In contrast, a total of 427 genes were found to be downregulated by TTX administration, as shown in Figure 1. The genes downregulated three-fold and more were extracted and are listed in Table 3. The highest downregulated gene in the TTX-administered group was elongation factor G2, and its FC value was −13.0. As shown in Figure 3, gene ontology classification of the downregulated genes showed that those involved in protein binding and enzyme and cofactors (metabolism) accounted for 20.8% and 13.1%, respectively, whereas those involved in the transcription factor, receptor activity and transporter activity accounted for 15.9, 10.1 and 6.6%, respectively.

To confirm the validity of the microarray data, real-time PCR was performed for the highest upregulated gene, chymotrypsin-like elastase family member 2A (Figure 4). There was a significant difference in the mRNA expression level between the TTX-administered (27.22 ± 4.18) and control group (1.00 ± 0.77) (p = 0.0019). This result is well correlated with the data of microarray analysis, FC 37.6 (Table 2).

Figure 3. Functional classification of 427 genes significantly downregulated in the liver of *Takifugu rubripes* in the TTX-administered group.

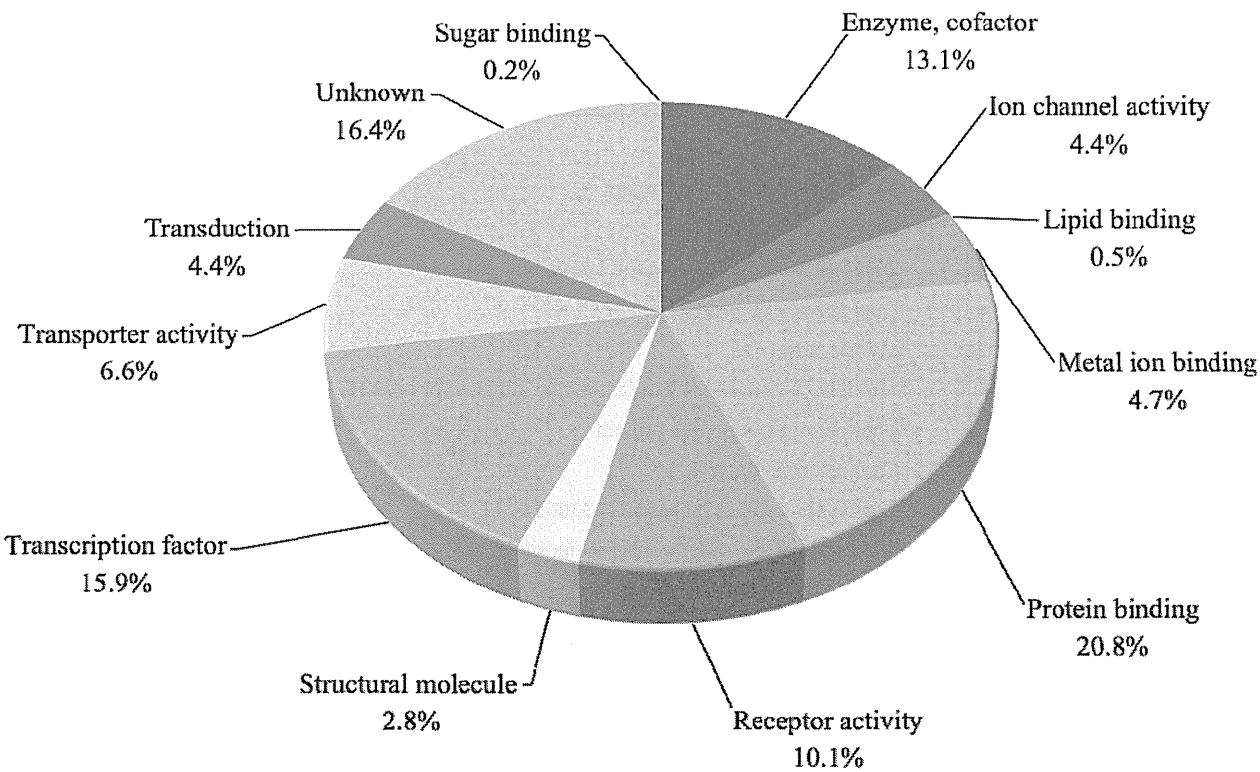


Table 3. List of genes downregulated in the liver of the TTX-administered pufferfish *Takifugu rubripes* group compared to those in the buffer-administered control group (FC [1] > 3.0).

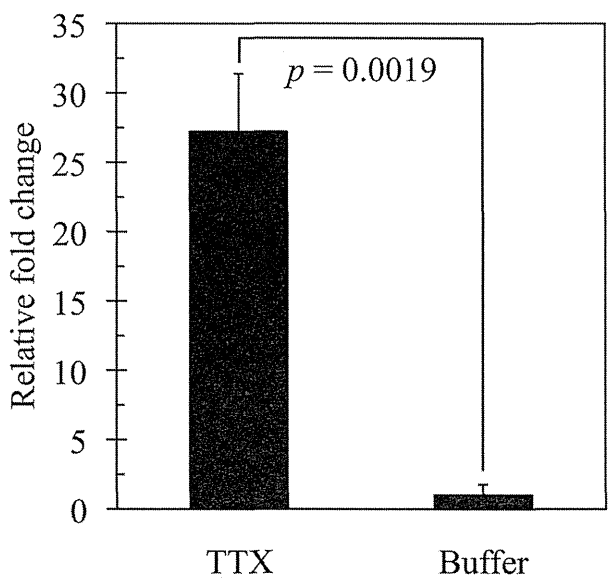
Ensemble ID	Gene name	Predicted description	Functional classification	FC
ENSTRUT00000047556	Fusa2	Elongation factor G 2	Transcription factor	−3.0
ENSTRUT00000009260	Rspo3	R-spondin-3	Transduction	−7.8
ENSTRUT00000029878	Ncoa2	Nuclear receptor coactivator 2	Transcription factor	−7.3
ENSTRUT00000005082	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.

