Report

Change in Tetrodotoxin Content of the Puffer Fish *Takifugu rubripes* during Seed Production from Fertilized Eggs to Juveniles

(Received June 6, 2009)

Yuji NAGASHIMA^{1,*}, Isao MATAKI¹, Maho TOYODA¹, Hiroshi NAKAJIMA²,
Kingo TSUMOTO², Kuniyoshi SHIMAKURA¹ and Kazuo SHIOMI¹

¹Department of Food Science and Technology, Tokyo University of Marine Science and
Technology: 4-5-7 Konan, Minato, Tokyo 108-8477, Japan;

²Mie Prefecture Fisheries Research Institute: 3564-3 Hamajima, Hamajima,
Shima, Mie 517-0404, Japan; * Corresponding author

Changes in tetrodotoxin (TTX) content of the puffer fish *Takifugu rubripes* during seed production were examined. Two mature female puffer fish *T. rubripes* (samples 1 and 2) that contained TTX were used. The toxic eggs were artificially fertilized, and hatchlings were reared in an indoor tank for 50 days and then in a netcage at sea for an additional 48 or 38 days. The TTX content of the fertilized eggs of sample 1 was initially 13.0 μg TTX/g, transiently increased to 67.6 μg TTX/g at 4 days after hatching, and then gradually decreased to 0.28 μg TTX/g at 98 days. In contrast, the total TTX content in an individual was 0.016 μg TTX at the fertilization stage and 0.01–0.03 μg TTX at the larval stage until 30 days after hatching. Thereafter, the total TTX content increased remarkably during culture in the netcage at sea, reaching 4.80 μg TTX at 98 days. Change in the TTX content of sample 2 showed a similar tendency to that of sample 1. The present study showed that the TTX content per gram of puffer fish body weight decreased during progression from fertilized eggs to juveniles, whereas the total TTX content increased.

Key words: puffer fish; *Takifugu rubripes*; seed production; tetrodotoxin; fertilized egg; larva; juvenile

Introduction

Puffer fish of the family Tetraodontidae contain a potent neurotoxin, tetrodotoxin (TTX), with high levels mainly in liver and ovary, and often cause human intoxication resulting from ingestion of the toxic portions¹. It is currently considered that TTX in puffer fish is exogenous and puffer fish accumulate TTX through the food chain by ingestion of TTX-bearing organisms². When innocuous cultured puffer fish *Takifugu rubripes* and *Takifugu niphobles* were fed TTX-containing diets, they became toxic and predominantly accumulated TTX in liver^{3–7}. Our previous studies *in vitro* confirmed the preferential accumulation of TTX in liver of puffer fish. When liver tissue slices were incubated with medium containing TTX, liver slices of puffer fish *T. rubripes* and *Takifugu pardalis* accumulated considerable amounts of TTX, but the liver tissue slices of other non-toxic species, parrotbass *Oplegnathus fasciatus*, greenling *Hexagrammos otakii*, and filefish *Thamnaconus modestus* did not^{8, 9}. Based on these findings, non-toxic puffer fish *T. rubripes* have been successfully produced by netcage or land culture with feeding of TTX-free diets¹⁰. However, aquaculture in an inappropriate way affords *T. rubripes* contaminated with TTX, because TTX is widely distributed in the aquatic environment, not only in marine organ-

isms, including bacteria, ribbonworms, flatworms, gastropods, and starfish, but also in marine sediments and sinking particles from coastal waters^{11–13}. Lin *et al.*¹⁴ observed toxicity in the liver and ovary of cultured *T. rubripes* from a farm in northern Taiwan in which the seawater was directly pumped from the adjacent sea into the culture ponds without any treatment, but not in those of the *T. rubripes* cultured in other farms using seawater filtered through sea sand and stored in a reservoir. Sato *et al.*¹⁵ also detected a small amount of TTX in the intestines of cultured *T. rubripes* specimens at the ages of 60 days and 1 year old, although the toxicity was as low as 0.2 mouse unit (MU)/g. These results indicate that the toxicity of cultured puffer fish *T. rubripes* varies greatly, depending on the culture conditions.

In the case of seed production of *T. rubripes*, wild mature puffer fish have been often employed to spawn¹⁶, though they have considerable amounts of TTX in the ovary. Although the toxin amount in the eggs is initially negligible, it is not clear whether the amount decreases with growth or not. Moreover, little has been reported about the toxicity of *T. rubripes* in the early stage of growth. In this study, we investigated the change in TTX content of hatchery-reared puffer fish *T. rubripes* for approximately 100 days from the stage of fertilized eggs to juveniles.

