Table 3. List of genes downregulated in the liver of the TTX-administered pufferfish *Takifugu rubripes* group compared to those in the buffer-administered control group (FC [1] > 3.0).

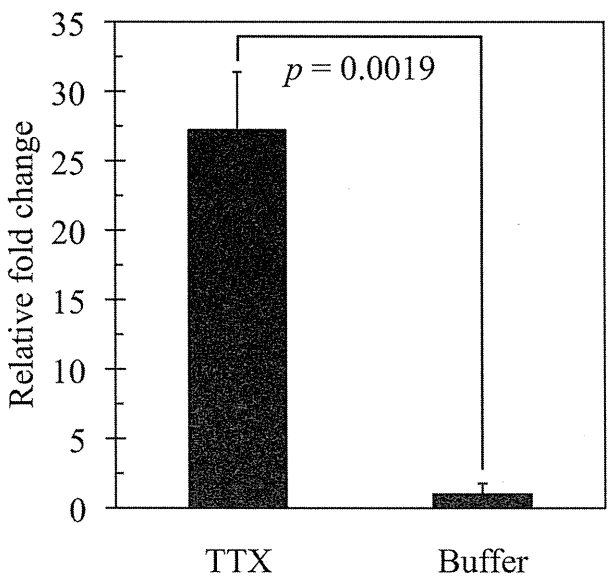
Ensemble ID	Gene name	Predicted description	Functional classification	FC
ENSTRUT00000047556	Fusa2	Elongation factor G 2	Transcription factor	−3.0
ENSTRUT00000009260	Rspo3	R-spondin-3	Transduction	−7.8
ENSTRUT00000029878	Ncoa2	Nuclear receptor coactivator 2	Transcription factor	−7.3
ENSTRUT00000005082	Sasb	Fatty acyl-CoA hydrolase precursor, medium chain	Enzyme, cofactor	−6.2
ENSTRUT00000042623	Kcnj3	G protein-activated inward rectifier potassium channel 1	Ion channel activity	−5.9
ENSTRUT00000029012	Galnt1	Polypeptide N-acetylgalactosaminyltransferase 1	Enzyme, cofactor	−5.7
ENSTRUT00000034755	Stk11ip	Serine/threonine kinase 11-interacting protein	Protein binding	−5.5
ENSTRUT00000007314	Unc5d	Netrin receptor UNC5D	Receptor activity	−5.3
ENSTRUT00000024559	Dnmt3a	DNA (cytosine-5)-methyltransferase 3A	Transcription factor	−4.7
ENSTRUT00000028271	finTRIM	Fish virus induced TRIM protein	Metal ion binding	−4.7
ENSTRUT00000021561	Synpo2	Synaptopodin-2	Protein binding	−4.4
ENSTRUT00000035155	Klhl8	Kelch-like protein 8	Protein binding	−4.4
ENSTRUT00000013283	Lnx2	Ligand of Numb protein X2	Metal ion binding	−4.3
ENSTRUT00000005282	Sox5	*T. rubripes* transcription factor SOX5 (AY277973.1)	Transcription factor	−4.1
ENSTRUT00000004950	Egr3	Early growth response protein 3	Transcription factor	−4.0
ENSTRUT00000001876	Nme1	Nucleoside diphosphate kinase A	Transcription factor	−4.0
ENSTRUT00000037748	Cadm2	Cell adhesion molecule 2	Protein binding	−3.9
ENSTRUT00000029148	Cln3	Battenin	Enzyme, cofactor	−3.8
ENSTRUT00000018412	Hecd3	Probable E3 ubiquitin-protein ligase HECTD3	Protein binding	−3.7
ENSTRUT00000010231	Wipi2	WD repeat domain phosphoinositide-interacting protein 2	Enzyme, cofactor	−3.7
ENSTRUT00000038056	Angpt2	Angiopoietin-2	Receptor activity	−3.7
ENSTRUT00000011781	-	Putative F-type lectin	Sugar binding	−3.6
ENSTRUT00000025271	Tyro3	Tyrosine-protein kinase receptor TYRO3	Transduction	−3.6
ENSTRUT00000036834	Pcolce	*T. rubripes* procollagen C-endopeptidase enhancer 1 (AF016494.1)	Protein binding	−3.4
ENSTRUT00000009581	Rims1	Regulating synaptic membrane exocytosis protein 1	Protein binding	−3.4
ENSTRUT00000013940	Pdlim5	PDZ and LIM domain protein 5	Protein binding	−3.4
ENSTRUT00000041778	Foxa3	*T. rubripes* folkhead transcription factor FoxA3 (AB604763.1)	Transcription factor	−3.3
ENSTRUT00000026385	Fam70a	Protein FAM70A	Unknown	−3.3
ENSTRUT00000003229	Arhgef26	Rho guanine nucleotide exchange factor 26	Transcription factor	−3.2
ENSTRUT00000007982	Kif2c	Kinesin-like protein KIF2C	Protein binding	−3.2
ENSTRUT00000013282	Lnx2	Ligand of Numb protein X2	Protein binding	−3.2
ENSTRUT00000005850	Crybb2	Beta-crystallin A2	Protein binding	−3.2
ENSTRUT00000003860	Edaradd	Ectodysplasin-A receptor-associated adapter protein	Protein binding	−3.1
ENSTRUT00000015819	Pbxip1	Pre-B-cell leukemia transcription factor-interacting protein 1	Transcription factor	−3.1
ENSTRUT00000011222	Serpinh1	Serpin H1	Protein binding	−3.1

Table 3. *Cont.*

Ensemble ID	Gene name	Predicted description	Functional classification	FC
ENSTRUT00000020096	Atp2b3	Plasma membrane calcium-transporting ATPase 3	Transporter activity	−3.1
ENSTRUT00000009874	Plxdc1	Plexin domain-containing protein 1	Unknown	−3.1
ENSTRUT00000047512	Suox	Sulfite oxidase, mitochondrial	Enzyme, cofactor	−3.1
ENSTRUT00000020028	finTRIM	Fish virus induced TRIM protein	Metal ion binding	−3.0
ENSTRUT00000041535	Pxn	Paxillin	Protein binding	−3.0
ENSTRUT00000033313	Nox5	*T. rubripes* NADPH oxidase 5 (BR000279.1)	Enzyme, cofactor	−3.0
ENSTRUT00000027204	Pacs2	Phosphofurin acidic cluster sorting protein 2	Unknown	−3.0
ENSTRUT00000044222	Tgfbrap1	Transforming growth factor-beta receptor-associated protein 1	Protein binding	−3.0
ENSTRUT00000027017	Hnrnpk	Heterogeneous nuclear ribonucleoprotein K	Transcription factor	−3.0
ENSTRUT00000043644	Hsp90b1	Heat shock protein 90kDa beta member 1	Protein binding	−3.0
ENSTRUT00000043157	Tle3	*T. rubripes* transducin-like enhancer protein 3 (AB236415.1)	Transcription factor	−3.0
ENSTRUT00000022207	Vwa1	Von Willebrand factor A domain-containing protein 1	Unknown	−3.0
ENSTRUT00000026592	Tacr3	Neuromedin-K receptor	Receptor activity	−3.0
ENSTRUT00000028610	Dach1	Dachshund homolog 1	Protein binding	−3.0
ENSTRUT00000011629	Gas2l3	GAS2-like protein 3	Protein binding	−3.0
ENSTRUT00000018505	Epha2	Ephrin type-A receptor 4 precursor	Receptor activity	−3.0

[1] FC is the average fold change of the TTX-administered (n = 5) compared to buffer-administered control group (n = 5).

