Table 2. Anatomic distribution of toxicity in toxic specimens of *Ostracion immaculatus* "hakofugu" collected from Western Japan

No.	Place of collection	Month of collection	Body weight (g)	Body length (mm)	Lethal potency (MU/g)		
					Muscle	Liver	Viscera excluding liver
1	Arikawa Bay, Nagasaki Prefecture	Nov.–Dec. 2003	563	227	0.5*	ND	ND
2			575	206	0.5*	ND	ND
3			494	239	0.5**	ND	ND
4		Nov.–Dec. 2004	220	153	0.5*	0.5*	1.0*
5			115	145	0.5**	0.5*	1.0*
6			270	160	ND	0.5*	0.5*
7			130	152	ND	0.5*	0.5*
8			358	188	0.5*	ND	ND
9			634	235	0.5**	ND	ND
10			205	153	ND	ND	0.5*
11			455	195	ND	ND	0.5*
12			410	187	NE	NE	0.5*
13			279	178	ND	ND	0.5**
14	Shimaura Island, Miyazaki Prefecture	May 2004	300	133	0.5*	ND	ND
15			191	145	ND	ND	1.0**
16	Offshore of Mugi, Tokushima Prefecture	Nov. 2004	267	158	ND	ND	0.5*
17		Jun. 2005	237	160	ND	ND	1.0*
18			259	160	ND	ND	0.5*
19			315	183	ND	ND	0.5**
20			190	146	ND	ND	0.5**
21	Offshore of Shimonoseki, Yamaguchi Prefecture	Dec. 2004	317	168	ND	ND	0.5**
22			235	155	ND	ND	0.5**
23		Apr. 2005	603	216	ND	ND	0.5**
24			554	202	ND	ND	0.5**
25		Jun. 2005	224	142	0.5*	ND	0.5*
26			627	209	0.5*	ND	0.5**
27			395	180	ND	ND	0.5**
28		Jul. 2005	82.5	101	0.5*	ND	0.5*
29			153	127	ND	0.5**	0.5*
30			336	172	ND	ND	0.5*
31			117	123	ND	ND	0.5**
32			106	110	ND	ND	0.5**
33			208	142	ND	ND	0.5**
34		Aug. 2005	411	203	ND	ND	0.5*
35			405	179	ND	ND	0.5**
36		Oct. 2005	302	159	0.5**	ND	0.5*
37			552	204	0.5*	ND	ND
38			861	215	0.5**	ND	ND
39			469	187	ND	0.5*	ND
40			288	174	ND	ND	0.5**
41		Nov. 2005	535	192	ND	0.5*	0.5*
42			89.2	110	ND	ND	0.5**
43			777	206	ND	ND	0.5**
44		Dec. 2005	668	198	ND	ND	0.5*
45		May 2006	458	178	ND	0.5*	ND
46			750	208	ND	ND	0.5**
47			458	184	ND	ND	0.5**

ND: Not detected (< 0.5 MU/g).
NE: Not examined.
*: Delayed lethal potency to mice
**: Acute lethal potency to mice

Table 3. Anatomic distribution of toxicity in toxic specimens of *Lactoria diaphana* "umisuzume" collected from Western Japan

No.	Place of collection	Month of collection	Body weight (g)	Body length (mm)	Lethal potency (MU/g)		
					Muscle	Liver	Viscera excluding liver
1	Shimaura Island, Miyazaki Prefecture	May 2004	391	163	0.5**	ND	ND
2			711	240	ND	ND	0.5**
3			541	237	ND	ND	0.5**
4			272	190	ND	ND	0.5*
5	Offshore of Mugi, Tokushima Prefecture	Jun. 2005	439	235	ND	0.5**	2.0*
6		Mar. 2006	NE	133	ND	ND	1.0**
7			NE	178	ND	NE	1.0**

ND: Not detected (< 0.5 MU/g).
NE: Not examined.
*: Delayed lethal potency to mice
**: Acute lethal potency to mice

toxin; PTX) 様物質を同時に保有するが，前者の保有量はわずかで，主たる原因物質は後者であることが示唆されている[23]．主な中毒症状や発症/回復/致死時間はアオブダイ中毒やハコフグ中毒に酷似していることから[22]，これら3者の中毒は，いずれもPTX様の活性を持つ物質に起因するものと推察される．

2. ハコフグとウミスズメの毒性

供試した2種のハコフグ科魚類のうち，ハコフグでは129個体中47個体(36.4%)，ウミスズメでは18個体中7個体(38.9%)がマウスに対して急性もしくは遅延性の毒性を示した．急性毒性の場合，試験液を投与されたマウスは，数分で激しく疾走または跳躍後，しだいに運動性を失い，呼吸停止により死亡した．症状から，原因物質にTTXなどの麻痺毒が疑われたが，希釈倍率が異なる試験液を投与した場合の用量と致死時間の関係は，TTXやPSPの一種であるサキシトキシン(STX)の用量致死時間曲線[24),25)]には当てはまらず，HPLC分析においてもTTXやその関連成分は検出されなかった．一方，遅延性毒性の場合，マウスに対して長時間にわたり痙攣や嗜眠，衰弱を誘起し，おおむね18～36時間で死亡させた．

マウス毒性を示したハコフグ47個体の部位別毒力をTable 2に示す．5個体の筋肉，1個体の肝臓および19個体の肝臓を除く内臓に0.5～1.0 MU/gの急性毒性が検出された．また，9個体の筋肉，7個体の肝臓，18個体の肝臓を除く内臓は，遅延性毒性(0.5～1.0 MU/g)を示した．供試したハコフグ129個体のうち，肝臓を除く内臓からの毒性の検出率が28.7%と最も高く，次いで筋肉(10.9%)，肝臓(6.2%)の順であった．同様に，ウミスズメ1個体の筋肉と肝臓，および4個体の肝臓を除く内臓に急性毒性(0.5～1.0 MU/g)が，2個体の肝臓を除く内臓に遅延性毒性(0.5～2.0 MU/g)認められた(Table 3)．筋肉，肝臓，肝臓を除く内臓からの毒性の検出率は，それぞれ5.6%，5.6%，33.3%で，ハコフグのパターンと類似していた．一方，両種ともに各部位の毒性に明瞭な地域差や季節変動は認められず，中毒が発生していない下関沖産でも多くの個体が有毒であった．

事例5～7の原因魚と同時期同海域で採捕されハコフグ(それぞれ島裏島産8個体，同9個体，有川湾産16個体)の毒性調査では，有川湾産8個体(Table 2の試料番号4～8および10～12)が0.5～1.0 MU/gと低いながらも遅延性毒性を示した．アオブダイ中毒では，中毒検体からの遅延性毒の検出例があるが[16]，毒力は0.6～0.9 MU/gと低く，また中毒に関連して行われたアオブダイの毒性スクリーニングでも，有毒個体の毒力は総じて低かった[14),16),20)]．Noguchiら[16]はアオブダイ中毒検体から遅延性の毒としてPTX様物質を分離し，このものがアオブダイ中毒の原因物質であると推定しているが，ハコフグ中毒の症例やハコフグ抽出液の毒力を考慮すると，ハコフグの遅延性毒性の本体も本物質もしくはその類縁体である可能性が高いと思われる．

