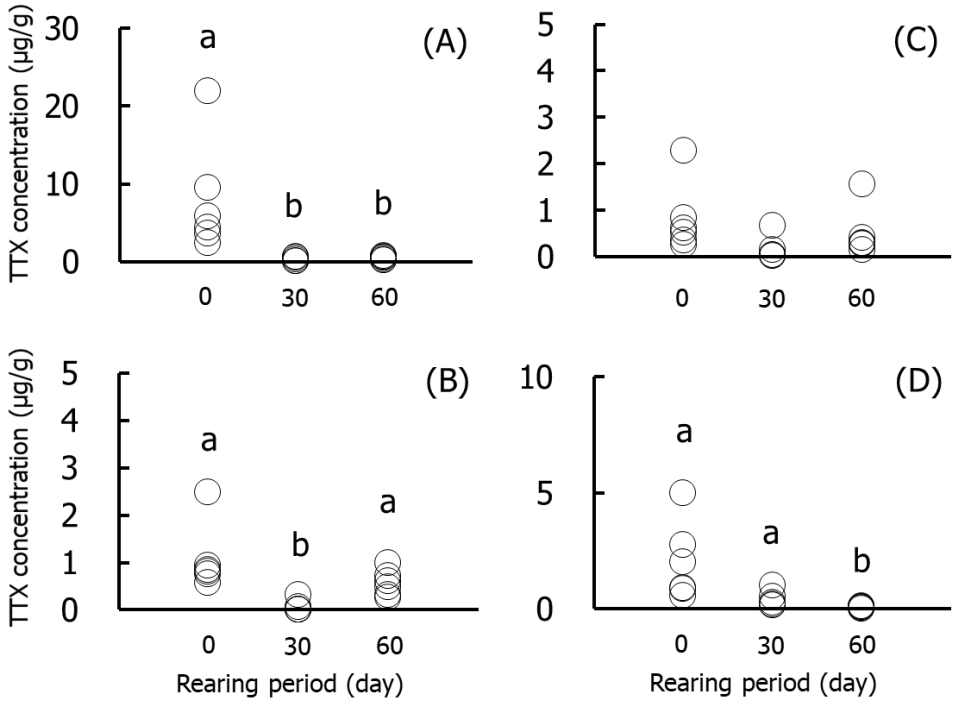

Figure 3. TTX concentrations in the skin (**A**), muscle (**B**), liver (**C**), and gonads (**D**) during rearing in Group II. Data are individual values (circles). a > b, $p < 0.05$.

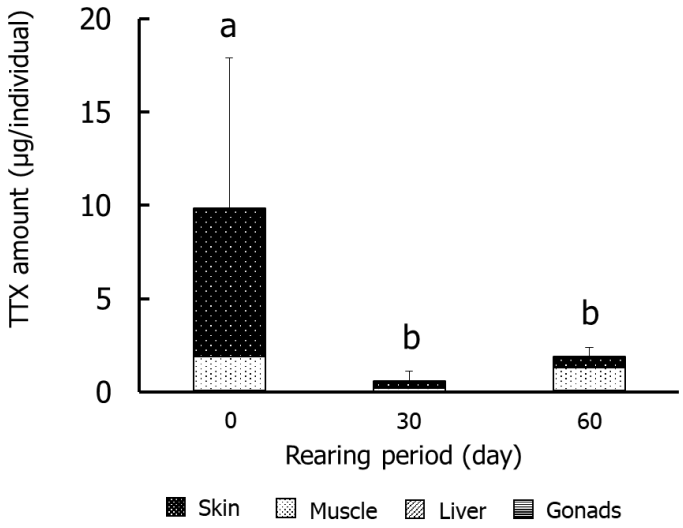

Figure 4. Amounts of TTX in tissues during rearing in Group II. Data are means (column) and standard deviations (error bar). a > b, $p < 0.05$.