Figure 4. Real-Time PCR of the gene encoding chymotrypsin-like elastase family member 2A in the liver of *Takifugu rubripes* from both TTX- and buffer-administered groups. Each value represents the mean ± SE of three individuals, each performed in duplicate.



4. Discussion

In this study, we performed a single intramuscular administration of TTX to cultured marine pufferfish specimens of *T. rubripes* and DNA microarray gene expression analysis on Day 5 after the administration to identify genes possibly related to TTX accumulation in the liver.

TTX was detected in the liver, skin and ovary, but not in the muscle and testis of the pufferfish specimens in the TTX-administered group. The amount of TTX was highest in the liver and skin. The skin accumulated 28 ± 5% of the administered dose at the same level as that of the liver (28 ± 6%) on Day 5. We previously reported that the liver and skin of cultured pufferfish specimens of *T. rubripes* (940–1120 g body weight) accumulated up to 63 ± 5% and 9 ± 3% of the administered dose of 0.25 mg TTX/kg body weight at 60 min after intravascular administration, respectively [17]. In this connection, the examination of the tissue distribution of ^{3}H-labeled TTX in cultured *T. rubripes* (90 and 110 g body weight) revealed that the total radioactivity was distributed mainly in the skin (45.1% and 54.1%, respectively), muscle (7.4% and 8.0%, respectively) and liver (19.0% and 15.7%, respectively) on Day 6 after intraperitoneal administration of ^{3}H-labeled TTX [34]. In addition, Honda *et al.* [5] performed the feeding experiments, in which zero-year- and one-year-old pufferfish specimens of cultured *T. rubripes* were reared for 60 days with various types of TTX-containing diets, and revealed that the test fish accumulated a small amount of TTX (less than 3 MU (mouse unit)/g in most cases) mainly in the skin and liver at low doses (0.1 MU/g body weight/day) and a large amount (up to 57 MU) mostly in the liver and ovary at higher doses (0.2–1.0 MU/g body weight/day). Moreover, Ikeda *et al.* [35] examined the transfer profile of intramuscularly administered 50 MU of TTX to the cultured young immature pufferfish *T. rubripes* (approximately four-months-old, 13.2 ± 3.4 g body weight). They reported that TTX tends to be transferred to the skin from the other tissues, such as the liver and circulating blood, and that the total amount of TTX remaining in the entire body at 72–168 h after administration was approximately 60%–80%. These results suggest that TTX was transferred to skin tissues regardless of the administration routes and would be released from the skin tissues to excrete excess TTX or as a biologic defense substance against predators [36–38].

Lee *et al.* [22] previously reported three fibrinogen-like protein genes expressed in toxic liver of two different pufferfish, akamefugu *T. chrysops* and kusafugu *T. niphobles*. However, the expressions of these genes were not observed in this study. Little is known about the timing of expression of these genes after the toxification of pufferfish liver. The other possibility is that these genes were tremendously expressed, and their transcripts were too highly labeled with Cy3 to be measured by microarray analysis. Further investigations are required about the relationship between the hepatic toxicity and the expression mechanism to understand the functions of these genes.

Matsumoto *et al.* [23] examined the hepatic gene expression profile of cultured *T. rubripes* at 12 h after intramuscular administration of TTX by suppression subtractive hybridization and found that upregulated genes encoded acute-phase response proteins, including hepcidin, complement components, serotransferrin, apolipoprotein A-1, high temperature adaptation protein Wap65-2, fibrinogen beta chain and 70 kDa heat-shock protein 4, in the liver. In this study, the increased expression of these genes were not detected, suggesting that these proteins subsided within five days after intramuscular administration of TTX.

Feroudj *et al.* [24] performed DNA microarray analysis with total RNAs from toxic and non-toxic wild pufferfish, demonstrating that 1108 transcripts were more than two-fold higher in toxic than nontoxic specimens. The expression levels of nine genes were upregulated more than 10-fold in toxic and proteins encoded by these genes were related to vitamin D metabolism and immunity.

Yotsu-Yamashita *et al.* [39] reported liver-specific expression of pufferfish saxitoxin and tetrodotoxin binding protein (PSTBP) in the marine pufferfish, *T. pardalis*. In addition, Tatsuno *et al.* [40] found four genes (Tr1–Tr4) encoding PSTBP homologs from the publicly available Fugu genome database and revealed the constitutive expression of two distinct isoforms (Tr1 and Tr3) in the liver of cultured non-toxic *T. rubripes* specimens, declining in their toxin-triggered gene expression. In this study, the expression change of genes encoding PSTBP homologs was hardly observed. PSTBP and its homologs may have functions to bind toxic substances other than TTX, when TTX is absent.

In the TTX-administered group, 59 and 427 genes were significantly upregulated and downregulated, respectively, in comparison with the buffer-administered control group (two-fold change, $p < 0.05$). The highest upregulated gene was chymotrypsin-like elastase family 2A (Cela2a), known as elastase-2A, with 37.6 FC. The validity of this value was confirmed by real-time PCR analysis, indicating a good quantitative performance of the microarray analysis. A homologous gene encoding elastase 2A was first cloned from the human pancreas [41]. Further details about human pancreatic elastase has recently been revealed through the human gene project, and the expression of human elastase 2A gene encoding "neutrophil elastase" has been found to be regulated by hematopoietic transcription factors, such as AML1, C/EBPα, PU.1 and c-Myb transcription factors [42,43]. However, there is still limited information on secretion in pancreatic juice [44]. Although *T. rubripes* Cela2a is estimated to correspond to elastase 2A in hepatopancreatic juice, which digests elastic and fibrous proteins, little information is available on the gene expression and regulation mechanism of *T. rubripes* Cela2a. One possibility is that the hepatopancreatic digestion is activated during enterohepatic metabolism of TTX in the pufferfish liver.

This study demonstrated the upregulation of the sodium channel beta-2 subunit gene (Scn2b, FC value of 4.0) by TTX administration. The sodium channel beta-2 subunit modulates the kinetics of channel gating, as well as the stabilization and location of TTX-sensitive voltage-gated sodium channels [45–47]. Pertin *et al.* [48] reported a marked upregulation of the beta-2 subunit in the spared nerve injury model of rat. Lopez-Santiago *et al.* [49] also reported that beta-2 subunit modulates mRNA and protein expression of TTX-sensitive voltage-gated sodium channels. These findings suggest that TTX accumulation in pufferfish liver affects the expression and composition of voltage-gated sodium channels.