References

1. Halstead, B.W. *Poisonous and Venomous Marine Animals of the World*, 2nd ed.; The Darwin Press Inc.: Princeton, NJ, USA, 1988; pp. 525–644.
2. Lee, C.H.; Ruben, P.C. Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin. *Channels* **2008**, *2*, 407–412.
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 Gakk.* **1981**, *47*, 535–537.
4. Saito, T.; Maruyama, J.; Kanoh, S.; Jeon, J.K.; Noguchi, T.; Harada, T.; Murata, O.; Hashimoto, K. Toxicity of the cultured pufferfish *Fugu rubripes rubripes* along with their resistibility against tetrodotoxin. *Nippon Suisan Gakk.* **1984**, *50*, 1573–1575.
5. Honda, S.; Arakawa, O.; Takatani, T.; Tachibana, K.; Yagi, M.; Tanigawa, A.; Noguchi, T. Toxification of cultured puffer fish *Takifugu rubripes* by feeding on tetrodotoxin-containing diet. *Nippon Suisan Gakk.* **2005**, *71*, 815–820.
6. Noguchi, T.; Arakawa, O.; Takatani, T. Toxicity of pufferfish *Takifugu rubripes* cultured in netcages at sea or aquaria on land. *Comp. Biochem. Physiol. Part D: Genom. Proteom.* **2006**, *1*, 153–157.
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* **2008**, *51*, 1269–1273.
8. Noguchi, T.; Arakawa, O. Tetrodotoxin-distribution and accumulation in aquatic organisms, and cases of human intoxication. *Mar. Drugs* **2008**, *6*, 220–242.
9. Noguchi, T.; Arakawa, O.; Daigo, K.; Hashimoto, K. Local differences in toxin composition of a xanthid crab *Atergatis floridus* inhabiting Ishigaki Island, Okinawa. *Toxicon* **1986**, *24*, 705–711.
10. Yasumoto, T.; Yasumura, D.; Yotsu, M.; Michishita, T.; Endo, A.; Kotaki, Y. Bacterial production of tetrodotoxin and anhydrotetrodotoxin. *Agric. Biol. Chem.* **1986**, *50*, 793–795.
11. Simidu, U.; Noguchi, T.; Hwang, D.F.; Shida, Y.; Hashimoto, K. Marine bacteria which produce tetrodotoxin. *Appl. Environ. Microbiol.* **1987**, *53*, 1714–1715.
12. Tamplin, M.L. A bacterial source of tetrodotoxins and saxitoxins. *ACS Symp. Ser.* **1990**, *418*, 78–106.

13. Wu, Z.; Xie, L.; Xia, G.; Zhang, J.; Nie, Y.; Hu, J.; Wang, S.; Zhang, R. A new tetrodotoxin-producing actinomycete, *Nocardiopsis dassonvillei*, isolated from the ovaries of puffer fish *Fugu rubripes*. *Toxicon* **2005**, *45*, 851–859.
14. Yu, V.C.; Yu, P.H.; Ho, K.C.; Lee, F.W. Isolation and identification of a new tetrodotoxin-producing bacterial species, *Raoultella terrigena*, from Hong Kong marine puffer fish *Takifugu niphobles*. *Mar. Drugs* **2011**, *9*, 2384–2396.
15. Yasumoto, T.; Yotsu-Yamashita, M. Chemical and etiological studies on tetrodotoxin and its analogs. *J. Toxicol. Toxin Rev.* **1996**, *15*, 81–90.
16. Miyazawa, K.; Noguchi, T. Distribution and origin of tetrodotoxin. *Toxin Rev.* **2001**, *20*, 11–33.
17. Matsumoto, T.; Nagashima, Y.; Kusuhara, H.; Ishizaki, S.; Shimakura, K.; Shiomi, K. Evaluation of hepatic uptake clearance of tetrodotoxin in the puffer fish *Takifugu rubripes*. *Toxicon* **2008**, *52*, 369–374.
18. Matsumoto, T.; Nagashima, Y.; Kusuhara, H.; Ishizaki, S.; Shimakura, K.; Shiomi, K. Pharmacokinetics of tetrodotoxin in puffer fish *Takifugu rubripes* by a single administration technique. *Toxicon* **2008**, *51*, 1051–1059.
19. Nagashima, Y.; Toyoda, M.; Hasobe, M.; Shimakura, K.; Shiomi, K. *In vitro* accumulation of tetrodotoxin in pufferfish liver tissue slices. *Toxicon* **2003**, *41*, 569–574.
20. Matsumoto, T.; Nagashima, Y.; Takayama, K.; Shimakura, K.; Shiomi, K. Difference between tetrodotoxin and saxitoxins in accumulation in puffer fish *Takifugu rubripes* liver tissue slices. *Fish Physiol. Biochem.* **2005**, *31*, 95–100.
21. Matsumoto, T.; Nagashima, 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* **2007**, *50*, 173–179.
22. Lee, J.H.; Kondo, H.; Sato, S.; Akimoto, S.; Saito, T.; Kodama, M.; Watabe, S. Identification of novel genes related to tetrodotoxin intoxication in pufferfish. *Toxicon* **2007**, *49*, 939–953.
23. Matsumoto, T.; Ishizaki, S.; Nagashima, Y. Differential gene expression profile in the liver of the marine puffer fish *Takifugu rubripes* induced by intramuscular administration of tetrodotoxin. *Toxicon* **2011**, *57*, 304–310.
24. Feroudj, H.; Matsumoto, T.; Kurosu, Y.; Kaneko, G.; Ushio, H.; Suzuki, K.; Kondo, H.; Hirono, I.; Nagashima, Y.; Akimoto, S.; *et al.* DNA microarray analysis on gene candidates possibly related to tetrodotoxin accumulation in pufferfish. *Toxicon* **2014**, *77*, 68–72.
25. Kodama, K.; Sato, S. Puffer fish toxin. In *Standard Methods of Analysis in Food Safety Regulation, Chemistry*, 3rd ed.; Ministry of Health Labour and Welfare, Ed.; Japan Food Hygiene Association: Tokyo, Japan, 2005; Volume 1, pp. 661–666.
26. Nagashima, Y.; Maruyama, J.; Noguchi, T.; Hashimoto, K. Analysis of paralytic shellfish poison and tetrodotoxin by ion-pairing high-performance liquid-chromatography. *Nippon Suisan Gakk.* **1987**, *53*, 819–823.
27. Shoji, Y.; Yotsu-Yamashita, M.; Miyazawa, T.; Yasumoto, T. Electrospray ionization mass spectrometry of tetrodotoxin and its analogs: Liquid chromatography/mass spectrometry, tandem mass spectrometry, and liquid chromatography/tandem mass spectrometry. *Anal. Biochem.* **2001**, *290*, 10–17.