4. Discussion

In Group I, the amounts and concentrations of TTX decreased over time, albeit non-significantly. The mean and maximum concentrations were highest on day 0, at 12.6 and 21.1 μg/g, respectively. The concentrations in specimens reared on a nontoxic diet decreased with time, and the mean and maximum on day 60 were 1.0 and 2.0 μg/g, respectively. The amounts of TTX also decreased with time, and the average on day 60 was ~10% of that on day 0. In previous studies, after feeding nontoxic specimens of *T. alboplumbeus* a toxic diet for 30 days and then a nontoxic diet until the end of the experiment (170 or 210 days after rearing started), 50–80% of the administered TTX (equivalent to the TTX accumulated during the first 30 days) was retained until 60 or 70 days, and 30–60% (equivalent to 60–80% of the accumulated TTX on day 30) remained at the

end of the experiment [18,20]. Therefore, *Y. criniger* has lower TTX retention compared to *T. alboplumbeus*.

In Group II, the TTX concentrations in skin were significantly lower on days 30 and 60 than on day 0. *Y. criniger* accumulates high concentrations of TTX in skin and eliminates it from the body on external stimulation [8,9,12]. In this study, *Y. criniger* was not exposed to external stimuli during rearing, so the decrease in TTX concentrations is unlikely to have been caused by external stimulation. Therefore, the skin of *Y. criniger* accumulates high concentrations of TTX, which is discharged (exuded) rapidly as a result of its low retention ability. In previous studies, *T. rubripes* and *T. alboplumbeus* retained identical or higher levels of TTX as that at the time of cessation of TTX feeding after 45 to 210 days of rearing on a nontoxic diet [18–20], indicating that TTX retention is lower in the skin of *Y. criniger* than in that of marine pufferfish of the genus *Takifugu*.

In our study, TTX was detected in the muscle of *Y. criniger* not only on day 0 but also on days 30 and 60. Therefore, *Y. criniger* muscle retains TTX. TTX is rarely detected in the muscle of *Takifugu*. In previous studies on nontoxic *T. rubripes* and *T. alboplumbeus* fed a toxic diet, TTX was almost undetectable in muscle [18–20]. TTX is present in the muscle of *C. patoca* [11], so *Y. criniger* muscle may have a TTX retention mechanism similar to that of *C. patoca*.

TTX concentrations in the liver on day 0 did not differ significantly from those after rearing. In previous studies on *T. alboplumbeus*, the liver accumulated the largest quantity of TTX at initiation of a nontoxic diet, which decreased with increased feeding duration [18,20]. However, the amounts of TTX in the skin and gonads increased in time, implying that TTX was transferred from the liver to the skin and gonads. In our study, the TTX concentrations in the gonads of *Y. criniger* were significantly lower on day 60 than on days 0 and 30 and were lower than those in the other three tissues on day 60. Therefore, TTX retention in the gonads was lower in *Y. criniger* than in *T. alboplumbeus*, and the toxin was not transferred from the liver to the gonads.

In Group II, the amounts of TTX were significantly lower in reared specimens (days 30 and 60) than in non-reared specimens (day 0), implying that TTX is not retained in *Y. criniger*. TTX-bearing fish consume TTX through the food chain, which begins with TTX-producing bacteria [1]. Such bacteria have been detected in *Y. criniger* and may supply TTX [24]. However, our findings indicate that TTX-producing bacteria did not supply enough TTX to render *Y. criniger* toxic. TTX in *Y. criniger* likely originates from TTX-bearing prey. Genetic analysis of the contents of the goby digestive tract revealed a highly toxic flatworm gene [25]. The *Y. criniger* used in that study were collected from the same river as the fish in our study, so our specimens likely ingested TTX from TTX-bearing flatworms.

Y. criniger retained TTX, albeit to a markedly lesser degree than *Takifugu*. *Takifugu* species possess pufferfish saxitoxin and TTX-binding protein (PSTBP), which is implicated in TTX absorption, transportation, and accumulation [26–29]. In a previous study, Western blotting using an antibody to PSTBP detected a protein homologous to PSTBP in *C. patoca* (in which TTX is present in muscle). However, the protein had a molecular weight of 50 kDa, less than half the PSTBP isoforms of *Takifugu* [30]. We plan to investigate this *Y. criniger* protein, with the aim of clarifying the molecular mechanism of TTX retention in goby and pufferfish.

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