九州ではハコフグの食習慣が半世紀以上あるにもかかわらず，中毒の発生は2000年以降に偏っており，本魚類は近年になって毒化し始めたものと推察される．この点は，海洋環境の変化に伴い無毒の魚類が突然毒化して起こるシガテラ[15]に類似している．また，ハコフグ，ウミスズメともにすべての個体が毒を保有しているわけではない．したがって，ハコフグ科魚類の毒化は，シガテラ毒魚[15]やアオブダイ[20]，TTXを持つ一般的なフグ[26]同様，食物連鎖を介する外因性のものと推察される．毒の起源生物としては，アオブダイの場合，PTX類縁体を産生する*Ostreopsis*属渦鞭毛藻が疑われている[20]．本藻は本来，熱帯ないし亜熱帯性の種で，日本では南西諸島での分布が確認されていたものであるが[27),28)]，近年，毒産生能を有する株が徳島県をはじめ長崎県や宮崎県沿岸にも分布することが分かってきた[20),29)]．したがって，ハコフグ類の毒化にも本藻の関与が疑われるが，この点については，ハコフグ類の毒本体の解明と併せて現在検討中である．

ま と め

ハコフグ中毒の実態調査を行ったところ，1990年から

2008年にかけて，長崎県[2),9)]，宮崎県[6),8),9)]，鹿児島県[4)]，および三重県[7)]で，聞き取り調査から新たに判明した1件を含め，計9事例の発生が確認された．中毒患者は計13名で，うち1名が死亡していた[4)~9)]．2事例については，中毒検体の形態的な特徴から原因魚種をハコフグと同定した．さらに，聞き取り調査の過程でウミスズメも原因魚種となる可能性が示唆された．一方，既報[5)]ではハマフグでの中毒が推察されている．いずれの事例においても，中毒症状や，喫食から発症，回復または死に至るまでの時間経過はTTXによる一般的なフグ中毒[12)]とは異なり，アオブダイ中毒[13)~20)]に酷似していた．他方，西日本近海に生息するハコフグ129個体およびウミスズメ18個体の毒性を調査したところ，両種共に約4割が有毒で，喫食頻度の高い筋肉や肝臓にも毒性が認められた．その毒力は総じて低いものの，アオブダイ中毒の中毒検体[16)]と同レベルであった．以上の結果から，日本近海産ハコフグ類，特にハコフグとウミスズメは食中毒の原因となりうる食品衛生上要警戒種であると結論した．

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QUIZ PAGE
OCTOBER 2009

Neurologic Symptoms in a Dialysis Patient After Ingesting Seafood

CLINICAL PRESENTATION

A 60-year-old man with end-stage renal disease caused by diabetic nephropathy treated with hemodialysis for 14 months was admitted to our hospital reporting neurologic symptoms. The prior evening, he ingested 8 gastropods (whelks) harvested from the sea of northern Japan (Fig 1) that were cooked in their own shells. He went to bed as usual, but awoke 6 hours later with nausea, drowsiness, dyspnea, limb weakness, facial palsy, and diplopia. He could not raise his head or get out of bed. His medications include antiplatelet and anticoagulant agents, isosorbide mononitrate for angina pectoris, and 1α-hydroxyvitamin D_3 for secondary hyperparathyroidism. On presentation, he was afebrile with blood pressure of 149/92 mm Hg and pulse rate of 70 beats/min. Barre and Mingazzini signs were positive, and his mouth opened only 15 mm. There was no neck stiffness, muscle atrophy, or involuntary movements, and Babinski reflex was not observed. Deep tendon reflexes were diminished in the lower extremities. Blood work showed the following values: creatine kinase, 116 U/L; C-reactive protein, 0.10 mg/dL (1.0 mg/L); and white blood cell count, $6.2 \times 10^3/\mu L$ ($6.2 \times 10^9/L$). Computed tomography and magnetic resonance imaging of the head showed only chronic small ischemic changes. Although his friends who did not have end-stage renal disease had ingested the same gastropods, they did not experience similar symptoms.

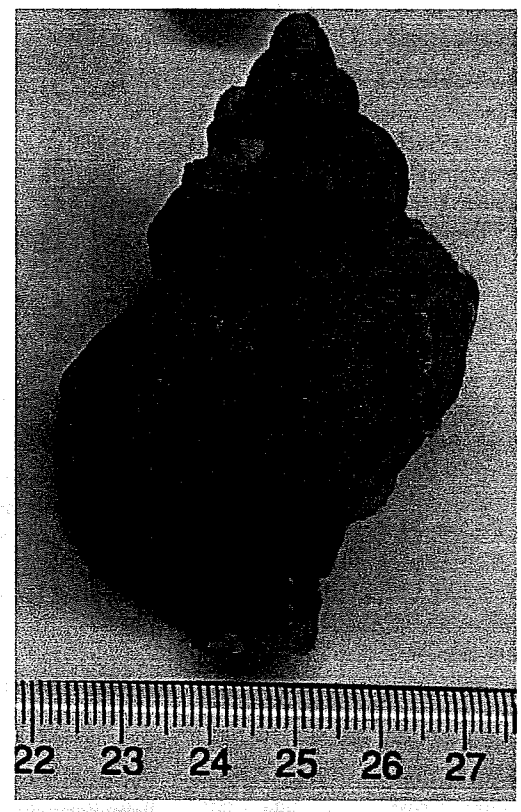

Figure 1. *Neptunea arthritica*. The gastropod in this photograph was purchased at the same time as the gastropods that the patient ingested.

- **What is the most likely diagnosis?**
- **How might the diagnosis be confirmed?**
- **What treatment might be initiated?**

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QUIZ PAGE OCTOBER 2009 ANSWERS

DISCUSSION

What is the most likely diagnosis?

After ingesting gastropods 16 hours before, tetramine poisoning was suspected. Tetramine (tetramethylammonium ion) is a natural neurotoxin that exists in the salivary gland of gastropods of the family Buccinidae, *Neptunea arthritica* and *Neptunea intersculpta*, living in cold temperate seas.[1] It is a quaternary ammonium compound that has an acetylcholine-like structure that stimulates both nicotinic and muscarinic receptors.[2] Tetramine poisoning occurs by consumption of these gastropods without removal of the salivary gland. A variety of symptoms, such as headache, dizziness, seasickness, visual impairment, and, in severe cases, motor paralysis and respiratory arrest, are observed. Symptoms of tetramine poisoning typically appear within 30 minutes of gastropod ingestion. However, in this case, symptoms of poisoning were not observed until 12 hours after gastropod ingestion (Fig 2). Tetramine absorption may have been delayed because of diabetic gastroparesis, resulting in the increased time between ingestion and the appearance of toxicity.

How might the diagnosis be confirmed?

Although measurement of plasma tetramine has not been reported previously, we applied the method of Kawashima et al[3] for determining tetramine concentration in gastropod tissues. When the patient arrived at the hospital, plasma tetramine concentration was 2.16 μg/mL. Measurement of plasma tetramine is feasible and useful for the diagnosis of tetramine poisoning.

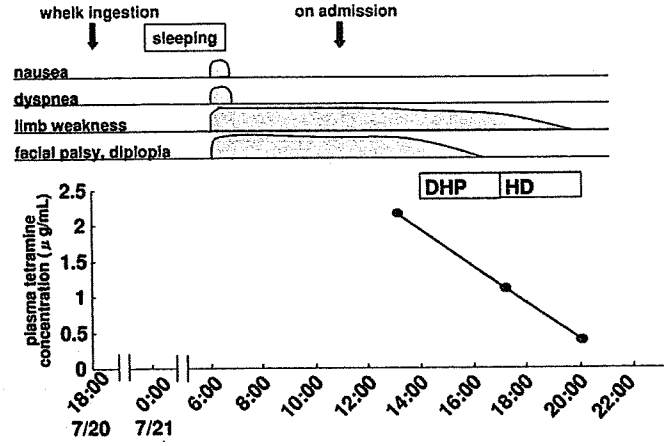

Figure 2. Plasma tetramine concentration and clinical course of the patient.

What treatment might be initiated?

Without definitively identifying the toxic agent on presentation, hemoperfusion using an activated charcoal column was performed, followed by hemodialysis (Fig 2). After hemoperfusion, dysarthria improved and the patient was able to maintain a sitting position. After hemodialysis, he was able to stand without assistance.