28. Agilent eArray Application—eArray User Login Site in Agilent Technologies Homepage. Available online: https://earray.chem.agilent.com/earray/ (accessed on 22 October 2014).
29. Fugu Genome Project. Available online: http://www.fugu-sg.org/ (accessed on 22 October 2014).
30. Feature Extraction ver. 10.7.3—download software site in Agilent Technologies Homepage. Available online: http://www.genomics.agilent.com/article.jsp?pageId=2059&_requestid=928092 (accessed on 22 October 2014).
31. GeneSpring Support—user guides site in Agilent Technologies Homepage. Available online: http://genespring-support.com/support/documentation (accessed on 22 October 2014).
32. Primer Express Version 3.0—Getting Started Guide in Applied Biosystems Homepage. Available online: https://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041902.pdf (accessed on 22 October 2014).
33. Gene Ontology Consortium. Available online: http://geneontology.org/ (accessed on 22 October 2014).
34. Watabe, S.; Sato, Y.; Nakaya, M.; Nogawa, N.; Oohashi, K.; Noguchi, T.; Morikawa, N.; Hashimoto, K. Distribution of tritiated tetrodotoxin administered intraperitoneally to pufferfish. *Toxicon* **1987**, *25*, 1283–1289.
35. Ikeda, K.; Murakami, Y.; Emoto, Y.; Ngy, L.; Taniyama, S.; Yagi, M.; Takatani, T.; Arakawa, O. Transfer profile of intramuscularly administered tetrodotoxin to non-toxic cultured specimens of the pufferfish *Takifugu rubripes*. *Toxicon* **2009**, *53*, 99–103.
36. Kodama, M.; Ogata, T.; Sato, S. External secretion of tetrodotoxin from puffer fishes stimulated by electric shock. *Mar. Biol.* **1985**, *87*, 199–202.
37. Kodama, M.; Sato, S.; Ogata, T.; Suzuki, Y.; Kaneko, T.; Aida, K. Tetrodotoxin secreting glands in the skin of puffer fishes. *Toxicon* **1986**, *24*, 819–829.
38. Saito, T.; Noguchi, T.; Harada, T.; Murata, O.; Hashimoto, K. Tetrodotoxin as a biological defense agent for puffers. *Nippon Suisan Gakk.* **1985**, *51*, 1175–1180.
39. Yotsu-Yamashita, M.; Okoshi, N.; Watanabe, K.; Araki, N.; Yamaki, H.; Shoji, Y.; Terakawa, T. Localization of pufferfish saxitoxin and tetrodotoxin binding protein (PSTBP) in the tissues of the pufferfish, *Takifugu pardalis*, analyzed by immunohistochemical staining. *Toxicon* **2013**, *72*, 23–28.
40. Tatsuno, R.; Yamaguchi, K.; Takatani, T.; Arakawa, O. RT-PCR- and MALDI-TOF mass spectrometry-based identification and distribution of isoforms homologous to pufferfish saxitoxin- and tetrodotoxin-binding protein in the plasma of non-toxic cultured pufferfish (*Takifugu rubripes*). *Biosci. Biotechnol. Biochem.* **2013**, *77*, 208–212.
41. Kawashima, I.; Tani, T.; Shimoda, K.; Takiguchi, Y. Characterization of pancreatic elastase II cDNAs: Two elastase II mRNAs are expressed in human pancreas. *DNA* **1987**, *6*, 163–172.
42. Lausen, J.; Liu, S.; Fliegauf, M.; Lübbert, M.; Werner, M.H. ELA2 is regulated by hematopoietic transcription factors, but not repressed by AML1-ETO. *Oncogene* **2006**, *25*, 1349–1357.
43. Friedman, A.D. Transcriptional regulation of granulocyte and monocyte development. *Oncogene* **2002**, *21*, 3377–3390.
44. Whitcomb, D.C.; Lowe, M.E. Human pancreatic digestive enzymes. *Dig. Dis. Sci.* **2007**, *52*, 1–17.
45. Isom, L.L.; Ragsdale, D.S.; De-Jongh, K.S.; Westenbroek, R.E.; Reber, B.F.; Scheuer, T.; Catterall, W.A. Structure and function of the beta 2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. *Cell* **1995**, *83*, 433–442.