Dysferlin gene (Dysf, an FC value of 4.1) was also upregulated in the liver of the TTX-administered group. Dysferlin is a ubiquitously expressed transmembrane protein involved in Ca^{2+}-mediated plasma membrane repair, vesicle fusion and Ca^{2+} homeostasis in skeletal muscle, regulating cell adhesion in human monocytes [50–52]. Oulhen *et al.* [53] suggested that dysferlin is essential for endocytosis oogenesis and embryogenesis in the sea star, *Patiria miniata*. TTX accumulation may damage plasma membranes, and thus, the upregulation of dysferlin found in this study may be related to the upregulation of the sodium channel beta-2 subunit gene, because channel proteins fuse with the plasma membrane.

Kitamura *et al.* [54] investigated gene expression changes in the cerebral cortical cells from E18 rat embryos by DNA microarray analysis in the presence and absence of TTX. They identified genes involved in the postsynaptic scaffold, regulation of actin dynamics, synaptic vesicle exocytosis and regulation of G-protein signaling as those downregulated in the presence of TTX and upregulated in the absence of TTX. In the present study, Rho GTPase-activating protein 29 gene (Arhgap29) was upregulated with 12.1 FC on Day 5 after TTX administration. Arhgap29 is a negative regulator of the Rho GTPase signaling pathway, which controls cytoskeletal rearrangement in human and other organisms [55–57]. This study also demonstrated the upregulation of the probable G-protein-coupled receptor 22 gene (GPR22, 3.3 FC), the GTP-binding protein Rheb gene (Rheb, 3.2 FC), Arf GAP with GTPase domain ankyrin repeat and the PH domain 2 gene (Agap2, 3.0 FC). Recently, Adams *et al.* [58] have found that the GPR22 gene is selectively expressed in the brain and heart of human and rodents, suggesting a possible role of GPR22 protein in the regulation of cardiac contraction. However, natural ligands for this receptor remain to be understood, and its function in other animal tissues is also unclear. Rheb protein is a molecular switch in many cellular processes, such as cell volume increase, cell cycle progression, neuronal axon regeneration, autophagy regulation, nutritional deprivation, cellular stress resistance and cellular energy control [59,60]. On the other hand, genes encoding the G-protein-activated inward rectifier potassium channel 1 gene (Kcnj3, FC value of −5.9) and the Rho guanine nucleotide exchange factor 26 gene (Arhgef26, FC value of −3.2) were downregulated in the liver of the TTX-administered group. As is well known, these genes are related to a G-protein-coupled receptor signal transduction system, suggesting that receptors and signaling pathways involved in cellular response to TTX may exist in pufferfish liver to reduce the toxic effect and to accumulate TTX.

It was demonstrated in the present study that the intramuscular administration of TTX influences the hepatic gene expression involved in gene transcription, the signaling pathway via receptors and channels and metabolic pathways. It has been reported that genes related to immunity and acute-phase responses were found to be upregulated in cultured *T. rubripes* on the intraperitoneal injection of TTX by SSH [23], in wild *T. chrysops* and *T. niphobles* by RAP RT-PCR [22] and in wild *T. rubripes* microarray analysis [24]. It is noted that samples were taken on Day 5 after TTX administration in the present study, differing from those taken at 12 h after administration of TTX for SSH, although both investigations adopted cultured *T. rubripes*. It may be important to have a G-protein-related signaling pathway for shifting acute-phase to steady-state metabolic responses. Alternatively, health conditions of pufferfish may cause varied gene expression patterns relating accumulation of TTX. Further investigation is needed to understand the biological significance of TTX in pufferfish.

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Author Contributions

Ta.M designed the study. S.W. arranged and oversaw the project. Ta.M., H.F., R.K., To.M., M.K., and Y.N. planned and performed the TTX administration test and TTX analysis. Ta.M., H.F., Y.K., H.K., and I.H. performed DNA microarray analysis. Ta.M. performed real-time PCR analysis. Ta.M., H.F., G.K., and H.U. undertook the data analysis. Ta.M. and S.W. wrote the manuscript with support from all authors.

Conflicts of Interest

The authors declare no conflict of interest.

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DNA microarray analysis on gene candidates possibly related to tetrodotoxin accumulation in pufferfish

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ABSTRACT

Pufferfish accumulate tetrodotoxin (TTX) at high levels in liver and ovary through the food chain. However, the mechanisms underlying TTX toxification in pufferfish have been poorly understood. In order to search gene candidates involved in TTX accumulation in the torafugu pufferfish *Takifugu rubripes*, a custom 4x44k oligonucleotide microarray slide was designed by the Agilent eArray program using oligonucleotide probes of 60 bp in length referring to 42,724 predicted transcripts in the publicly available Fugu genome database. DNA microarray analysis was performed with total RNA samples from the livers of two toxic wild specimens in comparison with those from a nontoxic wild specimen and two nontoxic cultured specimens. The mRNA levels of 1108 transcripts were more than 2-fold higher in the toxic specimens than in the nontoxic specimens. The levels of 613 transcripts were remarkably high, and 16 transcripts encoded by 9 genes were up-regulated more than 10-fold. These genes included those encoding forming structural filaments (keratins) and those related to vitamin D metabolism and immunity. It was also noted that the levels of the transcripts encoding serpin peptidase inhibitor clade C member 1, coagulation factor X precursor, complement C2, C3, C5, C8 precursors, and interleukin-6 receptor were high in the toxic liver samples.

1. Introduction

Tetrodotoxin (TTX) is one of the most potent non-proteinous neurotoxins blocking voltage-gated sodium channels (Narahashi et al., 1967; Kao, 1982) and commonly present in various tissues of pufferfish (Hashimoto, 1979). Pufferfish generally show the highest toxicity in the liver and ovary, followed by the intestine and skin, although their TTX accumulation patterns are species-specific (Watabe et al., 1987; Noguchi and Arakawa, 2008). TTX had been

Abbreviations: APP, acute phase protein; flp, fibrinogen-like protein; IL-6, interleukin-6; LC–FLD, liquid chromatography–fluorescence detection; RAP RT–PCR, mRNA arbitrarily primed reverse transcription–polymerase chain reaction; SSH, suppression subtractive hybridization; TTX, tetrodotoxin.

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believed to be present only in pufferfish for a long time. In 1960s and 1970s, however, TTX was found in other vertebrates including California newt *Taricha torosa* (Mosher et al., 1964), the goby *Yongeichthys criniger* (Noguchi and Hashimoto, 1973), and Costa Rican frogs *Atelopus* spp. (Kim et al., 1975). Since then, TTX has been detected in various organisms of different phylogenic classes including invertebrates such as trumpet shell *Charonia sauliae* (Narita et al., 1987), xanthid crab *Atergatis floridus* (Noguchi et al., 1984), blue-ringed octopus *Hapalochlaena maculosa* (Sheumack and Howden, 1978; Yotsu-Yamashita et al., 2007) and flatworm *Planocera multitentaculata* (Miyazawa et al., 1986). Finally, it was found that TTX is produced in intestinal bacterial strains of *Vibrio* sp. from pufferfish and toxic crabs (Noguchi et al., 1986, 1987) and in other bacteria such as *Shewanella alga* and *Alteromonas tetraodonis* from red calcareous alga *Jania* sp. (Yasumoto et al., 1986a,b). Thus it is likely that TTX-bearing pufferfish accumulate TTX in their body through the food chain. This scheme is supported by the fact that non-toxic cultured pufferfish were never toxic when reared with non-toxic diets (Matsui et al., 1981), but became toxic upon ingesting the toxic ovary of wild pufferfish (Saito et al., 1984). However, there is another possibility that TTX is synthesized in pufferfish themselves, because *in vivo* cultured TTX-producing bacteria do not produce enough quantities of TTX that explain toxicity of wild pufferfish (Matsui et al., 1990). The problem is that genes participating in TTX biosynthesis have not been found yet in any TTX-bearing organisms.