There is no specific therapy for tetramine poisoning. In a healthy individual, tetramine is excreted rapidly in urine and symptoms commonly disappear within a few hours.[2] Excretion of tetramine may be delayed, and symptoms of tetramine poisoning sometimes may be serious and prolonged in patients with decreased kidney function because more than 95% of this poison is excreted through the kidney.[4] Kidney failure in our patient resulted in severe limb weakness and diplopia for 8 hours until hemoperfusion was started. Intensive hemodialysis may promote rapid improvement of tetramine poisoning symptoms.

When the patient arrived at the hospital, plasma tetramine concentration was 2.16 μg/mL. After direct hemoperfusion and hemodialysis, plasma tetramine concentration had decreased to 1.11 μg/mL and 0.38 μg/mL, respectively. Tetramethylammonium ion has a molecular weight of 74 Da and thus low plasma protein binding,[5] indicating that plasma tetramine could be removed sufficiently by means of hemodialysis. In anuric patients, emergent hemodialysis may be essential.

FINAL DIAGNOSIS

Tetramine poisoning.

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Maturation-associated changes in toxicity of the pufferfish *Takifugu poecilonotus*

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ABSTRACT

From October 2006 to December 2007, wild specimens of the pufferfish *Takifugu poecilonotus* (93 females, 45 males) were collected from the Ariake Sea. Tissue toxicity was examined by mouse bioassay, and tetrodotoxin (TTX) content in the blood plasma by enzyme-linked immunosorbent assay. The relationship between toxicity and maturation was investigated based on changes in the gonadosomatic index: December-March in females and November-March in males, the 'maturation period'; April, 'just after spawning'; and the other months, the 'ordinary period'. Toxicity of both sexes was high throughout the year, but sharply declined in April. In all tissues examined (skin, liver, and ovary) other than testis, toxicity exceeded 1000 MU/g or 10,000 MU/individual in many individuals. Seasonal profiles of tissue toxicity differed markedly between sexes. In females, liver toxicity was high during the ordinary period, and ovary toxicity was high during the maturation period. In males, little maturation-associated change in the toxin distribution was observed. Plasma TTX levels were similar between the sexes (1.59–15.1 MU/ml), and fluctuated largely throughout the year without corresponding changes in tissue toxicity. The percentage of TTX binding to high molecular-weight substances in the plasma varied in association with maturation; the binding ratio fluctuated at relatively low levels during the ordinary period, and stabilized at a high level during the maturation period.

1. Introduction

Many marine pufferfish of the family Tetraodontidae possess a potent neurotoxin, tetrodotoxin (TTX). In toxic species inhabiting Japanese coastal waters, the liver and ovary usually have strong toxicity, whereas the muscle and testis are weakly toxic or non-toxic (Noguchi and Arakawa, 2008), indicating sexual differences in pufferfish toxicity, and that maturation may affect toxin kinetics in the pufferfish body. TTX is originally produced by marine bacteria and distributes over a wide variety of animals, including pufferfish, gobies, blue-ringed octopuses, carnivorous gastropods, starfish, toxic crabs, horseshoe crabs, flat worms, and ribbon worms (Miyazawa and Noguchi, 2001). TTX is exogenous in pufferfish and is derived from the food chain that consists of these TTX-bearers (Noguchi and Arakawa, 2008). The transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. Various types of toxin administration experiments performed with pufferfish have revealed important information on uptake and inter-tissue transfer of TTX in the pufferfish body (Matsui et al., 1981, Watabe et al., 1987, Yamamori et al., 2004, Honda et al., 2005, Kono et al., 2008, Ikeda et al., 2009). In these experiments, however, non-matured, non-toxic cultured fish were used,

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and the influence of aging or maturation was not considered. Although TTX-binding proteins have been found in the blood plasma of toxic pufferfish (Matsui et al., 2000; Yotsu-Yamashita et al., 2001), and may be involved in the transportation mechanism, little information is available on their distribution, seasonal variation, or functions other than TTX binding. In our studies to clarify the roles of TTX-binding high molecular-weight substances in the accumulation mechanisms of TTX in pufferfish and the effect of maturation, we collected the pufferfish *Takifugu poecilonotus* periodically from the Ariake Sea and investigated maturation-associated changes in tissue toxicity, as well as the amount and forms of TTX in the blood plasma.

2. Materials and methods

2.1. Pufferfish specimens

From October 2006 to December 2007, wild specimens of the pufferfish *T. poecilonotus* (93 females and 45 males) (Table 1) were collected from the Ariake Sea (off Minamishimabara, Nagasaki Prefecture, Japan), and transported live to the laboratory of Nagasaki University. After blood was withdrawn from the portal vein using a syringe precoated with sodium heparin, each fish was dissected to obtain the skin, liver, and gonads (ovary/testis), which were then extracted with 0.1% acetic acid according to the official guidelines of the Japan Food Hygiene Association (2005), and analyzed with a toxicity assay using mice.

2.2. Assessment of gonadosomatic index (GSI)

GSI (%) of each fish was calculated from its gonad weight (GW) and body weight (BW) using the following equation: $GSI = 100 \times GW/BW$.

2.3. Toxicity assay

Toxicity of each tissue extract from *T. poecilonotus* was determined by a mouse bioassay according to the official guidelines of the Japan Food Hygiene Association (2005). Lethal potency was expressed in mouse units (MU), where 1 MU was defined as the amount of toxin required to kill a 20-g male ddY strain mouse within 30 min after intraperitoneal administration.

2.4. Quantification of TTX in blood plasma

The blood collected from each fish was centrifuged at 6000 *g* for 7 min (4 °C), and the blood plasma obtained (200 µl) was ultrafiltered through a Microcon YM-50 membrane (cut-off 50,000 Da, Amicon). Phosphate buffered saline (10 mM, 200 µl) was added to the residue, and the mixture was ultrafiltered again through the same membrane. The operation was repeated one more time. The combined supernatant (low molecular-weight fraction) and the residue (high molecular-weight fraction) contain free TTX molecules (designated f-TTX) and the TTX molecules binding to high molecular-weight substances (designated b-TTX), respectively (Matsui et al., 2000). The low molecular-weight fraction was directly submitted to an enzyme-linked immunosorbent assay (ELISA) to determine the amount of f-TTX. To cut the binding between TTX and high molecular-weight substances, 0.1% acetic acid (400 µl) was added to the high molecular-weight fraction (Yamamori, 2002), and then the mixture was submitted to ELISA to quantify the amount of b-TTX. Preliminary experiments demonstrated that 0.1% acetic acid or TTX-binding substances in the high molecular-weight fraction did not affect the ELISA results (data not shown).

Table 1
Specification of *T. poecilonotus* specimens.