46. Srinivasan, J.; Schachner, M.; Catterall, W.A. Interaction of voltage-gated sodium channels with the extracellular matrix molecules tenascin-C and tenascin-R. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 15753–15757.
47. Malhotra, J.D.; Kazen-Gillespie, K.; Hortsch, M.; Isom, L.L. Sodium channel beta subunits mediate homophilic cell adhesion and recruit ankyrin to points of cell-cell contact. *J. Biol. Chem.* **2000**, *275*, 11383–11388.
48. Pertin, M.; Ji, R.R.; Berta, T.; Powell, A.J.; Karchewski, L.; Tate, S.N.; Isom, L.L.; Woolf, C.J.; Gilliard, N.; Spahn, D.R.; *et al.* Upregulation of the voltage-gated sodium channel beta2 subunit in neuropathic pain models: Characterization of expression in injured and non-injured primary sensory neurons. *J. Neurosci.* **2005**, *25*, 10970–10980.
49. Lopez Santiago, L.F.; Pertin, M.; Morisod, X.; Chen, C.; Hong, S.; Wiley, J.; Decosterd, I.; Isom, L.L. Sodium channel beta2 subunits regulate tetrodotoxin-sensitive sodium channels in small dorsal root ganglion neurons and modulate the response to pain. *J. Neurosci.* **2006**, *26*, 7984–7994.
50. Lek, A.; Evesson, F.J.; Sutton, R.B.; North, K.N.; Cooper, S.T. Ferlins: Regulators of vesicle fusion for auditory neurotransmission, receptor trafficking and membrane repair. *Traffic* **2012**, *13*, 185–194.
51. De Morrée, A.; Flix, B.; Bagaric, I.; Wang, J.; van den Boogaard, M.; Grand Moursel, L.; Frants, R.R.; Illa, I.; Gallardo, E.; Toes, R.; van der Maarel, S.M. Dysferlin regulates cell adhesion in human monocytes. *J. Biol. Chem.* **2013**, *288*, 14147–14157.
52. Kerr, J.P.; Ward, C.W.; Bloch, R.J. Dysferlin at transverse tubules regulates Ca^{2+} homeostasis in skeletal muscle. *Front. Physiol.* **2014**, doi:10.3389/fphys.2014.00089.
53. Oulhen, N.; Onorato, T.M.; Ramos, I.; Wessel, G.M. Dysferlin is essential for endocytosis in the sea star oocyte. *Dev. Biol.* **2014**, *388*, 94–102.
54. Kitamura, C.; Takahashi, M.; Kondoh, Y.; Tashiro, H.; Tashiro, T. Identification of synaptic activity-dependent genes by exposure of cultured cortical neurons to tetrodotoxin followed by its withdrawal. *J. Neurosci. Res.* **2007**, *85*, 2385–2399.
55. Saras, J.; Franzen, P.; Aspenstrom, P.; Hellman, U.; Gonez, L.J.; Heldin, C.H. A novel GTPase-activating protein for Rho interacts with a PDZ domain of the protein-tyrosine phosphatase PTPL1. *J. Biol. Chem.* **1997**, *272*, 24333–24338.
56. Hakoshima, T. Structural basis of the rho GTPase signaling. *J. Biochem.* **2003**, *134*, 327–331.
57. Myagmar, B.E.; Umikawa, M.; Asato, T.; Taira, K.; Oshiro, M.; Hino, A.; Takei, K.; Uezato, H.; Kariya, K. PARG1, a protein-tyrosine phosphatase-associated RhoGAP, as a putative Rap2 effector. *Biochem. Biophys. Res. Commun.* **2005**, *329*, 1046–1052.
58. Adams, J.W.; Wang, J.; Davis, J.R.; Liaw, C.; Gaidarov, I.; Gatlin, J.; Dalton, N.D.; Gu, Y.; Ross, J., Jr.; Behan, D.; Chien, K.; Connolly, D. Myocardial expression, signaling, and function of GPR22: A protective role for an orphan G protein-coupled receptor. *Am. J. Physiol. Heart Circ. Physiol.* **2008**, *295*, H509–H521.
59. Karassek, S.; Berghaus, C.; Schwarten, M.; Goemans, C.G.; Ohse, N.; Kock, G.; Jockers, K.; Neumann, S.; Gottfried, S.; Herrmann, C.; Heumann, R.; Stoll, R. Ras homolog enriched in brain (Rheb) enhances apoptotic signaling. *J. Biol. Chem.* **2010**, *285*, 33979–33991.

60. Sciarretta, S.; Zhai, P.; Shao, D.; Maejima, Y.; Robbins, J.; Volpe, M.; Condorelli, G.; Sadoshima, J. Rheb is critical regulator of autophagy during myocardial ischemia: pathophysiological implications in obesity and metabolic syndrome. *Circulation* **2013**, *125*, 1134–1146.

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

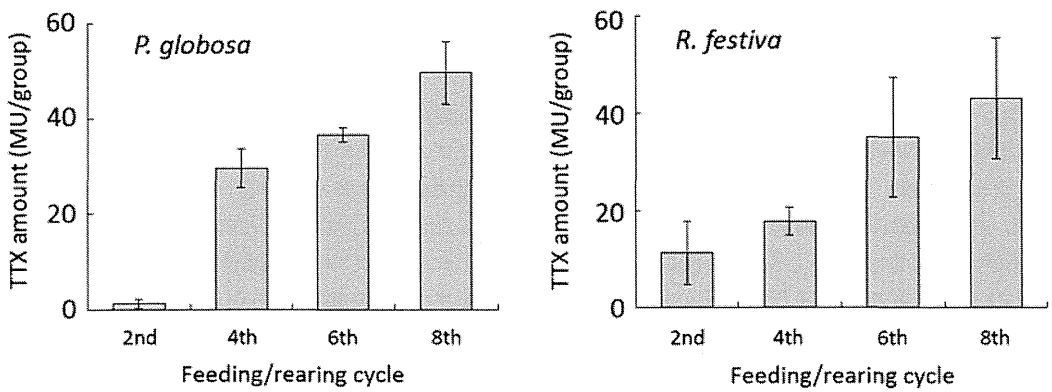

Fig. 1. Change in the estimated amount of TTX ingested during the experimental period
Data are shown as the mean (column) and standard deviation (error bar) of every 2 cycles.

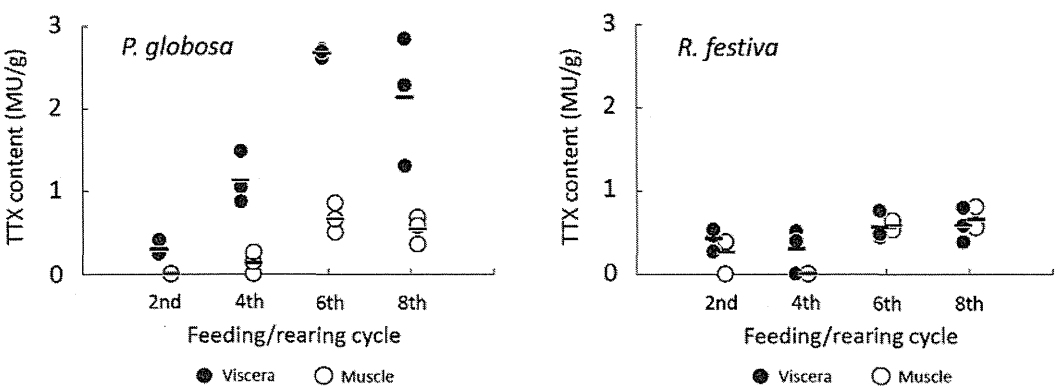

Fig. 2. Change in the toxin content (TTX amount per gram tissue) of viscera (closed circle) and muscle (open circle) during the experimental period.
Data are shown as individual values (closed/open circles) and the mean of every 2 cycles (horizontal bars).