Table 1. Tetrodotoxin content of puffer fish *Takifugu rubripes* (sample 1) during seed production

Stage/days after hatching	Number of specimens	Average body weight (g)	Tetrodotoxin content ($\mu\text{g TTX/g}$)	Total tetrodotoxin content ($\mu\text{g TTX/individual}$)
Fertilized egg	549	1.26×10^{-3}	13.0	0.016
Larvae reared in an indoor tank				
3 days	204	0.39×10^{-3}	64.5	0.025
4 days	141	0.45×10^{-3}	67.6	0.030
5 days	94	0.30×10^{-3}	19.9	0.006
9 days	98	0.56×10^{-3}	15.0	0.008
10 days	141	0.94×10^{-3}	12.0	0.012
12 days	73	1.76×10^{-3}	8.38	0.014
16 days	65	2.88×10^{-3}	6.58	0.019
30 days	41	39.0×10^{-3}	0.74	0.029
40 days	5	206×10^{-3}	1.17	0.650
50 days	3	957×10^{-3}	0.64	0.632
Juveniles reared in a netcage at sea				
70 days	3	2.72	0.44	1.77
80 days	3	7.19	0.40	2.84
98 days	3	17.1	0.28	4.80

Materials and Methods

Fish samples

Two mature female puffer fish *T. rubripes* were caught at off-shore of Mie Prefecture in April 12, 1999 (sample 1, body length of 54.4 cm, body weight of 3.6 kg) and April 16, 1999 (sample 2, body length of 52.6 cm, body weight of 2.4 kg) and transported to Mie Prefectural Fish Farming Center, Mie Prefecture. Eggs were collected, artificially fertilized, and reared at an indoor tank ($9 \times 5 \times 1.5$ m) with sea water filtered through sand for 50 days after hatching. Thereafter, the fish were reared in a netcage ($5 \times 5 \times 5$ m) at sea adjacent to Mie Prefectural Fish Farming Center for an additional period of approximately 50 days. The puffer fish were fed on rotifers (2–30 days after hatching), brine shrimp (12–40 days), commercial formula feed consisting of mainly krill, fish meal, and squid meal (25–50 days), and commercial pellets (50 days ~release)¹⁶⁾.

Determination of TTX

The fertilized eggs, larvae and juveniles of puffer fish were sampled at the designated times. Each group of samples was pooled, transported to the laboratory of Tokyo University of Marine Science and Technology, and frozen at -20°C until use. The samples were ground in a mortar with a pestle and homogenized with 0.1% acetic acid. TTX was extracted by heating in a boiling water bath for 10 min according to the official guideline of the Japan Food Hygiene Association¹⁷⁾. The extract was ultra-filtered using an Ultrafree-MC (nominal cut-off molecular weight 10 kDa, Millipore, Bedford, MA, U.S.A.) and analyzed for TTX by HPLC-post column derivatization, using a Mightysil RP-18 column (4.6×250 mm, Kanto Kagaku, Tokyo, Japan) and a mobile phase consisting of 2 mmol/L heptanesulfonic acid-10 mmol/L phosphate buffer (pH 7.0) at a flow rate of 0.8 mL/min¹⁸⁾. A mouse bioassay was performed for the fertilized eggs and the 12-day-old

larvae using the official method for TTX¹⁷⁾. The toxicity in the mouse bioassay was expressed as mouse units (MU), where 1 MU is defined as the amount of toxin that kills a male mouse of ddY strain weighing 20 g in 30 min after intraperitoneal injection. One MU is equivalent to $0.2 \mu\text{g}$ of TTX, based on the specific toxicity of TTX (5,000 MU/mg).

Results and Discussion

Changes in body weight and TTX content of the puffer fish during seed production for approximately 100 days are summarized in Tables 1 and 2. The body weight shown is an average of the pooled samples. The TTX content of the puffer fish decreased with growth, though total TTX content increased 300–600 times. In the case of sample 1 in Table 1, the fertilized egg weighed 1.26 mg and showed a TTX content of $13.0 \mu\text{g TTX/g}$ (equivalent to $0.016 \mu\text{g TTX/egg}$). After hatching, the body weight of the larvae decreased to 0.30 mg after 5 days and then increased with rearing time; 1 g after 50 days and 17.1 g after 98 days. The TTX content of the larvae and juveniles transiently increased to $67.6 \mu\text{g TTX/g}$ at 4 days after hatching and then gradually decreased to $0.28 \mu\text{g TTX/g}$ after 98 days. In contrast, the total TTX content of the larvae was as low as 0.01 – $0.03 \mu\text{g TTX}$ until 30 days after hatching and then increased to $0.6 \mu\text{g TTX}$ after 50 days. Total TTX content of juveniles increased during netcage culture at sea and reached $4.80 \mu\text{g}$ after 98 days. When the TTX content and the total TTX content of the 98-day-old juveniles were compared with those of the fertilized eggs, the TTX content per gram was decreased, while the total TTX content increased 300-fold. Toxicity of the eggs and the 12-day-old larvae was measured by mouse assay, and determined to be 82.2 MU/g (equivalent to $16.4 \mu\text{g TTX/g}$) and 44.5 MU/g (equivalent to $8.9 \mu\text{g TTX/g}$), respectively. The results of HPLC analysis and mouse assay agreed with each other. Typical toxin profiles of the eggs and the 12-day-old larvae are shown

in Fig. 1. TTX was detected at a retention time of 18.0 min (Fig. 1, left). A clear peak of TTX was identified in the eggs (Fig. 1, center) and the larvae (Fig. 1, right). Although unknown peaks were also observed in the samples, they did not interfere with the quantitation of TTX. We did not examine the anatomical distribution of TTX because of the small sample size in the present study.