Lee et al. (2007) first attempted to investigate genes related to the biosynthesis or accumulation of TTX in pufferfish. In their study, mRNA expression patterns in the liver of pufferfish, akamefugu *Takifugu chrysops* and kusafugu *Takifugu niphobles*, were compared by mRNA arbitrarily primed reverse transcription–polymerase chain reaction (RAP RT–PCR) with pufferfish bearing different concentrations of TTX and its derivatives in the liver. RAP RT–PCR provided three fibrinogen-like protein (flp) genes that were expressed higher in toxic than non-toxic pufferfish liver. Relative mRNA levels of flp-1, flp-2, and flp-3 genes showed a linear correlation with toxicity in the liver of two pufferfish species. Later, Matsumoto et al. (2011) examined changes in the gene expression profile in the liver of the torafugu pufferfish, *Takifugu rubripes*, induced by an intramuscular administration of TTX by employing suppression subtractive hybridization (SSH). The SSH study revealed that genes encoding acute-phase response proteins such as hepcidin precursors (Krause et al., 2000; Park et al., 2001), complement C3 and warm-temperature-acclimation-related 65 kDa protein (Wap65) (Hirayama et al., 2003) increased their transcript levels in the liver.

The objective of this study was to find gene candidates possibly involved in TTX accumulation by DNA microarray analysis with wild torafugu specimens that have different concentrations of TTX in the liver.

2. Materials and methods

2.1. Materials

Three wild torafugu specimens (526, 650, and 975 g body weight) were collected from Tokyo Bay off Miura Peninsula in December, 2009 (Table 1). Two cultured torafugu specimens (782 and 815 g body weight) were obtained alive from a local fish farmer in Ehime Prefecture, Japan, which had been cultured by a conventional method in net cages at Uwa Sea, and used as non-toxic control samples. Crystalline TTX (Wako Pure Chemicals Industries, Osaka, Japan) was used as a standard for the liquid chromatography–fluorescence detection (LC–FLD) analysis. All other chemicals were of a reagent grade.

2.2. TTX extraction and determination

TTX was extracted from the liver with 0.1% acetic acid by heating in a boiling water bath for 10 min according to the standard assay procedures for TTX (Kodama and Sato, 2005). The extracts were then applied to a Bio-Gel P-2 column (3 cm × 15 cm, Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with water after adjusting pH of the extract to 6.5 with 0.1 N NaOH. TTX quantitation was performed by LC–FLD analysis according to the methods of Nagashima et al. (1987) and Yotsu (1989).

2.3. Design of torafugu microarray

The nucleotide sequences of predicted transcripts in fugu genome were cited from the ensemble database version 55. Among 48,623 transcripts, 42,724 annotated were used for designing probes. Oligonucleotide probes of 60 bp in length were designed by eArray program (https://earray.chem.agilent.com/earray/) and spotted on a slide glass by Agilent Technologies (Santa Clara, CA, USA; design ID, 025856).

2.4. Microarray analyses

Total RNA was extracted from the liver using RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA was labeled with Cy3 using One Color Quick Amp Labeling Kit (Agilent Technologies) and hybridized on the microarray using Agilent Gene Expression Hybridization Kit (Agilent Technologies) according to the manufacturer's instructions. After hybridization, the slide glass was washed using Agilent Gene Expression Wash Buffer Kit (Agilent Technologies) and scanned with Genepix 400B (Axon Instruments, Foster City, CA, USA). Fluorescence intensity was calculated by using Feature Extraction software version 9.5 (Agilent Technologies) and the data was analyzed with GeneSpring GX software version 11.0 (Agilent Technologies). The data was normalized among the arrays and the transcripts showing more than 2-fold differences between toxic and

Table 1
Toxicity of the liver of wild torafugu *Takifugu rubripes*.

No.	Body length (cm)	Body weight (g)	Liver weight (g)	TTX conc. (μg/g)	Toxicity (MU/g)
1	36	650	14.5	90.7	452
2	33	526	11.1	24.5	123
3	39	975	26.5	N.D.	–

N.D. indicates a sample where TTX was not detected (<0.15 μg TTX/g tissue). MU indicates mouse unit.

non-toxic pufferfish samples at a certain statistical cut-off were searched.

3. Results and discussion

TTX in the liver of wild specimens was analyzed by LC–FLD, and the concentrations of TTX determined are listed in Table 1. TTX was detected in the liver from wild pufferfish No. 1 and No.2, but not in the liver of No. 3 (<0.15 μg TTX/g tissue). The liver of the cultured specimens used in this study did not contain any detectable amount of TTX.

Based on these results, DNA microarray analysis was performed with total RNA from the liver of two toxic wild specimens in comparison with those from a non-toxic wild specimen and two cultured specimens. The DNA microarray analysis revealed that the mRNA levels of 1108 transcripts showed more than 2-fold differences in the liver between toxic and non-toxic pufferfish. The levels of 613 transcripts were higher in the toxic liver than in the non-toxic liver, and the levels of 16 transcripts encoded by 9 genes were up-regulated more than 10-fold in the toxic liver (Table 2). These are involved in the formation of structural filaments (keratins), vitamin D metabolism, and immunity. Lee et al. (2007) claimed that, in two different pufferfish, akamefugu *T. chrysops* and kusafugu *T. niphobles*, the mRNA levels of fibrinogen-like and hepcidin-like genes that are likely to be involved in immunity were high in the liver containing high concentrations of TTX. Matsumoto et al. (2011) also identified various genes in the liver of cultured torafugu induced by an intramuscular administration of TTX, with a possible relation to immunity. In this study, we identified several genes involved in blood coagulation and complement pathway (Table 3). Because the mRNA levels of certain genes identified so far, such as hepcidin, were too high, the fluorescent intensity was saturated and we could not detect any differences in the mRNA levels for these genes between toxic and non-toxic livers.