Collection month	Sex	Number of specimens	Mean body weight (g)	Mean tissue weight		
				Skin (g)	Liver (g)	Gonad (g)
2006 Oct	♀	1	198	24	12	2.0
	♂	3	211	24	10	2.2
Nov	♀	3	273	30	16	4.0
	♂	3	227	26	14	13
Dec	♀	2	176	21	9.2	4.8
	♂	3	210	21	9.4	21
2007 Jan	♀	8	278	31	16	19
	♂	3	248	25	8.8	31
Feb	♀	10	267	29	13	31
	♂	6	280	32	15	34
Mar	♀	8	243	22	9.2	39
	♂	3	126	14	3.9	13
Apr	♀	12	121	15	4.1	3.3
	♂	5	113	16	3.4	4.1
Jun	♀	17	124	15	6.2	1.1
	♂	2	143	18	4.5	1.4
Aug	♀	7	156	17	6.5	1.2
	♂	3	155	19	4.7	0.9
Sep	♀	8	121	13	4.4	0.9
	♂	4	116	13	6.5	0.5
Oct	♀	11	146	16	8.7	1.4
	♂	5	123	15	6.6	1.3
Dec	♀	6	194	22	8.9	3.7
	♂	5	166	18	5.3	8.5

ELISA was performed according to the previously reported method (Ngy et al., 2008) using a monoclonal anti-TTX antibody developed by Kawatsu et al. (1997). The amount of TTX (ng) determined by ELISA was converted to MU based on the specific toxicity of TTX (220 ng/MU). The sum of f-TTX and b-TTX was considered as the total TTX amount in plasma (designated p-TTX), and the percentage of b-TTX in p-TTX (designated the binding ratio) was calculated using the following equation:

$$\text{Binding ratio} = 100 \times \text{b-TTX}/(\text{f-TTX} + \text{b-TTX}) = 100 \times \text{b-TTX}/\text{p-TTX}$$

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was applied to the toxicity data (both in MU/g and MU/individual) of each tissue, the amount of TTX in the plasma (f-TTX, b-TTX, and p-TTX), and the binding ratio. Tukey–Kramer post hoc test was used to determine significant differences between females and males, and/or the ordinary period and maturation period when ANOVA detected significant differences ($p < 0.05$). Student's t-test was also applied to the data as appropriate.

3. Results

3.1. Seasonal changes in the gonadosomatic index (GSI)

Seasonal changes in GSI are shown in Fig. 1. In female specimens, GSI began to increase in December, peaked (average ± SD: 15.5 ± 3.4%) in March, and then decreased abruptly in April, except for one brooding fish. In male specimens, GSI began to increase 1 month earlier than females in November, reached a maximum (12. 7 ± 1.2%) in January, and gradually decreased thereafter till April. Based on the results, we considered December-March in females and November-March in males as the 'maturation period', April as 'just after spawning', and the other months as the 'ordinary period', and used this seasonal classification to investigate the relationship between toxicity and maturation, as described below.

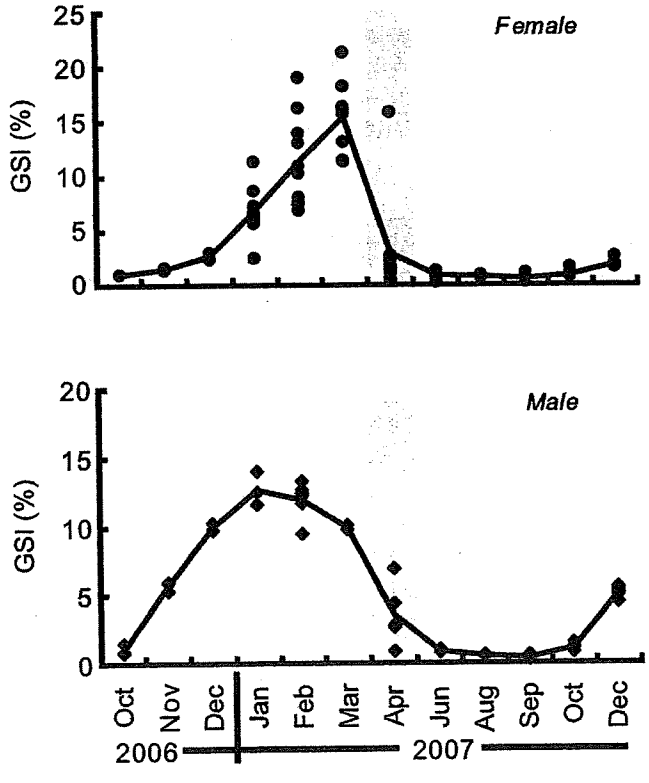

Fig. 1. Seasonal changes in the gonadosomatic index (GSI) in female (upper) and male (lower) specimens of *T. poecilonotus*. Data are shown by individual values (symbols) and mean of each month (bend of sequential line). White, light gray, and gray zones indicate 'ordinary period', 'maturation period', and 'just after spawning', respectively (common in all figures).

3.2. Seasonal changes in toxicity per gram of each tissue

3.2.1. Females

Seasonal changes in the toxicity (MU/g) of each tissue in the female specimens are shown in Fig. 2. All tissues on the whole showed very high toxicity; the mean toxicity score exceeded 1000 MU/g in 4 of 12 months in the skin, 5 of 12 months in the liver, and 500 MU/g in 6 of 12 months in the ovary. Especially in the liver, the score exceeded 3000 MU/g in June and August.

Toxicity of each tissue exhibited a change associated with maturation, i.e., it was significantly higher (Tukey–Kramer post hoc test, $p < 0.05$) in the liver during the ordinary period than during the maturation period, and

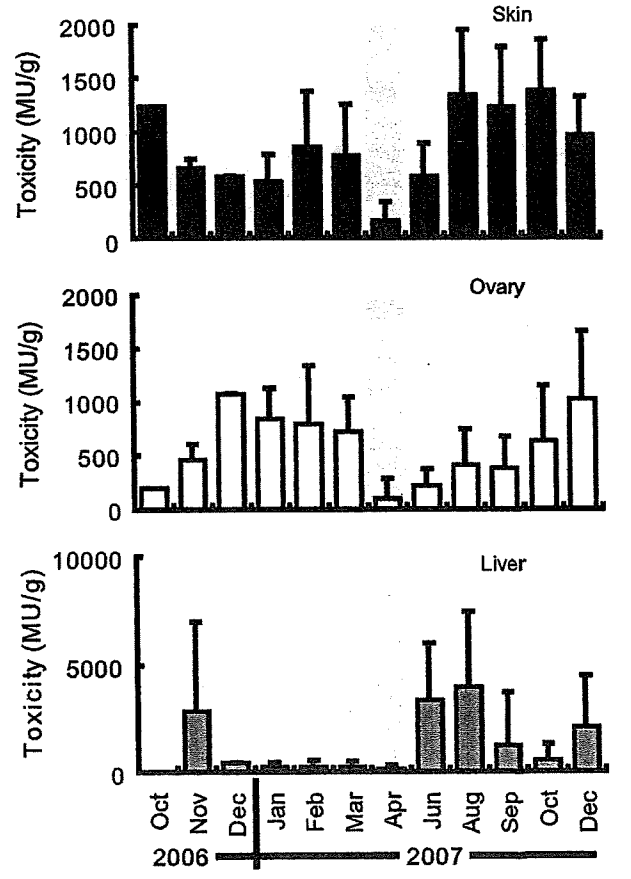

Fig. 2. Seasonal changes in the toxicity (MU/g) of skin (upper), ovary (middle), and liver (lower) in the female specimens of *T. poecilonotus*. Data are shown by mean (column) and standard deviation (SD, error bar) of each month.

Table 2
Result of statistical analysis for each tissue toxicity test (compared based on seasonal classification).

Sex	Seasonal classification†	n	Toxicity					
			Liver		Ovary/testis		Skin	
			(MU/g)	(MU/individual)	(MU/g)	(MU/individual)	(MU/g)	(MU/individual)
♀	O	47	2320 ± 2770[a]	16,300 ± 24,600[a]	385 ± 348[b]	580 ± 691[b*]	1010 ± 563[a*]	16,700 ± 12,400
	M	34	584 ± 1190[b]	6140 ± 11,500[b]	849 ± 440[a]	18,200 ± 15,000[a]	761 ± 429[ab*]	19,900 ± 12,600
♂	O	17	909 ± 2320[a]	3830 ± 8350[ab]	41.9 ± 90.8[c*]	29.2 ± 72.2[b*]	730 ± 402[ab]	11,200 ± 5740
	M	23	267 ± 393[b]	2450 ± 4520[b]	1.04 ± 1.77[c*]	13.3 ± 24.6[b]	572 ± 410[b]	13,000 ± 9630

Data are shown as mean ± standard deviation (SD).
Different alphabetical superscripts indicate significant differences among the measured values in each column (Tukey–Kramer post hoc test, $p < 0.05$).
Asterisks indicate significant differences between the two measured values in each column (Student's *t*-test, $p < 0.05$).
†O: ordinary period; M: maturation period.

vice versa in the ovary (Fig. 2 and Table 2). Skin toxicity in general was maintained at high levels throughout the year, but also fell significantly (Student's *t*-test, $p < 0.05$) during the maturation period, though the fluctuation range was much smaller than that of liver toxicity (Fig. 2 and Table 2). In all three tissues, toxicity declined markedly just after spawning in April (Fig. 2).