Changes in body weight and TTX content of the puffer fish sample 2 were closely similar to those of sample 1 (Table 2). The body weight was 1.17 mg for the egg, increased with the growth, and reached 12.2 g for the juvenile after 88 days. The TTX content of the puffer fish increased from 4.7 μg TTX/g at the fertilization to 16.0 μg TTX/g at the larval stage after 3 days, thereafter it gradually decreased to 0.25 μg TTX/g at the juvenile stage after 88 days. However, the total

Fig. 1. Toxin profiles of cultured puffer fish *Takifugu rubripes*
Left, TTX standard; center, the fertilized eggs;
right, the larvae 12 days after hatching

TTX content increased by 600-fold, from 0.005 μg TTX for the egg to 3.04 μg TTX for 88-day-old juvenile. It is most likely that the change in the total TTX content of the puffer fish during seed production correlated with that in the body weight.

The present study revealed that the TTX content of the puffer fish *T. rubripes* hatched from toxic eggs was decreased at the larval and juvenile stages. However, the total TTX content of the puffer fish began to increase at the late larval stage 40 or 50 days after hatching, although it is unclear how the puffer fish obtained and accumulated TTX during seed production. HPLC analysis demonstrated that the feeds used in this study contained no detectable amount of TTX ($<0.04 \mu\text{g}$ TTX/g). It is possible that the puffer fish might have eaten TTX-bearing animals adhering to the netcages, because ribbonworms adhering to cultured oysters on floating culture rafts were found to contain a large quantity of TTX, more than 10,000 MU/g¹⁹⁾. In addition, TTX-producing bacteria in the intestines of puffer fish and in seawater might be involved in toxification of the puffer fish during seed production^{11), 14), 20)}. Yamamori suggested the presence of TTX-bearing plankton in Okkirai Bay, Iwate Prefecture and proposed biosynthesis of TTX in puffer fish *T. niphobles* from TTX-precursor in the plankton*¹. The route and mechanism involved in toxification of puffer fish remain to be identified.

Finally, it should be noted that the toxicity of the puffer fish detected in this study is not a matter of concern from the viewpoint of food hygiene, because the safety of puffer fish consumption is evaluated on the basis of toxicity (MU/g or μg TTX/g), but not total toxin content in a specimen.

Acknowledgements

We thank Mie Prefectural Fish Farming Center, Owase for kindly providing specimens of the puffer fish *T. rubripes*.

Table 2. Tetrodotoxin content of puffer fish *Takifugu rubripes* (sample 2) during seed production

Stage/days after hatching	Number of specimens	Average body weight (g)	Tetrodotoxin content (μg TTX/g)	Total tetrodotoxin content (μg TTX/individual)
Fertilized egg	639	1.17×10^{-3}	4.72	0.005
Larvae reared in an indoor tank				
3 days	60	0.41×10^{-3}	16.0	0.006
4 days	121	0.43×10^{-3}	7.04	0.003
5 days	119	0.42×10^{-3}	2.42	0.001
8 days	122	1.11×10^{-3}	1.00	0.001
20 days	71	6.76×10^{-3}	0.52	0.003
50 days	3	746×10^{-3}	0.69	0.526
Juveniles reared in a netcage at sea				
61 days	3	2.29	0.46	1.07
71 days	3	4.12	0.27	1.13
81 days	3	5.99	0.24	1.42
88 days	3	12.2	0.25	3.04

*¹ Yamamori, K. Growth and toxification of juvenile puffer fish. Nippon Suisan Gakkaishi, **75**, 712–713 (2009).

