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Toxic *Takifugu pardalis* eggs found in *Takifugu niphobles* gut: Implications for TTX accumulation in the pufferfish

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ABSTRACT

Pufferfish (*Takifugu* spp.) possess a potent neurotoxin, tetrodotoxin (TTX). TTX has been detected in various organisms including food animals of pufferfish, and TTX-producing bacteria have been isolated from these animals. TTX in marine pufferfish accumulates in the pufferfish via the food web starting with marine bacteria. However, such accumulation is unlikely to account for the amount of TTX in the pufferfish body because of the minute amounts of TTX produced by marine bacteria. Therefore, the toxification process in pufferfish still remains unclear. In this article we report the presence of numerous *Takifugu pardalis* eggs in the intestinal contents of another pufferfish, *Takifugu niphobles*. The identity of *T. pardalis* being determined by direct sequencing for mitochondrial DNA. LC-MS/MS analysis revealed that the peak detected in the egg samples corresponded to TTX. Toxification experiments in recirculating aquaria demonstrated that cultured *Takifugu rubripes* quickly became toxic upon being fed toxic (TTX-containing) *T. rubripes* eggs. These results suggest that *T. niphobles* ingested the toxic eggs of another pufferfish *T. pardalis* to toxify themselves more efficiently via a TTX loop consisting of TTX-bearing organisms at a higher trophic level in the food web.

1. Introduction

Tetrodotoxin (TTX) is known to be the substance of pufferfish toxin and one type of potent neurotoxin specific to voltage-gated sodium channels of excitable membranes of muscle and nerve tissues (Colquhon et al., 1972; Narahashi, 2001; Noguchi et al., 2006a). TTX was believed to occur only in pufferfish (Tetraodontidae) until 1960's, when it was detected in Californian newt *Taricha torosa* (Mosher et al., 1964). Subsequently, TTX (along with some analogs) was also detected from potential pufferfish food organisms belonging to various disparate groups, including starfish (e.g., *Astropecten* spp.; Maruyama et al., 1984, 1985), gastropods (e.g., *Babylonia japonica*; Noguchi et al., 1981), crustaceans (e.g., the xanthid crab, *Atergatis floridus*; Noguchi et al., 1983), flatworms and ribbonworms (e.g., *Cephalothrix simula*; Asakawa et al., 2013), apart from several species of bacteria that are symbiotic with the pufferfish and the potential food organisms (Noguchi et al., 1987; Wu et al., 2005; Noguchi and Arakawa, 2008). In addition, TTX has been detected in some free-living bacteria, including those in deep sea sediments (Simidu et al., 1987; Do et al., 1990), although it is not clear if these bacteria form part of the food chain leading to pufferfish. In any case, it appears plausible that the TTX in pufferfish is a result of accumulation through the food chain, which consists of several steps, starting with bacteria, as suggested by several reports (Noguchi et al., 2006a; Noguchi and Arakawa, 2008). These speculations have actually been supported by several studies: non-toxic pufferfish have been produced when grown from hatching with a non-toxic diet, and furthermore, these cultured non-toxic pufferfish have become toxic when administered orally with TTX (Matsui et al., 1981, 1982; Noguchi et al., 2006b; Saito et al., 1984; Yamamori et al., 2004; Honda et al., 2005).

TTX has been detected not only in pufferfish and their prey, but also in organisms ecologically unrelated to pufferfish, such as the Costa Rican frogs of the genus *Atelopus* (Kim et al., 1975) and some land planarians (Stokes et al., 2014), besides Californian newt *T. torosa* (Mosher et al., 1964). It has also been suggested that TTX in the rough-skin newt, *Taricha granulosa*, is obtained endogenously

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(Hanifin et al., 2002; Cardall et al., 2004). These findings suggest that TTX accumulates in pufferfish by means other than through the classical food chain; since in vivo cultured TTX-producing bacteria are unable to produce enough quantities of TTX to account for the amount of TTX in wild pufferfish (Miyazawa and Noguchi, 2001; Food Safety Commission of Japan, 2005; Wu et al., 2005; Wang et al., 2008; Yang et al., 2010). This also indicates that TTX-bearing pufferfish prey are abundant. In any case, the origin of TTX and the acquisition process are both likely to vary across different animal species, and it remains unclear exactly where pufferfish acquire the large quantities of TTX that they possess.

Recently, our lab unexpectedly found numerous eggs among the intestinal contents of the pufferfish, *Takifugu niphobles*, and partial mitochondrial DNA sequences from these eggs identified them to be those of another pufferfish species, namely, *Takifugu pardalis*. We have demonstrated the toxification process using cultured pufferfish *Takifugu rubripes* in this study by means of experimentally reproducing the serendipitous finding of eggs in the pufferfish gut, thus indicating that pufferfish toxification manifests from the accumulated TTX at relatively higher trophic levels in the food chain.

2. Materials and methods

2.1. The intestinal contents of wild pufferfish

Wild specimens of the pufferfish, *T. niphobles* (body weight: 60.3 ± 25.7 g and 48.9 ± 21.2 g for females and males, respectively; detailed in Table 1) were collected from coastal waters in Nagai, Kanagawa, Japan (35°12′N, 139°36′E), on 13 March 2012 (water temperature: 13.9 °C; salinity: 31.4 practical salinity unit, psu), 08 March 2013 (16.0 °C; 32.4 psu) and 14 March 2013 (14.2 °C; 30.6 psu), 07 March 2014 (12.5 °C; 32.3 psu) and 13 March 2014 (12.5 °C; 31.8 psu), and 09 March 2015 (11.7 °C; 34.3 psu), 16 March 2015 (12.3 °C; 34.8 psu) and 19 March 2015 (14.6 °C; 35.0 psu). The gonadosomatic index (GSI) was 2.2 ± 0.9 for females and 2.0 ± 1.4 for males (detailed in Table 1). The unexpected eggs that were found among the gut contents of the pufferfish were also collected. Five eggs from each fish were immediately subjected to DNA extraction and the remaining eggs were stored at −30 °C until TTX extraction.