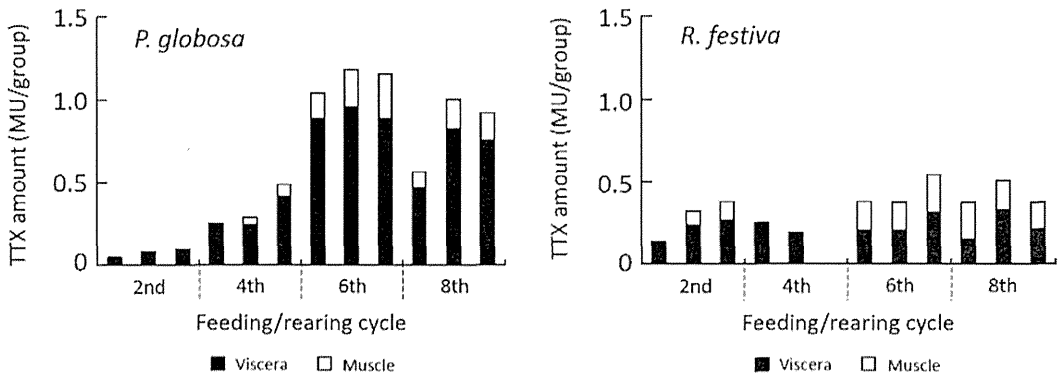

Fig. 3. Change in the toxin amount (TTX amount per group) during the experimental period. Bars indicate values in viscera (closed column) and muscle (open column).

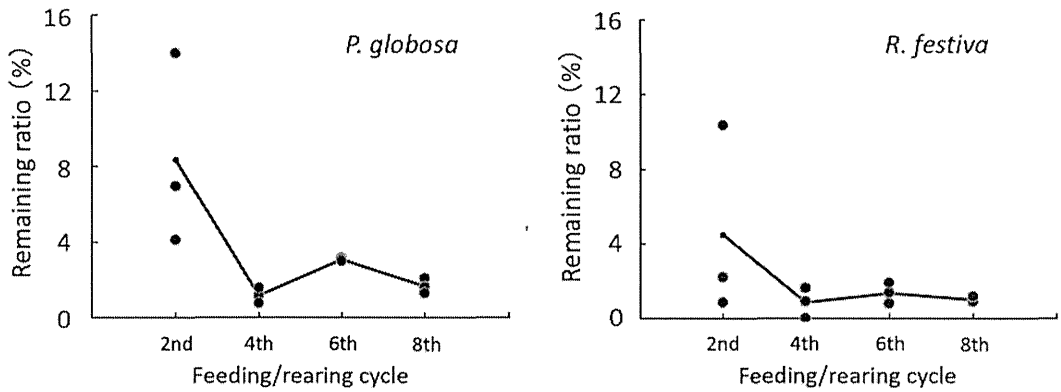

Fig. 4. Change in the remaining ratio of TTX during the experimental period. Data points are shown as circles and the mean (dots joined by the solid line) of every 2 cycles.

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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文　献

1) Sui, L. M., Chen, K., Hwang, P. A., Hwang, D. F. Identification of tetrodotoxin in marine gastropods implicated in food poisoning. J. Nat. Toxins, **11**, 213–220 (2002).
2) Shui, L. M., Chen, K., Wang, J. Y., Mei, H. Z., Wang, A. Z., Lu, Y. H., Hwang, D. F. Tetrodotoxin-associated snail poisoning in Zhoushan: A 25-year retrospective analysis. J. Food Prot., **66**, 110–114 (2003).
3) 高谷智裕，荒川　修，野口玉雄．中国で頻発している小型巻貝による食中毒について．食衛誌，**46**, J-208–J-209 (2005).
4) Noguchi, T., Onuki, K., Arakawa, O. Tetrodotoxin poisoning due to pufferfish and gastropods, and their intoxication mechanism. International Scholarly Reseach Network, **2011**, 1–10 (2011).
5) Hwang, D. F., Cheng, C. A., Tsai, H. T., Shih, D. Y. C., Ko, H. C., Yang, R. Z., Jeng, S. S. Identification of tetrodotoxin and paralytic shellfish toxins in marine gastropods implicated in food poisoning. Fish. Sci., **61**, 675–679 (1995).
6) Hwang, D. F., Shiu, Y. C., Hwang, P. A., Lu, Y. H. Tetrodotoxin in gastropods (snails) implicated in food poisoning in northern Taiwan. J. Food Prot., **65**, 1341–1344 (2002).
7) Yong, C. C., Han, K. C., Lin, T. J., Tsai, W. J., Deng, J. F. An outbreak of tetrodotoxin poisoning following gastropod mollusc consumption. Human and Experimental Toxicology, **14**, 446–450 (1995).
8) Shiu, Y. C., Lu, Y. H., Tsai, Y., Chen, S. K., Hwang, D. F. Occurrence of tetrodotoxin in the causative gastropod *Polinices didyma* and another gastropod *Natica lineate* collected from western Taiwan. J. Food Drug Analysis, **11**, 159–163 (2003).
9) Hwang, P. A., Tsai, Y. H., Lu, Y. H., Hwang, D. F. Paralytic toxins in three new gastropod (Olividae) species implicated in food poisoning in southern Taiwan. Toxicon, **41**, 529–533 (2003).
10) Hwang, P. A., Tsai, Y. H., Deng, J. F., Cheng, C. A., Ho, P. H., Hwang, D. F. Identification of tetrodotoxin in a marine gastropod (*Nassarius glans*) responsible for human morbidity and mortality in Taiwan. J. Food Prot., **68**, 1696–1701 (2005).
11) Hwang, P. A., Tsai, Y. H., Lin, S. J., Hwang, D. F. The gastropods possessing TTX and/or PSP. Food Reviews International, **23**, 321–340 (2007).
12) Liu, F. M., Fu, Y. M., Shih, D. Y. C. Occurrence of tetrodotoxin poisoning in *Nassarius papillosus* Alectrion and *Nassarius gruneri* Niotha. J. Food Drug Analysis, **12**, 189–192 (2004).
13) Jen, H. C., Lin, S. J., Lin, S. Y., Huang, Y. W., Liao, I. C., Arakawa, O., Hwang, D. F. Occurrence of tetrodotoxin and paralytic shellfish poisons in a gastropod implicated in food poisoning in southern Taiwan. Food Addit. Contam., **24**, 902–909 (2007).
14) Taniyama, S., Isami, Y., Matsumoto, T., Nagashima,Y., Takatani, T., Arakawa, O. Toxicity and toxin profile of tetrodotoxin detected in the scavenging gastropod *Nassarius (Alectrion) glans* "kinshibai". J. Food Hyg. Soc. Japan, **50**, 22–28 (2009).
15) Arakawa, O., Shiomi, K. Marine gastropod toxins: tetramine and tetrodotoxin. Food Sanitation Reserch, **60**, 15–25 (2010).
16) Taniyama, S., Takatani, T., Sorimachi, T., Sagara, T., Kubo, H., Oshiro, N., Ono, K., Xiao, N., Tachibana, K., Arakawa, O. Toxicity and toxin profile of scavenging and carnivorous gastropods from the coastal waters of Okinawa prefecture, Japan. Food Hyg. Saf. Sci., **54**, 49–55 (2013).
17) 児玉正昭，佐藤　繁．第7章自然毒・A動物毒・1．フグ毒．食品衛生検査指針理化学編（厚生労働省監修），日本食品衛生学会，2005, p.661–666.
18) Nakashima, K., Arakawa, O., Taniyama, S., Nonaka, M., Takatani, T., Yamamori, K., Fuchi, Y., Noguchi, T. Occurrence of saxitoxins as a major toxin in the ovary of a marine puffer Arothron firmamentum. Toxicon, **43**, 207–212 (2004).
19) Noguchi, T., Maruyama, J., Ueda, Y., Hashimoto, K., Harada, T. Occurrence of tetrodotoxin in the Japanese ivory shell *Babylonia japonica*. Bull. Japan. Soc. Sci. Fish., **47**, 909–913 (1981).
20) Jeon, J. K., Narita, H., Nara, M., Noguchi, T., Maruyama, J., Hashimoto, K. Occurrence of tetrodotoxin in a gastropod mollusk, "Araregai" *Niotha clathrata*. Bull. Japan. Soc. Sci. Fish., **50**, 2099–2102 (1984).
21) Hwang, D. F., Chueh, C. H., Jeng, S. S. Occurrence of tetrodotoxin in the gastropod mollusk *Natica lineata* (Lined Moon Shell). Toxicon, **28**, 21–27 (1990).
22) Narita, H., Noguchi, T., Maruyama, J., Ueda, Y., Hashimoto, K., Watanabe, Y., Hida, K. Occurrence of tetrodotoxin in a trumpet shell, "Boshubora" *Charonia sauliae*. Bull. Japan. Soc. Sci. Fish., **47**, 935–941 (1981).
23) Narita, H., Nara, M., Baba, K., Ohgami, H., Ai, T., Noguchi, T., Hashimoto, K. Effect of feeding a trumpet shell, *Charonia sauliae* with toxic starfish. J. Food Hyg. Soc. Japan, **25**, 251–255 (1984).
24) Narita, H., Noguchi, T., Maruyama, J., Nara, M., Hashimoto, K. Occurrence of a tetrodotoxin-associated substance in a gastropod, "Hanamushirogai" *Zeuxis siquijorensis*. Bull. Japan. Soc. Sci. Fish., **50**, 85–88 (1984).