3.2.2. Males

Seasonal changes in the toxicity (MU/g) of each tissue in the male specimens are shown in Fig. 3. As a whole, the skin showed high toxicity throughout the year; the mean toxicity score, although significantly lower (Student's *t*-test, $p < 0.05$) than that in the females (Table 3), exceeded 500 MU/g in 8 of 12 months. The liver toxicity score, which was also significantly lower (Student's *t*-test, $p < 0.05$) than that in the females (Table 3), was exceptionally high (~5000 MU/g) in June, but less than 300 MU/g in 8 of 12 months. The testis toxicity was usually very low; the mean score was less than 10 MU/g except for June, August, and September.

Like females, males also showed a decline in toxicity in April (Fig. 3). Although decreases in the liver and skin toxicity during the maturation period was also observed in the male specimens, the degree looked smaller than that in the females. Testis toxicity was significantly higher (Student's *t*-test, $p < 0.05$) during the ordinary period than during the maturation period (Fig. 3 and Table 2).

3.3. Seasonal changes in toxicity per each individual tissue and plasma TTX content

3.3.1. Female

Seasonal changes in toxicity (MU/individual) in each tissue, and in the plasma TTX content in female specimens are shown in Fig. 4. The skin toxicity level was similar to the sum of ovary and liver toxicity levels, both of which (skin toxicity and sum of ovary and liver toxicity) fluctuated up and down with approximately 30,000 to 40,000 MU/individual as the upper limit.

The maturation-associated change in liver and ovary toxicity described in Section 3.2.1 became more distinct when observed as toxicity per individual, i.e., liver toxicity was high and ovary toxicity very limited during the ordinary period, whereas during the maturation period, liver toxicity largely decreased, and ovary toxicity increased remarkably as the GSI increased [all these changes are statistically significant (Tukey–Kramer post hoc test, $p < 0.05$) (Table 2)]. This rise, however, depended on the increase in the ovary mass, and the toxin concentration did not largely change during the maturation period, or gradually increased during the ordinary period (Fig. 2). When observed as toxicity per individual, all three tissues also showed a marked decline in toxicity just after spawning in April (Fig. 4).

The plasma TTX content (p-TTX = b-TTX + f-TTX) ranged between 1.75 and 15.1 MU/ml, the levels being much lower than that in the other three tissues. Although p-TTX was significantly higher (Student's *t*-test, $p < 0.05$) in the ordinary period than in the maturation period, it generally showed large fluctuations throughout the year, which did not clearly correspond to changes in tissue toxicity; even the decline just after spawning in April was not observed in

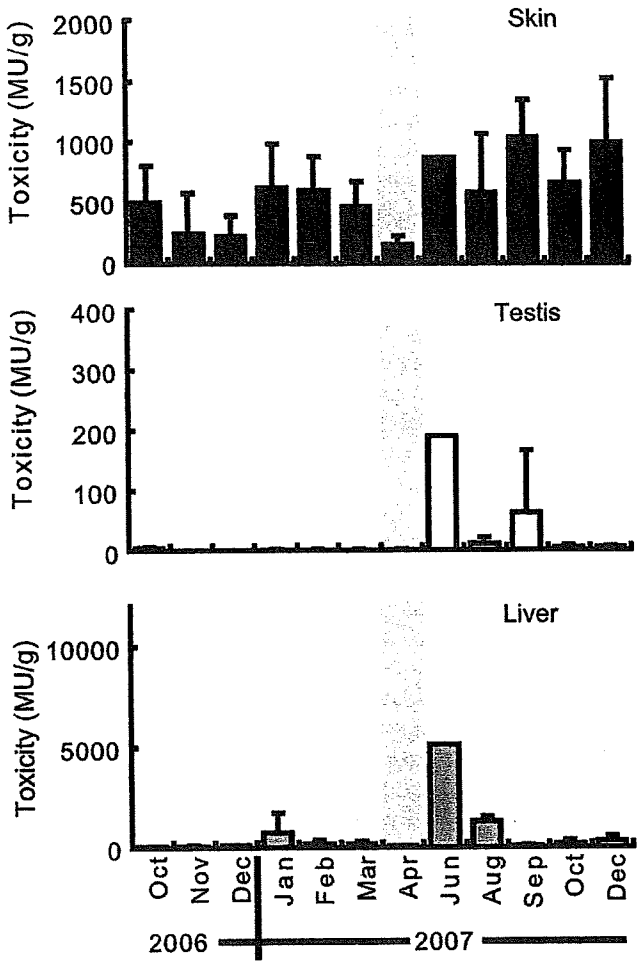

Fig. 3. Seasonal changes in the toxicity (MU/g) of skin (upper), testis (middle), and liver (lower) in the male specimens of *T. poecilonotus*. Data are shown by mean (column) and SD (error bar) of each month.

the plasma TTX (Fig. 4 and Table 4). b-TTX remained at a certain level irrespective of maturation, but the binding ratio, which fluctuated within relatively low levels during the ordinary period, was stabilized at a high level as f-TTX decreased during the maturation period (Fig. 4). The changes of both binding ratio and f-TTX were statistically significant (Tukey–Kramer post hoc test, $p < 0.05$) (Table 4).

3.3.2. Male

Seasonal changes in toxicity (MU/individual) of each tissue and in plasma TTX in males are shown in Fig. 5. Skin toxicity largely exceeded that of the other tissues, except for June, in which the liver toxicity was extremely high. As a whole, the tissue toxicities of males were significantly lower (Student's *t*-test, $p < 0.05$) than those of females; the level of liver, gonad, and skin toxicity was about 1/4, 1/400, and 1/1.5 that of the female specimens, respectively (Table 3).

The toxicity of each tissue again declined in April (Fig. 5). No other maturation-associated change, however, was observed, and there were some months in which liver toxicity increased during the maturation period (Fig. 5 and Table 2).

Plasma TTX (1.59–13.5 MU/ml) levels were almost the same between males and females, and fluctuated independently of the degree of maturation (Fig. 5 and Table 4). The binding ratio, however, showed a very similar fluctuation pattern to that in females; low during the ordinary period and high during the maturation period (Fig. 5 and Table 4).

4. Discussion

Seasonal changes in the GSI (Fig. 1) suggest that maturation of female *T. poecilonotus* inhabiting the Ariake Sea occurs during December-March and that of males occurs during November-March, and spawning occurs during March-April. The pufferfish *T. rubripes* that live in the Ariake Sea as their spawning ground also spawn from the second half of March to May at the entrance of the sea (Takita and Intong, 1991).

The toxicity of the Ariake specimens, both females and males of *T. poecilonotus*, was very high throughout the year, except that it sharply declined just after spawning in April (Figs. 2–5). In all tissues other than testis, toxicity in many individuals exceeded 1000 MU/g or 10,000 MU/individual. Compared with males, toxicity was generally higher in females, partly because testes, unlike ovaries, cannot actively accumulate TTX, and testis toxicity is much lower than that of ovary (Figs. 2–5, Table 2 and 3). Skin, liver, and ovary are strongly toxic (generally greater than 1000 MU/g), whereas muscle and testes are also weakly toxic in the *T. poecilonotus* specimens collected from the Pacific coast of the Tohoku Region, the Japan Sea, the Seto Inland Sea, and coastal waters of the Oita Prefecture (Kodama et al., 1984, Endo, 1984, Fuchi et al., 1999).