In addition to the blood coagulation factors/regulators and complement components, the mRNA level of the interleukin-6 (IL-6) receptor gene was high in the toxic liver (Table 3). IL-6 is a cytokine that affects various cells (Van Snick, 1990). Especially in mammals, IL-6 induces the expression of acute phase proteins (APPs) in the liver during injury, infection, and tissue trauma (Moshage, 1997). Some blood coagulation factors/regulators and complement components are APPs in other animals. Because the mRNA levels of many putative APP genes seemed to be high in the toxic liver, circulating IL-6 levels might be high in pufferfish. In mammal, IL-6 has a pleiotropic function, including the induction of keratinocyte proliferation (Grossman et al., 1989). In this study, 2 genes encoding keratin, which is abundant in keratinocytes, were up-regulated in the toxic liver. Therefore, these results possibly reflect high circulating IL-6 levels in the toxic liver. In torafugu, IL-6 transcripts have been detected in various tissues and increased by certain immune stimulants such as phytohemagglutinin, lipopolysaccharide, and polyinosinic–polycytidylic acid (Bird et al., 2005). Although there are no reports available whether or not IL-6 gene expression and/or IL-6 level in the blood is increased by TTX, Honda et al. (2005) showed that TTX acts as an immune stimulant in torafugu. Unfortunately, however, these markedly up-regulated genes are not likely candidate members that encode those related to biosynthesis or accumulation of TTX.

In this study, 3 wild specimens and 2 cultured specimens were used for the DNA microarray analysis, where two wild pufferfish were toxic and one non-toxic. The normalized relative mRNA levels of each transcript listed in Table 2 for different specimens are shown in Fig. 1. All transcripts showed higher levels in the toxic samples (fish 1 and 2) than in the non-toxic samples (fish 3–5). However, some of them showed lower levels in the liver of cultured pufferfish (fish 4 and 5) than in the wild non-toxic liver (fish 3). Rearing conditions may strongly affect the physiological condition of pufferfish, resulting in the mRNA levels of certain genes being different between the wild and cultured pufferfish. We are now performing DNA microarray analyses on the cultured pufferfish with or without

Table 2
Genes up-regulated more than 10 folds in the toxic liver.

No.	Group	Product name	Gene ID	Transcript ID	FC[a]
1	Structural protein	Type I cytokeratin, enveloping layer	ENSTRUG00000000933	ENSTRUT00000002225	39.2
2				ENSTRUT00000002226	28.8
3				ENSTRUT00000002227	22.6
4				ENSTRUT00000002229	48.8
5		Keratin, type I cytoskeletal 13	ENSTRUG00000002582	ENSTRUT00000006040	32.3
6				ENSTRUT00000006043	18.3
7	Vitamin D metabolism	Vitamin D 25-hydroxylase	ENSTRUG00000017301	ENSTRUT00000044482	39.3
8		25-hydroxyvitamin D-1 alpha hydroxylase, mitochondrial Precursor	ENSTRUG00000018507	ENSTRUT00000044483	33.9
9				ENSTRUT00000047536	44.7
10				ENSTRUT00000047537	63.0
11				ENSTRUT00000047538	52.9
12	Immunity	Major histocompatibility complex class I protein[b]	ENSTRUG00000000486	ENSTRUT00000001153	193.0
13			ENSTRUG00000002296	ENSTRUT00000005314	61.5
14	Other	Synaptosomal-associated protein, 91kDa homolog	ENSTRUG00000000481	ENSTRUT00000001139	11.3
15		Agmatinase, mitochondrial Precursor	ENSTRUG00000008312	ENSTRUT00000020887	76.9
16		Transmembrane protease, serine 13	ENSTRUG00000013292	ENSTRUT00000033970	11.7

[a] FC (fold change) of the gene with plural transcripts is represented by the mean value.
[b] The predicted product was localized in 2 genes in different scaffolds.

Table 3
Immune-related genes up-regulated in the toxic liver.

Group	Product name	Gene ID	Transcript ID	FC[a]
Coagulation factor/regulator	Serpin peptidase inhibitor, clade C (antithrombin), member 1	ENSTRUG00000010570	ENSTRUT00000026784	3.5
	Coagulation factor × precursor	ENSTRUG00000004411	ENSTRUT00000010534	4.5
			ENSTRUT00000010535	5.3
			ENSTRUT00000010536	4.4
			ENSTRUT00000010538	4.6
			ENSTRUT00000010539	4.4
			ENSTRUT00000010540	4.5
			ENSTRUT00000010541	4.6
			ENSTRUT00000010542	4.6
			ENSTRUT00000010543	4.5
Complement component	Complement C2 precursor	ENSTRUG00000008352	ENSTRUT00000020998	4.5
			ENSTRUT00000020999	4.8
			ENSTRUT00000021000	5.1
			ENSTRUT00000021001	5.3
			ENSTRUT00000021002	5.0
			ENSTRUT00000021003	4.6
	Complement C3 precursor	ENSTRUG00000002842	ENSTRUT00000006677	4.2
	Complement C5 precursor	ENSTRUG00000012757	ENSTRUT00000032416	4.3
			ENSTRUT00000032417	5.1
	Complement component C8 beta chain precursor	ENSTRUG00000006173	ENSTRUT00000015084	4.2
			ENSTRUT00000015085	3.6
			ENSTRUT00000015086	3.6
			ENSTRUT00000015087	3.6
Cytokine receptor	Interleukin-6 receptor subunit beta precursor	ENSTRUG00000011889	ENSTRUT00000030166	3.1
			ENSTRUT00000030168	3.1
			ENSTRUT00000030169	3.2
			ENSTRUT00000030171	3.4
			ENSTRUT00000030172	3.2
			ENSTRUT00000030173	3.2

[a] FC (fold change) of the gene with plural transcripts is represented by the mean value.

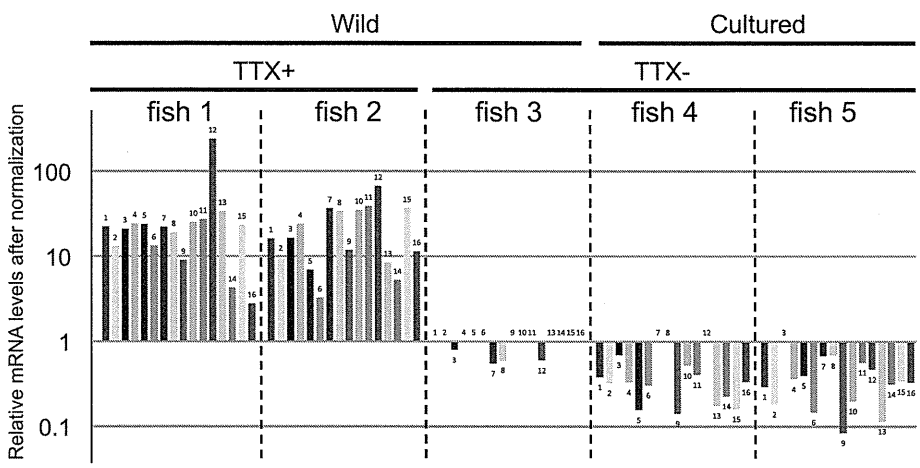

Fig. 1. The relative mRNA levels of the transcripts with more than 10-fold higher in the toxic than in non-toxic liver. The relative mRNA levels of the transcripts listed in Table 2 are shown for each fish (fish 1–5). The numbers on the bars correspond to those of the transcripts listed in Table 2. Fish 1–3 correspond to those listed in Table 1, whereas fish 4 and 5 do to cultured ones (782 and 815 g body weight, respectively) used as non-toxic control samples. Wild and cultured indicate the wild and the cultured pufferfish specimens, respectively. TTX+ and TTX− indicate the toxic and non-toxic specimens, respectively.