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REVIEW FOR IPFC9 SPECIAL ISSUE

Ichthyology and Indo-Pacific Fish Conferences from the 1980s to the 2010s

Taxonomy and systematics of tetraodontiform fishes: a review focusing primarily on progress in the period from 1980 to 2014

Keiichi Matsuura

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Abstract When the first Indo-Pacific Fish Conference (IPFC1) was held in Sydney in 1981, there were still many problems in the generic- and species-level taxonomy of all tetraodontiform families except for the recently reviewed Triacanthodidae and Triacanthidae. The period from IPFC1 to IPFC9 (1981–2013) was a time of great progress in the taxonomy and systematics of the Tetraodontiformes: many review and revisional papers have been published for various genera and species, with descriptions of many new taxa occurring mainly on coral reefs and in tropical freshwaters; and cladistic analyses of morphological characters have been performed to clarify phylogenetic relationships of various families and molecular analyses have greatly progressed to provide detailed phylogenetic relationships of families, genera, and even species. The purpose of this paper is to provide a review on developments in the taxonomy and systematics of the Tetraodontiformes, focusing primarily on contributions since 1980 (when James C. Tyler's monumental work was published) through the period of IPFCs, including pertinent publications before 1980. This paper recognizes 412 extant species in the 10 families of living Tetraodontiformes, with the allocation of species and genera as follows: Triacanthodidae including 23 species in 11 genera, Triacanthidae seven species in four genera, Balistidae 37 species in 12 genera, Monacanthidae 102 species in 27 genera, Aracanidae 13 species in six genera, Ostraciidae 22 species in five genera, Triodontidae monotypic, Tetraodontidae 184 species in 27 genera, Diodontidae 18 species in seven genera, and Molidae five species in three genera. Phylogenetic relationships of the families have been clarified by morphological and molecular analyses and have provided well-supported sister relationships of the families: Triacanthodidae and Triacanthidae, Balistidae and Monacanthidae, and Tetraodontidae and Diodontidae. However, there remain problems with the phylogenetic positions of the Triodontidae and Molidae due to conflicts of differing positions in morphological and molecular studies (e.g., Molidae has been placed differently among molecular studies).

Keywords Tetraodontiformes · Classification · Morphology · Molecular · Phylogenetic relationships

Introduction

Tetraodontiform fishes are distributed in tropical to temperate seas and freshwaters of the world. They show a remarkable diversity in shape, size, and way of life (Fig. 1). A small filefish of the genus *Rudarius* Jordan and Fowler 1902 and a small pufferfish of the genus *Carinotetraodon* Benl 1957 mature at about 2 cm in total length (TL) (Lim and Kottelat 1995; Tyler 1970), whereas ocean sunfishes of the genus *Mola* Koelreuter 1766 attain over 300 cm TL (Yoshita et al. 2009). Tetraodontiform fishes are characterized by a small mouth with either relatively few teeth that are often enlarged or massive beak-like tooth plates, a small gill opening restricted to the side of the body, scales usually modified as spines, enlarged plates, or