The seasonal profile of tissue toxicity was markedly different between females and males. In females, liver toxicity was high during the ordinary period, and that of ovary was high during the maturation period (Fig. 2 and Table 2). This finding suggests that 'turnover of toxins' occurs between the liver and ovary (Fig. 4 and Table 2). Skin toxicity also decreased slightly during maturation period

Table 3
Result of statistical analysis for each tissue toxicity test (independent of seasonal classification).

Sex	*n*	Toxicity					
		Liver		Ovary/testis		Skin	
		(MU/g)	(MU/individual)	(MU/g)	(MU/individua)	(MU/g)	(MU/individual)
♀	93	1410 ± 2290*	10,600 ± 19,700*	518 ± 454*	7080 ± 12,400*	809 ± 550*	16,100 ± 12,800*
♂	45	485 ± 1460*	2710 ± 6070*	16.1 ± 58.2*	18.0 ± 47.8*	585 ± 416*	11,100 ± 8300*

Data are shown as mean ± SD.
Asterisks indicate significant differences between the two measured values in each column (Student's *t*-test, $p < 0.05$).

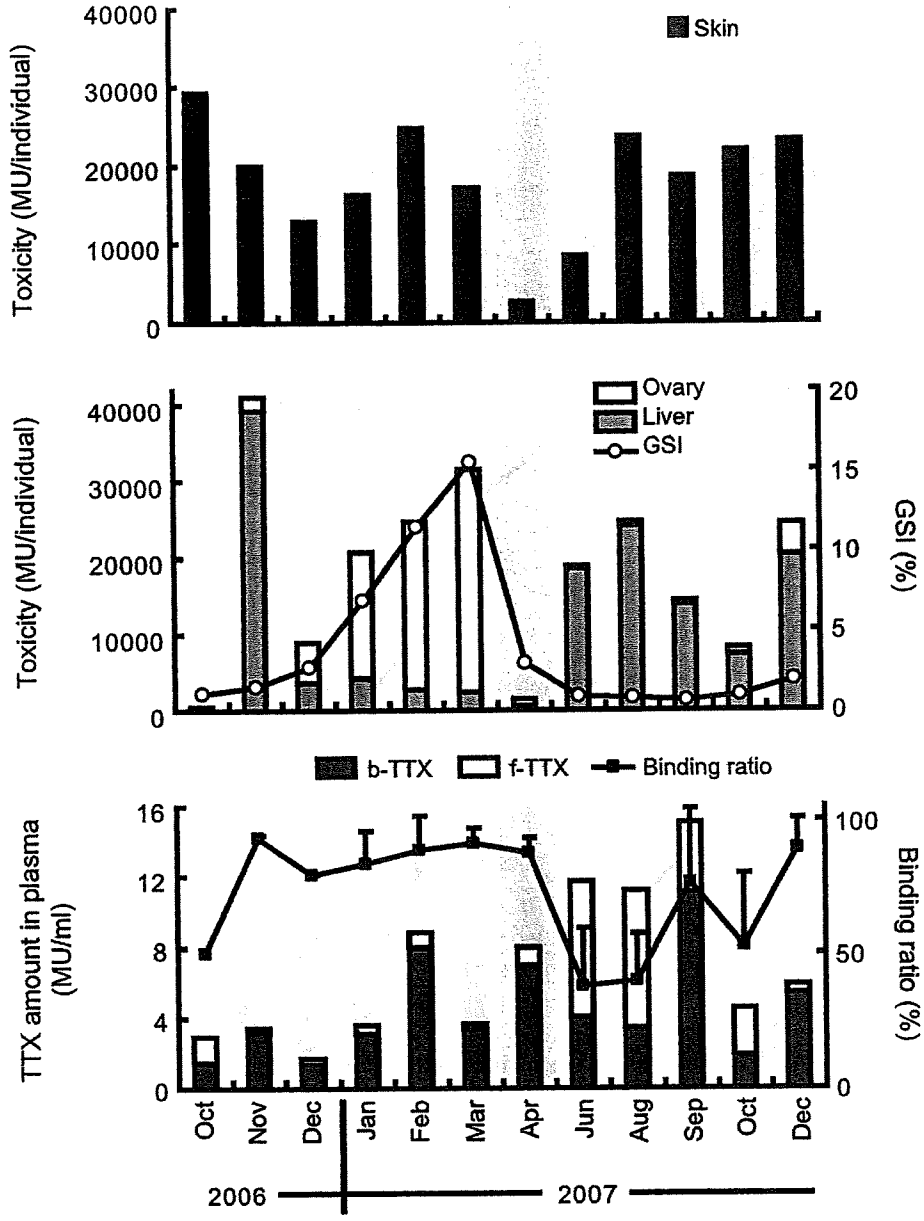

Fig. 4. Seasonal changes in the toxicity (MU/individual) of the skin (upper) and ovary/liver with GSI (middle), and in the TTX amount of blood plasma (lower) in the female specimens of *T. poecilonotus*. The sum of free TTX (f-TTX) and TTX binding to high molecular-weight substances (b-TTX) was considered as a total TTX amount in plasma (p-TTX), and the percentage of b-TTX in p-TTX was calculated as the binding ratio. Data are shown by mean of each month (column or symbol on sequential line). Error bars (SD) for data other than the binding ratio are omitted to avoid confusion.

Table 4
Result of statistical analysis for the amount of TTX in plasma and binding ratio.

Sex	Seasonal classification†	*n*	TTX amount			
			p-TTX (MU/ml)	f-TTX (MU/ml)	b-TTX (MU/ml)	Binding ratio (%)
♀	O	47	9.80 ± 10.45*	5.25 ± 6.29[a]	4.55 ± 5.90	52.2 ± 27.8[b]
	M	34	5.45 ± 5.71*	0.55 ± 0.65[b]	4.90 ± 5.34	87.7 ± 10.6[a]
♂	O	17	10.37 ± 6.85	6.19 ± 5.79[a]	4.18 ± 2.28	47.5 ± 26.1[b]
	M	23	7.78 ± 6.45	1.00 ± 1.28[b]	6.78 ± 6.21	84.0 ± 15.2[a]

Different alphabetical superscripts indicate significant differences among the measured values in each column (Tukey-Kramer post hoc test, $p < 0.05$).
Asterisks indicate significant differences between the two measured values in each column (Student's *t*-test, $p < 0.05$).
Data are shown as mean ± SD.
†O: ordinary period; M: maturation period.