TTX, using more samples for statistical analyses to confirm the present results.

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Conflict of interest

None.

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報 文

腐肉食性小型巻貝2種に対するフグ毒給餌実験

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Accumulation of Tetrodotoxin from Diet in Two Species of Scavenging Marine Snails

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A feeding experiment of TTX-containing diet was conducted using the small scavenging marine snails *Pliarcularia globosa* and *Reticunassa festiva*. Seventy-five specimens of each species were divided into 15 groups of 5 individuals, of which 3 groups were directly submitted, without feeding, to toxin quantification as described below. TTX was not detected. Each of the remaining 12 groups was accommodated in a plastic case (80×70×40 mm) filled with seawater, and fed for 24 hours with ovary tissue (0.1 g) of the pufferfish *Takifugu vermicularis*, whose TTX content had previously been determined. Then the seawater was exchanged for fresh seawater, the snails were reared for 4 days without feeding, and then the seawater was changed again. This feeding/rearing cycle (5 days) was repeated 8 times, and 3 groups were sampled every 2 cycles. The combined viscera and combined muscle of each group were each extracted with 0.1% aqueous acetic acid, and then TTX was quantified by liquid chromatography-mass spectrometry. The estimated amount of ingested TTX was calculated by multiplying the difference between the amounts of ovary tissue supplied and remaining by the toxin content (122–126 MU/g). Similar mean values of 5.1 MU/group/cycle in *P. globosa* and 5.3 MU/group/cycle in *R. festiva* were obtained. Toxin content (TTX amount per gram of tissue) and toxin amount (TTX amount per group) during the experimental period were 0.23–2.85 MU/g and 0.05–0.96 MU/group, respectively, in *P. globosa* viscera. Both values increased markedly from the 2nd cycle to the 6th cycle. In contrast, no such increase in toxin content/amount was observed throughout the experimental period in *P. globosa* muscle (<0.05–0.86 MU/g, <0.02–0.27 MU/group), *R. festiva* viscera (<0.05–0.8 MU/g, <0.02–0.33 MU/group), and *R. festiva* muscle (<0.05–0.81 MU/g, <0.02–0.23 MU/group). The remaining ratio of TTX (percentage of total toxin amount [sum of the toxin amount of viscera and muscle] to estimated TTX ingestion amount) was less than 4% in *P. globosa*, and less than 2% in *R. festiva* after the 4th cycle, suggesting that the possibility that these two species would accumulate TTX at levels high enough to raise food hygiene issues is low.

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緒　　言

ムシロガイ科やマクラガイ科に属する腐肉食性小型巻貝のうち数種は，フグ毒テトロドトキシン（TTX）を保有するが，なかには高毒化するものがあり，中国大陸や台湾で食中毒を招来してきた．すなわち，中国福建省や浙江省ではムシロガイ科オオハナムシロ *Zeuxis siquijorensis* の近縁種 *Zeuxis semiplicatus*[1)~4)]，台湾ではムシロガイ科キンシバイ *Nassarius glans*，アラレガイ *Niotha clathrata*，オキナワハナムシロ *Zeuxis scalaris*，サメムシロ *Nassarius papillosus*，およびマクラガイ科ジュドウマクラ *Oliva miniacea* の喫食により多数の食中毒事例が発生している[5)~13)]．日本でも，2007および2008年に，それぞれ長崎県と熊本県でキンシバイによる重篤なTTX中毒が発生しており，その後の調査では，中毒検体と同じ海域で採取された同種個体の半数以上で，筋肉から食品衛生上“強毒”もしくは“猛毒”に相当する毒性が検出されている[14), 15)]．また，沖縄県沿岸産巻貝の毒性調査では，ムシロガイ科イボヨフバイ *Nassarius coronatus*，カゲロウヨフバイ *Zeuxis* sp.，アワムシロ *Niotha albescens*，マクラガイ科サツマビナ *Oliva annulata* およびヘコミマクラ *Oliva concavospira*[16)] からTTXが検出されており，これら腐肉食性小型巻貝の潜在的な高毒化リスクが危惧される．本研究では，当該リスク評価の前提となる腐肉食性巻貝のTTX蓄積能を評価するため，無毒のムシロガイ科巻貝2種を用いてフグ毒の給餌による毒化モデル実験を行った．

実験方法

巻貝試料

2011年6月に沖縄県西表島で採取したコブムシロ *Pliarcularia globosa* 75個体（殻高11.2±1.10 mm，殻径8.93±0.72 mm，重量0.83±0.16 g）および2011年8月に広島県尾道市で採取したアラムシロ *Reticunassa festiva* 75個体（殻高14.4±1.86 mm，殻径7.22±0.64 mm，重量0.58±0.12 g）を試料とした．いずれも採集後，生体の状態で長崎大学水産学部水産食品衛生学研究室に持ち帰り，給餌実験に用いた．

餌料用ナシフグ卵巣

TTX含有餌料には，凍結保存しておいた有明海産ナシフグ *Takifugu vermicularis* 1個体分の卵巣を用いた．左葉と右葉からそれぞれ一部分を採り，あらかじめ後述のLC-MS法でTTX含量を測定のうえ，残りを給餌実験に使用した．

本研究では，便宜上，LC-MS法で測定したTTX量をマウス単位（MU；1 MUは体重20 gのddY系雄マウスを30分間で死亡させる毒力と定義され，TTX 220 ngに相当する[17)]）に換算して表示する．給餌実験に用いた卵巣左右葉のTTX含量（一部分を用いて測定した値）は，それぞれ122および126 MU/gで，11-*oxo*TTXは検出されなかった．卵巣内のTTXの分布は明らかではないが，左右葉ごとにほぼ均一と仮定し，以下，当該TTX含量をもって，それぞれ左右葉全体のTTX含量とみなした．

給餌実験

コブムシロおよびアラムシロをそれぞれ5個体ずつ15群に分け，うち3群は毒を投与せずに直接後述のLC-MS法でTTX量を測定し，残りの12群を以下の毒化モデル実験に用いた．すなわち，自然海水を入れたプラスチックケース（80×70×40 mm）に各群を収容し，通気した状態で，有毒ナシフグ卵巣からかき取った断片0.1 g（12.2または12.6 MU）を1日間給餌した．給餌期間終了時に飼育海水（約100 mL）を回収し，メンブレンフィルター（C020A047A, ADVANTEC）でろ過後，フィルター上の残餌をスパーテルで注意深く採取し，重量を測定した．試料巻貝については，新たな自然海水にて4日間通気しながら無給餌で飼育した．給餌開始から，無給餌期間終了時に再度飼育海水を新鮮海水と交換するまでを1サイクルとし，これを計8サイクル（40日間）繰り返した．この間，2サイクル（10日間）ごとに3群ずつ取り上げ，内臓と筋肉に含まれるTTX量を測定した．また，コブムシロについては，無給餌期間の飼育海水（約100 mL）を回収し，活性炭（10 g）に添加してTTXをいったん吸着させ，水洗後，吸着したTTXを1%酢酸含有20%エタノール（100 mL）で溶出して，LC-MS法で定量した．