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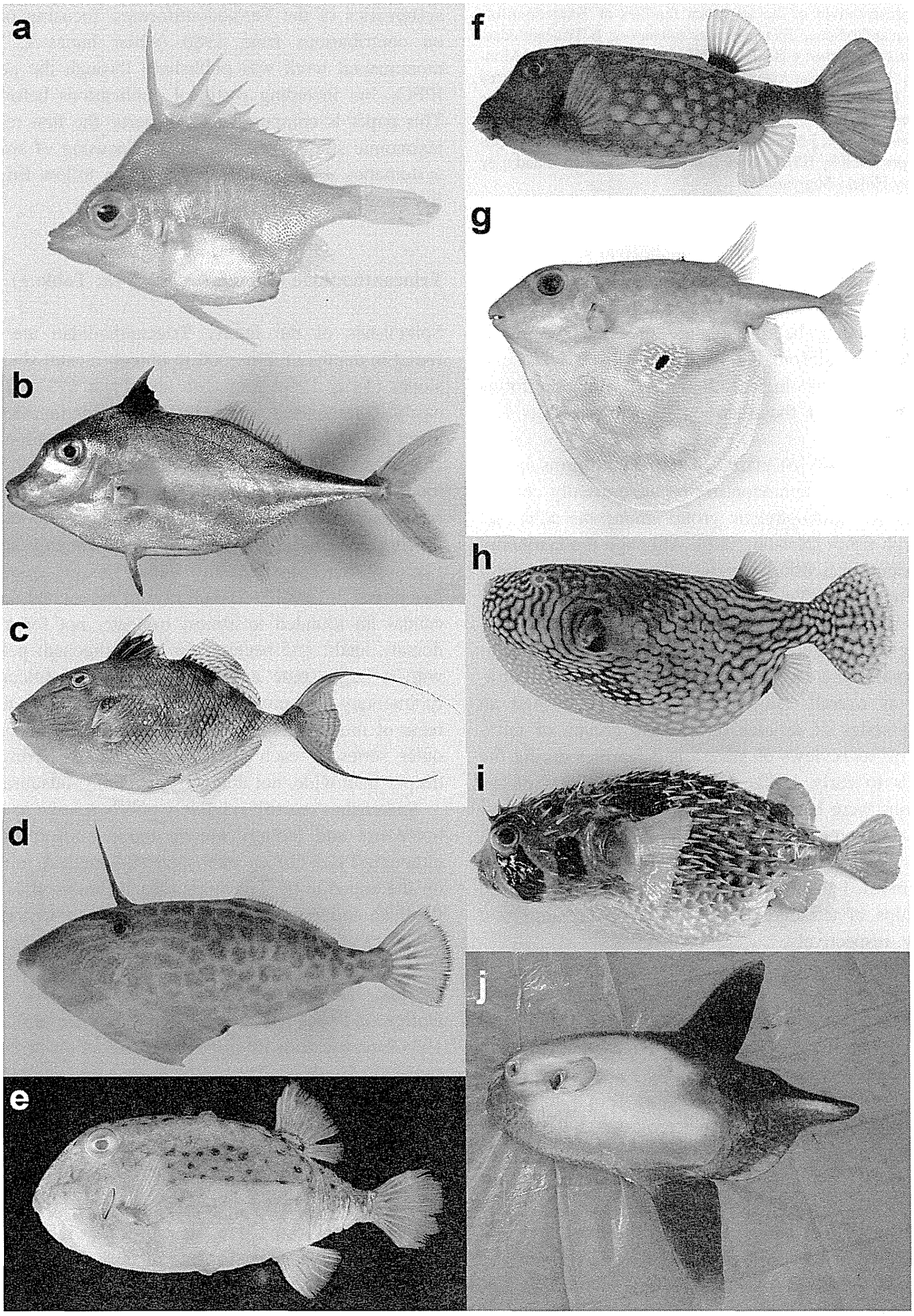
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◀**Fig. 1** Representatives of the 10 extant families of Tetraodontiformes. **a** Triacanthodidae, *Triacanthodes anomalus*; **b** Triacanthidae, *Triacanthus biaculeatus*; **c** Balistidae, *Abalistes filamentosus*; **d** Monacanthidae, *Thamnaconus hypargyreus*; **e** Aracanidae, *Kentrocapros aculeatus*; **f** Ostraciidae, *Ostracion immaculatus*; **g** Triodontidae *Triodon macropterus*; **h** Tetraodonitidae, *Arothron mappa*; **i** Diodontidae, *Diodon liturosus*; **j** Molidae, *Masturus lanceolatus*. Photographs of **a** and **e** provided by BSKU; **b**, **d**, **f**, **h**, and **i** by KAUM; **c** and **g** by NSMT; **j** by Hideki Sugiyama

a carapace, and pelvic fins that are reduced or absent (Tyler 1980; Nelson 2006). In addition, many pufferfishes are characterized by having a strong toxin in the viscera and skin, and even in the musculature of some species of *Lagocephalus* Swainson 1839 (Matsuura 1984). Because of their peculiar morphological characters, tetraodontiforms have long attracted the attention of ichthyologists and biologists.

Since Cuvier (1816) classified tetraodontiforms in the order Plectognathi, tetraodontiforms were usually considered to form a monophyletic group among the advanced percomorph fishes (Nelson 2006). Although the taxonomy of tetraodontiform fishes progressed greatly in the nineteenth century (see Tyler 1980, for a history of the classification of the order), information about tetraodontiform taxonomy was scattered in many articles, making it difficult to understand the taxonomic relationships of tetraodontiforms overall. Alec Fraser-Brunner published an important series of articles on various groups of tetraodontiforms as reviews of genera and families during the mid-1930s to early 1950s: however, many parts of his publications were based on cursory examinations of relatively few specimens. There were no comprehensive phylogenetic studies until Winterbottom (1974) and Tyler (1980) provided their interpretations of the phylogenetic relationships of tetraodontiforms based on myology and osteology, respectively.

When the first Indo-Pacific Fish Conference (IPFC1) was held in Sydney in 1981, many problems remained in the generic- and species-level taxonomy of all tetraodontiform families, except for the Triacanthodidae and Triacanthidae for which Tyler (1968) had provided a monographic revision. The period from IPFC1 to IPFC9 (1981–2013) was a time of great progress in the taxonomy and systematics of tetraodontiforms. Many review and revisional papers have been published for various genera and species, with descriptions of new taxa found mainly in coral reefs and tropical freshwaters; and cladistic analyses of morphological characters have clarified phylogenetic relationships of a number of families and molecular analyses greatly assisted our understanding of the detailed phylogenetic relationships of families, genera, and even species. The purpose of this paper is to present a review of the developments in the taxonomy and systematics of the Tetraodontiformes, focusing primarily on contributions from 1980 (when James C. Tyler's monumental work was published) through the period of IPFCs, but including pertinent publications before 1980. This paper is composed of two parts, the first reviewing taxonomic studies and the second focusing of studies on systematics. Institutional abbreviations follow Fricke and Eschmeyer (2014).