References

- 1) Noguchi, T., Arakawa, O. Tetrodotoxin-distribution and accumulation in aquatic organism, and cases of human intoxication. *Mar. Drugs*, **6**, 220–242 (2008).
- 2) Noguchi, T., Arakawa, O., Takatani, T. TTX accumulation in pufferfish. *Comp. Biochem. Physiol. D*, 145–152 (2006).
- 3) Matsui, T., Hamada, S., Konosu, S. Difference in accumulation of puffer fish toxin and crystalline tetrodotoxin in the puffer fish, *Fugu rubripes rubripes*. *Nippon Suisan Gakkaishi*, **47**, 535–537 (1981).
- 4) Matsui, T., Sato, H., Hamada, S., Shimizu, C. Comparison of toxicity of the cultured and wild puffer fish *Fugu niphobles*. *Nippon Suisan Gakkaishi*, **48**, 253 (1982).
- 5) Yamamori, K., Kono, M., Furukawa, K., Matsui, T. The toxification of juvenile cultured kusafugu *Takifugu niphobles* by oral administration of crystalline tetrodotoxin. *Shokuhin Eiseigaku Zasshi* (J. Food Hyg. Soc. Japan), **45**, 73–75 (2004).
- 6) Honda, S., Arakawa, O., Takatani, T., Tachibana, K., Yagi, M., Tanigawa, A., Noguchi, T. Toxicification of cultured puffer fish *Takifugu rubripes* by feeding on tetrodotoxin-containing diet. *Nippon Suisan Gakkaishi*, **71**, 515–520 (2005).
- 7) Kono, M., Matsui, T., Furukawa, K., Yotsu-Yamashita, M., Yamamori, K. Accumulation of tetrodotoxin and 4,9-anhydrotetrodotoxin in cultured juvenile kusafugu *Fugu niphobles* by dietary administration of natural toxic komonfugu *Fugu poecilonotus* liver. *Toxicon*, **51**, 1269–1273 (2008).
- 8) Nagashima, Y., Toyoda, M., Hasobe, M., Shimakura, K., Shiomi, K. *In vitro* accumulation of tetrodotoxin in pufferfish liver tissue slices. *Toxicon*, **41**, 569–574 (2003).
- 9) Matsumoto, T., Nagsahima, Y., Kusuhara, H., Sugiyama, Y., Ishizaki, S., Shimakura, K., Shiomi, K. Involvement of carrier-mediated transport system in uptake of tetrodotoxin into liver tissue slices of puffer fish *Takifugu rubripes*. *Toxicon*, **50**, 173–179 (2007).
- 10) Noguchi, T., Arakawa, O., Takatani, T. Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. *Comp. Biochem. Physiol. D*, 153–157 (2006).
- 11) Simidu, U., Noguchi, T., Hwang, D.-F., Shida, Y., Hashimoto, K. Marine bacteria which produce tetrodotoxin. *Appl. Environ. Microbiol.*, **53**, 1714–1715 (1987).
- 12) Miyazawa, K., Noguchi, T. Distribution and origin of tetrodotoxin. *J. Toxicol. Toxin Review*, **20**, 11–33 (2001).
- 13) Hamasaki, K., Kogure, K., Noguchi, T., Shida, Y., Ohwada, K. Tetrodotoxin in sinking particles from coastal waters. *Mar. Biol.*, **118**, 761–765 (1994).
- 14) Lin, S.-J., Chai, T.-J., Jeng, S. S., Hwang, D. F. Toxicity of the puffer *Takifugu rubripes* cultured in northern Taiwan. *Fish. Sci.*, **64**, 766–770 (1998).
- 15) Sato, S., Komaru, K., Ogata, T., Kodama, M. Occurrence of tetrodotoxin in cultured puffer. *Nippon Suisan Gakkaishi*, **56**, 1129–1131 (1990).
- 16) Iwamoto, A., Fujimoto, H. (Tabeta, O. ed.) Torafugunogyogotoshigenkanri (Fisheries and stock managements of ocellate puffer *Takifugu rubripes* in Japan). Tokyo, Koseisha Koseikaku, 1997, p. 94–109. (ISBN 4-7699-0843-1)
- 17) Yasumoto, T. Life Health Bureau, Ministry of Health and Welfare (ed.), Standard Methods of Analysis in Food Safety Regulation, Tokyo, Japan Food Hygiene Association, 1991, p. 296–305.
- 18) Nagashima, Y., Maruyama, J., Noguchi, T., Hashimoto, K. Analysis of paralytic shellfish poison and tetrodotoxin by ion-pairing high performance liquid chromatography. *Nippon Suisan Gakkaishi*, **53**, 819–823 (1987).
- 19) Asakawa, M., Toyoshima T., Shida, Y., Noguchi, T., Miyazawa, K. Paralytic toxins in a ribbon worm *Cephalothrix* species (Nemertean) adherent to cultured oysters in Hiroshima Bay, Hiroshima Prefecture, Japan. *Toxicon*, **38**, 763–773 (2000).
- 20) Noguchi, T., Hwang, D.-F., Arakawa, O., Sugita, H., Deguchi, Y., Shida, Y., Hashimoto, K. *Vibrio alginolyticus*, a tetrodotoxin-producing bacterium, in the intestines of the fish *Fugu vermicularis vermicularis*. *Mar. Biol.*, **94**, 625–630 (1987).