TTXの定量

各群の内臓と筋肉をそれぞれ合一し，食品衛生検査指針理化学編のフグ毒検査法[17)] に準じて毒の抽出を行った．抽出液は関東化学(株)製のHLC-DISK 13水系メンブランフィルター（0.45 μm）でろ過後，ZMD2000質量分析計を搭載したAlliance 2690 HPLCシステム（Waters社製）を用い，既報の方法[18)] に準拠してLC-MS分析を行った．カラムに関東化学社製のMightysil RP-18 GP (2.0×250 mm, 5 μm)，移動相には30 mmol/Lヘプタフルオロ酪酸を含む1 mmol/L酢酸アンモニウム緩衝液（pH 5.0）を使用し，流速を0.2 mL/minとした（移動相に用いた試薬はいずれも和光純薬工業(株)製）．デソルベーション温度350℃，ソースブロック温度120℃，コーン電圧50 Vに設定し，イオン化法はESIポジティブモードで，プロトン付加分子$[M+H]^+$のm/z（TTX: 320，11-*oxo*TTX: 336）をモニタリングし，MassLynx™オペレーションシステムにて解析した．標準物質として，和光純薬工業(株)製のTTX（生化学用）を用いた．

結果および考察

推定TTX摂取量

コブムシロとアラムシロの摂餌量を直接測定することは困難であったため，給餌量（0.1 g）と給餌期間終了時の残餌量の差をもって推定摂餌量とし，これに毒量（122または126 MU/g）を乗じた値を推定TTX摂取量とした．コブムシロの場合，推定TTX摂取量（積算値）の平均値は，2サイクル目では1.2 MU/群と低かったが，4サイクル目では29.6 MU/群と大きく増加し，8サイクル目では49.6 MU/群に達した（Fig. 1）．一方，アラムシロでは2～8サイクル目まで漸増し，8サイクル目では43.0 MU/群に達した．1サイクルあたりの平均値を求めると，コブムシロで5.1 MU/群/サイクル，アラムシロで5.3 MU/群/サイクルとなり，2種の巻貝は同程度のTTXを摂取したものと推定された．

組織別TTX量の推移

コブムシロ，アラムシロ共に毒を投与せず，給餌前に直接LC-MS分析に付した群からは，TTXは全く検出されなかった（検出限界0.05 MU/g）．

一方，TTX含有餌料を投与した群については，その多くで内臓と筋肉の両者からTTXが検出された．両種の巻貝における毒含量（組織1 gあたりのTTX量）の推移をFig. 2に示す．コブムシロの場合，内臓の毒含量は，2サイクル目から6サイクル目にかけて顕著に増加し，6～8サイクル目では，6群中5群で2 MU/gを超えたのに対し，筋肉の毒含量は，4サイクル目から6サイクル目にかけて若干上昇したものの，いずれの群も1 MU/gを超えることはなかった．他方，アラムシロの場合，筋肉のみならず内臓でも，コブムシロの筋肉同様，毒含量の顕著な増加は見られなかった．実験期間中の最高毒含量は，コブムシロで2.85 MU/g（内臓），アラムシロで0.81 MU/g（筋肉）で，小型巻貝バイ *Babylonia japonica*（15～53 MU/g）[19]，アラレガイ *Niotha clathrata*（4～35 MU/g）[20]，イボヨフバイ（5.64～11.1 MU/g），カゲロウヨフバイ（12.7 MU/g），アワムシロ（5.08 MU/g），サツマビナ（10.8 MU/g）およびヘコミマクラ（6.65 MU/g）[16] 天然個体の毒力のいずれも下回った．

次に，両種における毒量（1群あたりのTTX量）の推移をFig. 3に示す．コブムシロの場合，内臓の毒量は2サイクル目から6サイクル目にかけて顕著に上昇し，6サイクル目に0.9 MU/群前後に達した後，8サイクル目に若干減少した．一方，筋肉では，4サイクル目になって初めて毒の移行が見られ，以後その量は若干増加したが，総毒量（内臓と筋肉の毒量の和）に占める割合はおおむね2割程度にとどまった．他方，アラムシロの場合，内臓の毒量に顕著な増加は見られず，実験期間を通して0.2～0.3 MU/群程度であった．筋肉では，2サイクル目に毒の移行が見

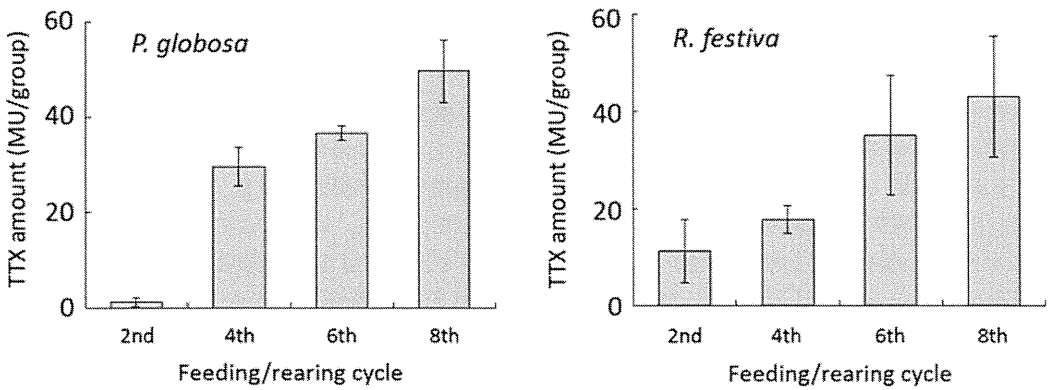

Fig. 1. Change in the estimated amount of TTX ingested during the experimental period
Data are shown as the mean (column) and standard deviation (error bar) of every 2 cycles.

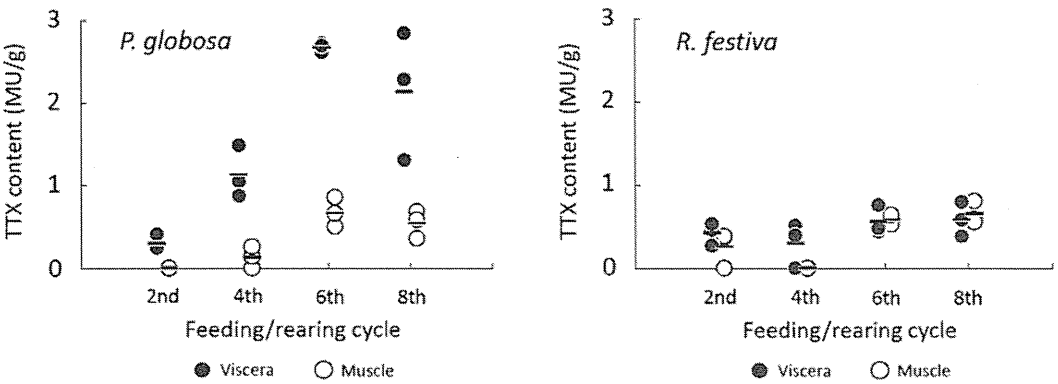

Fig. 2. Change in the toxin content (TTX amount per gram tissue) of viscera (closed circle) and muscle (open circle) during the experimental period.
Data are shown as individual values (closed/open circles) and the mean of every 2 cycles (horizontal bars).