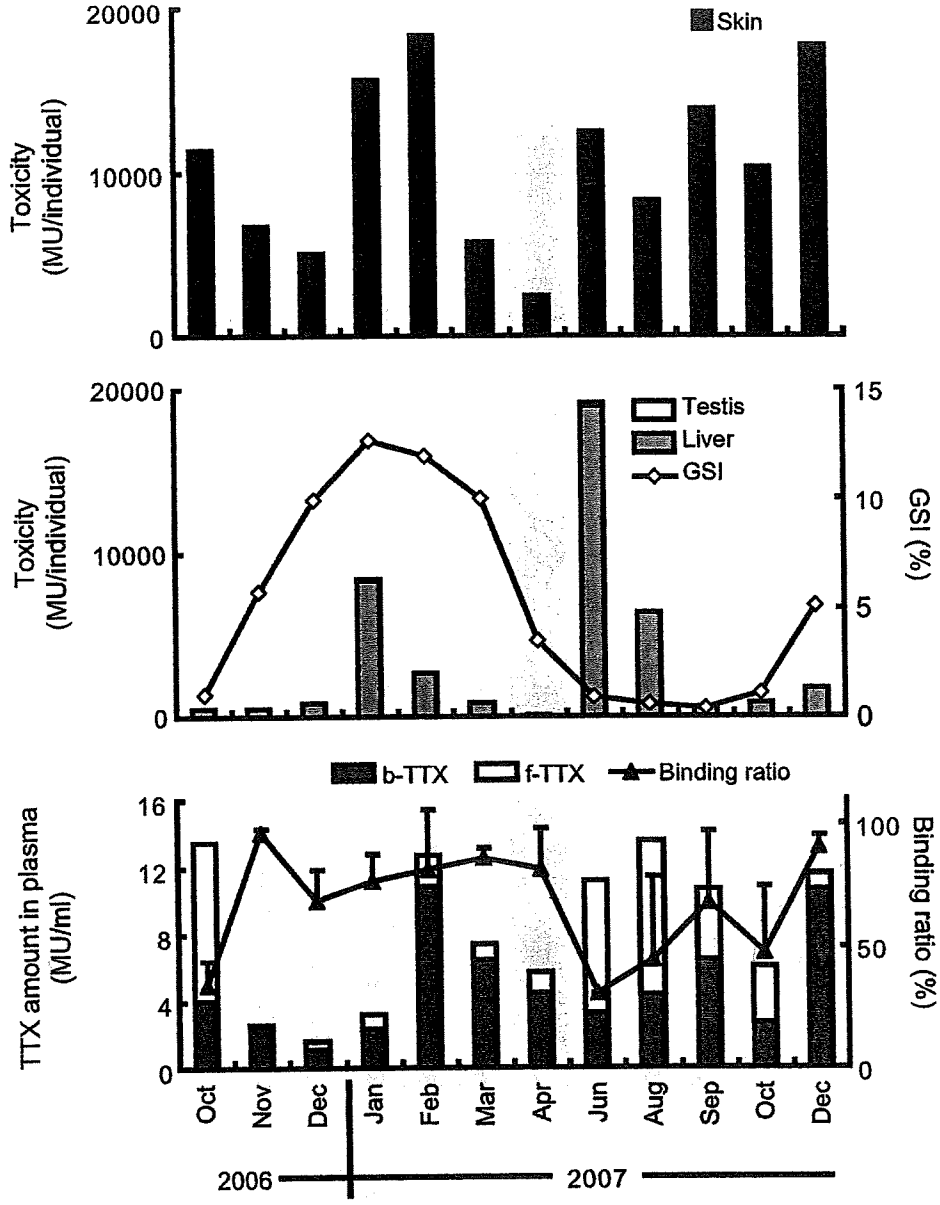

Fig. 5. Seasonal changes in the toxicity (MU/individual) of the skin (upper) and testis/liver with GSI (middle), and in the plasma TTX content in male specimens of *T. poecilonotus*. Refer to the caption of Fig. 4 for the meaning of b-TTX, f-TTX, and binding ratio. Data are shown by mean of each month (column or symbol on sequential line). Error bars (SD) for data other than the binding ratio are omitted to avoid confusion.

(Fig. 2 and Table 2). Therefore, it is presumed that the TTX absorbed from toxic food organisms into the pufferfish body is transferred mainly to the liver and skin during the ordinary period, but is actively transported and accumulated into the ovary during the maturation period. Matsumoto/Nagashima et al. demonstrated that the liver tissue of *T. rubripes* is equipped with a specific TTX-uptake mechanism (Nagashima *et al.*, 2003, Matsumoto et al., 2005, 2007), and using a pharmacokinetic model showed that TTX introduced into the pufferfish body is rapidly taken up into the liver via the blood (Matsumoto et al., 2008a, 2008b). We also found that TTX administered intramuscularly to non-toxic cultured specimens of *T. rubripes* was transferred first into the liver and then the skin via the blood (Ikeda *et al.*, 2009). A similar result was obtained in oral administration experiments (Kono *et al.*, 2008), suggesting that, under natural conditions as well, pufferfish take up most of the ingested TTX into the liver first. During the ordinary period, some of the TTX taken up into the liver is gradually transferred to the skin, where it accumulates in the basal cells and/or TTX-bearing secretory glands or cells (succiform cells) of the epithelia (Kodama et al., 1986, Tanu et al., 2002, Mahmud et al., 2003a, 2003b), and is excreted by external stimuli under certain circumstances (Kodama et al., 1985, Saito et al., 1985). During the maturation period, the toxin transfer to the skin decreases somewhat, and most of the TTX taken up into the liver would be transported to the ovary, presumably with the precursors of yolk proteins that are synthesized in the liver (Wallace, 1985, Specker and Sullivan, 1994). The majority of the toxin

kinetics after uptake into the liver, however, remains still unclear, and further detailed investigations, such as an approach using the model of Matsumoto et al. (2008a, 2008b) are needed to clarify this point.

Jang and Yotsu-Yamashita (2006) examined the distribution of TTX and its analogs among the tissues of *Takifugu* (*Fugu*) *pardalis*, and claimed that the ratio of 4,9-anhydroTTX and 4-Cysteinyl TTX to TTX in the liver was significantly higher than that of other tissues during the maturation period. Therefore, conversion of TTX into such almost non-toxic analogs might be another possible cause of decline in liver toxicity during the maturation period. To elucidate this point, investigations on the maturation-associated change in toxin profile of *T. poecilonotus* are now in progress.

In males, maturation-associated changes in the toxin distribution in the body were not clearly observed. Unlike ovaries, testes do not actively take up TTX. Therefore, even during the maturation period, as well as during the ordinary period, the TTX taken up into the liver is transferred mainly to the skin, and only a small portion to the testis.

In both females and males, the binding ratio of plasma TTX was low during the ordinary period, and high during the maturation period (Figs. 4, 5, and Table 4), suggesting that quantity, species, and/or activity of TTX-binding high molecular-weight substances are increased during the maturation period, which might be involved in the transportation of TTX from the liver to ovary. Alternatively, that b-TTX remained at a certain level irrespective of maturation, but free TTX decreased during the maturation period (Figs. 4, 5, and Table 4). In this view, the decreased portion of f-TTX is thought to correspond to the increased ovary toxicity. Although not conclusive, we lean toward the former possibility, because it is unlikely that most of f-TTX is specifically taken up only into the ovary, and because free TTX has nowhere to go in males during the maturation period in the latter hypothesis. TTX-binding proteins have been isolated from the blood plasma of marine pufferfish (Matsui et al., 2000; Yotsu-Yamashita et al., 2001), and may be involved in the transportation mechanism. The relationship between the binding ratio and these proteins or other high molecular-weight substances, especially those that appear with maturation remains to be elucidated. Further studies are in progress.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Toxins of Pufferfish That Cause Human Intoxications

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Abstract—Many marine pufferfish possess a potent neurotoxin, tetrodotoxin (TTX). In general, they have strong toxicity in the liver and ovary, leading to a frequent occurrence of human poisonings. TTX is originally produced by marine bacteria and distributes over a wide variety of aquatic animals. In pufferfish, TTX is derived from the food chain that consists of these TTX-bearing organisms (i.e., their prey). The transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via prey remain unclear. Recent studies have revealed that the liver of pufferfish has a specific TTX-uptake mechanism, and TTX introduced into the pufferfish body is first absorbed in the liver and then transferred to the skin through the circulatory system. This inter-tissue transfer and accumulation of TTX are greatly affected by the state of maturation. TTX-bearing organisms show extremely high resistance to TTX, and seem to possess TTX as a biological defense mechanism. Furthermore, TTX may involved in the control of information transmission in the central nervous system of pufferfish.