Triacanthodidae (Spikefishes, Fig. 1a; Table 1)

Spikefishes of the family Triacanthodidae are usually found in depths of 100–600 m on continental shelves and slopes (Tyler 1968; Matsuura and Tyler 1997). They are easily distinguished externally from other families of the order Tetraodontiformes by the following combination of characters: body deep and slightly compressed, covered by moderately thick skin with numerous small scales not readily distinguishable to the unaided eye, with each scale bearing upright spinules and having a roughly shagreen-like appearance; two separate dorsal fins, six spines in the first dorsal fin and 12–18 soft rays in the second dorsal fin; caudal fin rounded to almost truncate, not forked; most dorsal-, anal-, and pectoral-fin rays branched; pelvic fins with a large spine and one or two inconspicuous and rudimentary soft rays; mouth small and usually terminal; teeth of moderate size, usually conical, 10 or more in an outer series in each jaw; caudal peduncle compressed, deeper than wide, not distinctly tapered (Matsuura 2001).

Spikefishes are still relatively poorly known in terms of taxonomy and biology among tetraodontiform families, although Tyler (1968) published an excellent monograph on the superfamily Triacanthoidea including the Triacanthodidae and Triacanthidae. He recognized 19 triacanthodid species in 11 genera (Table 1): *Atrophacanthus* Fraser-Brunner 1950 (one species), *Bathyphylax* Myers 1934 (two species), *Halimochirurgus* Alcock 1899 (two species), *Hollardia* Poey 1861 (three species), *Johnsonina* Myers 1934 (one species), *Macrorhamphosodes* Fowler 1934 (two species), *Mephisto* Tyler 1966b (one species), *Parahollardia* Fraser-Brunner 1941b (two species), *Paratriacanthodes* Fowler 1934 (two species), *Triacanthodes* Bleeker 1857 (two species), and *Tydemania* Weber 1913 (one species). Because all these genera and species were described in detail by Tyler (1968), their taxonomic features are not repeated here, except for new information that provides a better understanding of taxa.

Many species of the Triacanthodidae are known from the tropical and warm regions of the Indo-Pacific. However, five species are distributed in the western Atlantic: *Hollardia hollardi* Poey 1861, *Hollardia meadi* Tyler 1966a, *Johnsonina eriomma* Myers 1934, *Parahollardia*

Table 1 A list of 23 species of the Triacanthodidae in the world

Subfamily	Species	Distribution
Hollardiinae	*Hollardia goslinei* Tyler 1968	Hawaii
	Hollardia hollardi Poey 1861	Bermuda and Florida Keys southward to Gulf of Mexico and Caribbean Sea
	Hollardia meadi Tyler 1966a	Bahamas, Cuba, and Barbados
	Paraholardia lineata (Longley 1935)	Atlantic coast of the USA from Virginia to Florida; Gulf of Mexico from Florida to Yucatan
	Paraholardia schmidti Woods 1959	Honduras to Panama in the western Caribbean Sea
Triacanthodinae	*Atrophacanthus japonicus* (Kamohara 1941)	Off Tanzania, Maldive Islands, Japan, and Celebes Sea
	Bathyphylax bombfrons Myers 1934	Off Kenya, China Sea (off Hong Kong), Australia, and New Caledonia
	Bathyphylax omen Tyler 1966c	Off Kenya
	Bathyphylax pruvosti Santini 2006	Marquesas Islands
	Halimochirurgus alcocki Weber 1913	Arabian Sea, Japan, Mariana Islands, Australia, and New Caledonia
	Halimochirurgus centriscoides Alcock 1899	Arabian Sea and Australia
	Johnsonia eriomma Myers 1934	Bahamas to Greater and Lesser Antilles and western Caribbean Sea
	Macrorhamphosodes platycheilus Fowler 1934	Bay of Bengal, Philippines, and Australia
	Macrorhamphosodes uradoi (Kamohara 1933)	South Africa, off Kenya, Japan, Korea, East China Sea, South China Sea, Australia, New Caledonia, and New Zealand
	Mephisto fraserbrunneri Tyler 1966b	Somalia, Arabian Sea, and Andaman Sea
	Paratriacanthodes abei Tyler 1997	South China Sea
	Paratriacanthodes herrei Myers 1934	Philippines
	Paratriacanthodes retrospinis Fowler 1934	East Africa, Japan, Kyushu-Palau Ridge, South China Sea, Australia, and New Caledonia
	Triacanthodes anomalus (Temminck and Schlegel 1850)	Japan, Korea, East China Sea, South China Sea
	Triacanthodes ethiops Alcock 1894	East Africa, Maldive Islands, Japan, East China Sea, Philippines, Indonesia, New Caledonia, and Australia
	Triacanthodes indicus Matsuura 1982	Saya de Malha Bank (western Indian Ocean)
	Triacanthodes intermedius Matsuura and Fourmanoir 1984	New Caledonia
	Tydemania navigatoris Weber 1913	East Africa, Bay of Bengal, Japan, China, Philippines, Indonesia, and Australia

lineata (Longley 1935), and *Parahollardia schmidti* Woods 1959. *Hollardia goslinei* Tyler 1968 are known only from nine specimens collected from the Hawaiian Islands.

Little information about the Atlantic and Hawaiian spikefishes has been published since Tyler's (1968) monograph. Matsuura (1983) provided a brief morphological account of *Hollardia hollardi* with a color photograph based on specimens collected from off Surinam. McEachran and Fechhelm (2005) also provided a brief account and line drawing of *Hollardia hollardi* and *Hollardia meadi* from the Gulf of Mexico. McEachran and Fechhelm (2005) and Hartel et al. (2008) reported briefly *Parahollardia lineata* from the Gulf of Mexico and off greater New England, respectively. Tyler et al. (2013) documented a large northern range extension for *Hollardia hollardi* to the northeast coast of the USA off Massachusetts, whereas this species had previously only been known from the Florida Keys, Bahamas, Bermuda, Gulf of Mexico, Caribbean, and south to Brazil, with the new northern occurence perhaps associated with warming currents along the east coast of North America.

In contrast to the Atlantic spikefishes, many papers on Indo-Pacific spikefishes were published after 1980. Matsuura (1982) described *Triacanthodes indicus* based on 13 specimens collected from the western Indian Ocean. This species is characterized by relatively large nasal organs compared to other species of *Triacanthodes*. Matsuura and Fourmanoir (1984) described *Triacanthodes intermedius* based on two specimens collected from New Caledonia. *Triacanthodes intermedius* is unique among spikefishes in having several intermediate characters between *Paratriacanthodes* and *Triacanthodes*. Tyler (1997) described *Paratriacanthodes abei* based on a single