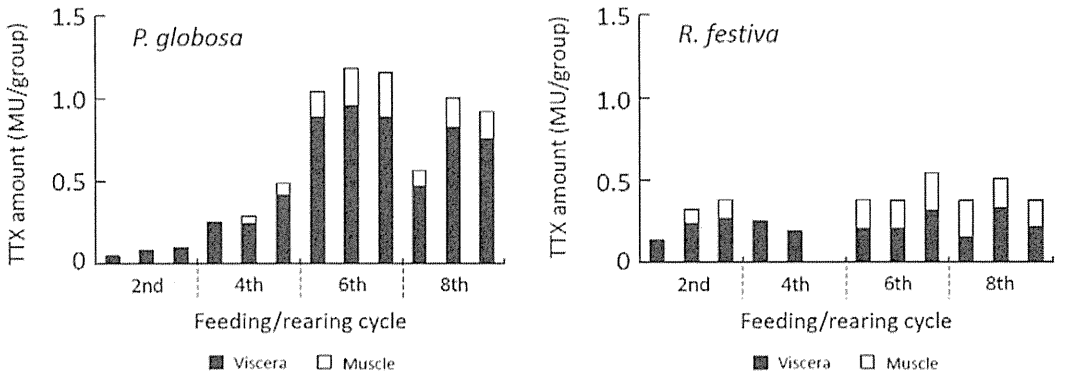

Fig. 3. Change in the toxin amount (TTX amount per group) during the experimental period.
Bars indicate values in viscera (closed column) and muscle (open column).

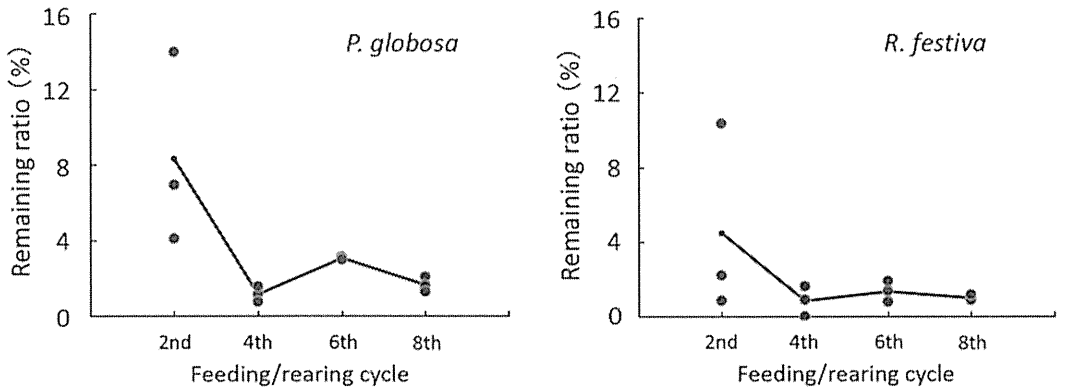

Fig. 4. Change in the remaining ratio of TTX during the experimental period.
Data points are shown as circles and the mean (dots joined by the solid line) of every 2 cycles.

られ，その量は6～8サイクル目に若干増加して内臓の毒量と同程度（総毒量に占める割合が5割前後）となった．コブムシロとアラムシロは，筋肉に少量の毒が移行する点では類似するが，内臓の毒の保持能力には若干差があり，コブムシロの方が内臓に毒が残存しやすいものと推察された．小型巻貝のうち，キンシバイ[10),14),16)]，マサメダマ *Natica lineata*[21)]，およびヘコミマクラ[16)]では，筋肉に毒が偏在する個体も見られるが，少なくともコブムシロでは，そのような個体が出現する可能性は低いものと推察される．

TTX残存率

TTX残存率（推定TTX摂取量（積算値）に対する総毒量の割合）の推移をFig. 4に示す．コブムシロ，アラムシロともに2サイクル目では10%を超える群も見られたが，それ以降はコブムシロで4%未満，アラムシロで2%未満と低い値を示した．時に高毒化するボウシュウボラ *Charonia sauliae*[22)]の無毒個体に対し，有毒トゲモミジガイ *Astropecten polyacanthus* を給餌した毒化モデル実験[23)]では，中腸腺の毒蓄積率（ここでのTTX残存率と同意）は18～91%であったと報告されている．これらの値は，コブムシロ，アラムシロのTTX残存率よりもはるかに高い．一方，前述のとおり，コブムシロ，アラムシロともに毒を投与しなかった群からTTXは全く検出されなかった．また，コブムシロについては既報[16)]でも無毒（10 MU/g未満）とされていることから，これら2種の巻貝に，ボウシュウボラのような高濃度のTTXを蓄積する能力はないものと推察される．

コブムシロのTTX排出について検討するため，無給餌期間中の飼育海水につき，活性炭処理後にLC-MS分析を行ったところ，ほとんどの群でTTXが検出されたものの，いずれも2 MU未満と微量であった．今回，給餌から毒の排出まで24時間以上かかることを想定していたため，給餌期間中の飼育海水については分析を行わなかったが，今後，同様の給餌実験を行う場合は，餌料からの漏出や海水中での分解を含め，この期間のTTXの動態について十分に検討する必要があろう．一方，これまでに数種の巻貝からTTX誘導体が検出されており[14),24)]，貝体内における毒成分の変換についても考慮しなければならない．なかでも，キンシバイで検出例のある11-*oxo*TTXは，TTXより毒性が強いため[14)]，TTXもしくは無毒／弱毒誘導体からの変換について特段の注意を払う必要がある．本研究では，LC-MSで少なくとも11-*oxo*TTX（*m/z* 336（$[M+H]^+$））の保持時間[14)]付近にピークは認められず，当該変換はなかったものと推察される．しかしながら，ほかの誘導体の分析は行っておらず，貝体内においてTTXが別の誘導体に変換，もしくは分解された可能性は否定できない．巻貝のTTX代謝機構については今後の検討課題である．

ま と め

腐肉食性小型巻貝であるコブムシロとアラムシロに

TTX含有餌料を投与すると，ともに内臓と筋肉が僅かに毒化するが，内臓のTTX保持能力には種間で若干差があり，コブムシロのほうが，毒が残存しやすいことが示された．しかしながら，コブムシロでも最高毒含量は3 MU/g程度で，かつTTX残存率は4%未満と非常に低く，自然界においてこれら2種が食品衛生上問題となるほど高毒化する可能性は低いものと推察された．

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