2.2. DNA extraction and PCR amplification

Total genomic DNA was extracted from each egg by the method of Sezaki et al. (1999). The fragments of partial mitochondrial DNA were amplified by PCR using two primer sets, 16S AR-L (forward: 5′-CGCCT GTTTA TCAAA AACAT-3′) and 16S BR-H (reverse: 5′-CCGGT CTGAA CTCAG ATCAC GT-3′) for the 16S rRNA gene (Palumbi et al., 1991), and TCytb-F1 (forward: 5′-ACCTR TGGCG TGAAA AACCA YCGTT GT-3′) and TCytb-R1 (reverse: 5′-CATYC GGTTT ACAAG ACCGR CGCTC TG-3′) for the cytochrome *b* gene. Primers for cytochrome *b* gene were designed based on the mitochondrial DNA sequences from multiple species of the family Tetraodontidae. PCR amplification was performed in a 20 μl reaction mixture containing genomic DNA as a template, 1 unit *ExTaq* DNA polymerase (Takara Bio, Shiga, Japan), 1.6 μl of 2.5 mM deoxynucleotide triphosphates (dNTP), 5 μl of 5 μM primers and 2 μl of 10 × *ExTaq* DNA polymerase buffer (Takara Bio). The thermal cycling program for the PCR consisted of an initial denaturation at 95 °C for 1 min followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s.

2.3. Direct sequencing and phylogenetic analyses

Prior to the direct sequencing of the amplified product, the DNA fragment was purified by chloroform extraction, followed by polyethylene glycol (PEG) 8000 precipitation and ethanol precipitation. Sequencing was performed for both strands using a 3130*xl* genetic analyzer (Applied Biosystems, Foster, CA, USA) and a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The concatenated nucleotide sequences of the 16S rRNA gene and cytochrome *b* gene of eggs were aligned using CLUSTAL W (Thompson et al., 1994) with those in the DDBJ/EMBL/GenBank databases obtained using a BLAST search (Altschul et al., 1997). The alignment was then subjected to phylogenetic inference by means of the maximum likelihood method using MEGA ver. 6.0.6 (Tamura et al., 2013), with the corresponding concatenated sequences from the yellow-stripe toadfish, *Torquigener brevipennis*

Table 1
Characteristics of the *Takifugu niphobles* specimens used in this study[a].

Sampling date	Sex	No. of specimen	No. of egg-fed specimen	Intestinal egg content (g)	Toxicity of the intestinal eggs (MU)	Standard length (mm)	Body weight (g)	GSI[b]
2012								
13 Mar	Female	n = 8	n = 8	N/D[c]	N/D	119.4 ± 3.8	64.1 ± 9.4	2.6 ± 0.5
	Male	n = 2	n = 2	N/D	N/D	111.0 ± 2.0	54.5 ± 1.5	1.5 ± 0.2
2013								
08 Mar	Female	n = 9	n = 8	0.28 ± 0.25	2.63 ± 4.42	112.7 ± 18.3	60.3 ± 22.8	2.1 ± 0.8
	Male	n = 13	n = 7	0.05 ± 0.09	0.25 ± 0.74	108.4 ± 17.7	55.2 ± 22.0	1.9 ± 1.1
14 Mar	Female	n = 6	n = 6	0.72 ± 0.48	10.38 ± 12.94	122.2 ± 16.7	56.2 ± 24.5	2.0 ± 0.4
	Male	n = 8	n = 8	0.26 ± 0.36	4.10 ± 6.48	110.8 ± 11.1	55.4 ± 12.2	2.7 ± 0.8
2014								
07 Mar	Male	n = 2	n = 2	0.44 ± 0.30	0.29 ± 0.17	128.0 ± 1.0	73.3 ± 3.6	2.6 ± 0.3
13 Mar	Female	n = 10	n = 7	0.38 ± 0.62	0.49 ± 0.66	121.7 ± 17.2	63.9 ± 23.8	2.4 ± 1.3
	Male	n = 6	n = 3	0.09 ± 0.12	0.06 ± 0.13	111.3 ± 12.5	50.0 ± 17.4	3.0 ± 2.1
2015								
09 Mar	Female	n = 13	n = 0	N/A[d]	N/A	118.7 ± 12.8	33.0 ± 9.7	1.9 ± 0.5
	Male	n = 10	n = 3	0.05 ± 0.08	2.72 ± 7.04	115.6 ± 17.4	30.8 ± 15.4	1.3 ± 1.3
16 Mar	Female	n = 11	n = 8	0.38 ± 0.57	15.84 ± 30.37	164.6 ± 17.6	87.3 ± 20.9	2.5 ± 0.7
	Male	n = 4	n = 2	0.08 ± 0.08	1.63 ± 2.32	140.0 ± 26.0	59.0 ± 28.0	2.1 ± 1.1
19 Mar	Female	n = 4	n = 4	0.47 ± 0.37	3.30 ± 2.46	130.0 ± 18.1	43.5 ± 16.0	2.5 ± 1.6
	Male	n = 4	n = 0	N/A	N/A	133.0 ± 22.5	45.5 ± 18.1	2.0 ± 1.2

[a] Data are represented by mean ± standard deviation.
[b] GSI represents gonadosomatic index: gonad weight/body weight × 100.
[c] N/D, not determined.
[d] N/A, not applicated.