TTX poisonings due to small scavenging gastropods have so far occurred in Taiwan and China. Recently, one such gastropod, *Nassarius glans*, caused food poisoning incidents in Kyushu, Japan. *N. glans* is highly toxic, and possesses a large amount of TTX not only in the viscera but also in the muscle. After 1990, a total of 9 poisoning incidents due to ingestion of boxfish (pufferfish of the family Ostraciidae) occurred in southwestern Japan, involving 13 patients and 1 death. The symptoms are very similar to those of parrotfish poisoning (a unique variety of food poisoning that has sporadically occurred in Japan), suggesting that the causative substance is a palytoxin (PTX)-like toxin as in the parrotfish poisoning. Freshwater pufferfish and some marine pufferfish possess paralytic shellfish poison (PSP) instead of or in addition to TTX, and may cause 'paralytic shellfish poisoning by pufferfish'.

The toxins of the above mentioned fish and shellfish are all exogenous, and their toxicity may be greatly affected by a change in the marine environment, such as elevations in water temperature due to global worming. We need to enhance the information/collaboration network among East Asian countries and vigilantly monitor how our changing climate is affecting the toxicity and distributions of these organisms.

Keywords: tetrodotoxin, paralytic shellfish poison, palytoxin, pufferfish, gastropod, *Nassarius glans*, boxfish, *Ostracion immaculatus*

1. INTRODUCTION

Many Japanese know that pufferfish possess a fatal toxin. Nevertheless, they have a historical preference for eating pufferfish, and established unique food culture associated with this organism. However, as food poisonings due to ingestion of pufferfish were occurring very frequently, the Japanese Ministry of Health and Welfare (presently the Ministry of Health, Labour, and Welfare) published a guideline for edible pufferfish in 1983, with updates in 1993 and 1995 (Noguchi and Ebesu, 2001; Noguchi and Arakawa, 2008). Since then, accidents in specialist restaurants have been almost eliminated, but many cases of pufferfish poisoning continue to occur every year due to the consumption of home-made dishes with toxic portions, such as liver and ovary, which are prepared using wild fish that are caught recreationally. On the other hand, food poisonings due to small gastropods that possess the same toxin as pufferfish have occurred very frequently in Taiwan and China, where consumption of pufferfish is completely prohibited (Hwang and Noguchi, 2007; Hwang *et al.*, 2007). In the present paper, we review the property of 'pufferfish toxin' tetrodotoxin (TTX), species and toxic portions of TTX-bearing organisms, the accumulation mechanism and physiological function of TTX in pufferfish, and cases of human poisoning due to pufferfish. We also provide an introduction to TTX poisonings caused by marine organisms other than pufferfish, which are presently posing a food hygiene issue in Japan, and pufferfish poisonings due to toxins other than TTX.

2. PROPERTY OF TETRODOTOXIN (TTX)

Tetrodotoxin, a pufferfish toxin named after its order name Tetraodontiformes, is a potent neurotoxin of low molecular weight, whose unique structure (Fig. 1) was determined by three groups in 1964 (Tsuda et al., 1964; Woodward, 1964; Goto et al., 1965). Various TTX derivatives have so far been separated from pufferfish, newts, frogs, and other TTX-bearing organisms (Yotsu-Yamashita, 2001). High-purity TTX is insoluble not only in all sorts of organic solvents but also in water, though it becomes soluble in water when an acid is added. The toxin is stable in neutral to weakly acidic solutions and does not decompose by cooking (i.e., the application of heat). TTX inhibits the conduction of action potential by selectively plugging sodium channels on the nerve/muscle membrane at extremely low concentrations (Narahashi, 2001). The lethal potency is 5000 to 6000 MU/mg [1 MU (mouse unit) is defined as the amount of toxin required to kill a 20-g male mouse within 30 min after intraperitoneal administration], and the minimum lethal dose (MLD) for humans is estimated to be approximately 10000 MU (≈2 mg) (Noguchi and Ebesu, 2001).

The main symptoms of human intoxication include numbness of lips, tongue and the limbs, paresthesia, dysarthria, respiratory distress; death can occur due to respiratory failure in most critical cases (Noguchi and Ebesu, 2001). When a poisoning occurs, it is essential to transport the patient immediately to a well-equipped hospital. At present, there is no antidote or specific medication for TTX, and no fundamental treatment besides facilitating elimination of the toxin from the body,

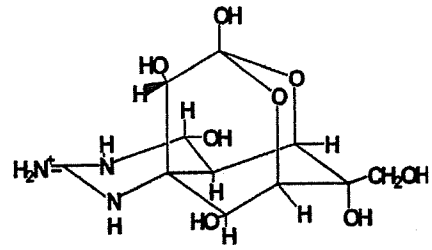

Fig. 1. Chemical structure of tetrodotoxin (TTX). Reprinted from *Marine Drugs* **6**, Noguchi and Arakawa, Tetrodotoxin—distribution and accumulation in aquatic organisms, and cases of human intoxication, 220–242, 2008, Fig. 1.

and managing the respiratory/circulatory system properly using an artificial respirator. Although a monoclonal anti-TTX antibody has recently been developed (Kawatsu et al., 1997) and utilized as a chemical tool for research, it has little effect in clinical use.

3. DISTRIBUTION OF TTX IN AQUATIC ORGANISMS

Among the marine pufferfish inhabiting coastal waters of Japan, the following 22 species are listed as toxic; "kusafugu" *Takifugu niphobles*, "komonfugu" *T. poecilonotus*, "higanfugu" *T. pardalis*, "shosaifugu" *T. snyderi*, "mafugu" *T. porphyreus*, "karasu" *T. chinensis*, "mefugu" *T. obscurus*, "mushifugu" *T. exascurus*, "nameradamashi" *T. pseudommus*, "akamefugu" *T. chrysops*, "nashifugu" *T. vermicularis*, "torafugu" *T. rubripes*, "shimafugu" *T. xanthopterus*, "gomafugu" *T. stictonotus*, "shiroamifugu" *Tetraodon alboreticulatus*, "senninfugu" *Pleuranacanthus sceleratus*, "okinawafugu" *Chelonodon patoca*, "hoshifugu" *Arothron firmamentum*, "kitamakura" *Canthigaster rivulata*, "dokusabafugu" *Lagocephalus lunaris*, "kanafugu" *L. inermis*, "sansaifugu" *Takifugu flavidus* (Noguchi and Arakawa, 2008). All belong to the Tetraodontidae family, and pufferfish of Diodontidae and Ostraciidae possess no TTX at all. The toxic parts are different depending on species, which can be categorized as (1) muscle, testis and skin that are non-toxic (less than 10 MU/g) and edible; *T. rubripes*, *T. xanthopterus*, "shirosabafugu" *Logocephalus wheeleri*, etc., (2) skin is toxic, but muscle and testis are edible; *T. snyderi*, *T. porphyreus*, *T. vermicularis*, etc., (3) testis is also toxic, and only muscle is edible; *T. niphobles*, *T. poecilonotus*, *T. pardalis*, etc. In general, viscera, especially the liver and ovary are highly toxic (the toxicity often exceeds 1,000 MU/g), and the Japanese Ministry of Health, Labour, and Welfare has prohibited these organs from being used for food from all species of pufferfish.

The toxicity of Taiwanese marine pufferfish was extensively studied by one of the present authors (Hwang et al., 1992a). Among the 23 species examined, only two species, "kurosabafugu" *Logocephalus gloveri* and *L. wheeleri*, which are likely used as the ingredients for producing dried dressed fish fillets, were non-toxic in all tissues, whereas *L. lunaris*, "takifugu" *Takifugu oblongus*, and *T. nihpbles* that occasionally

cause food poisonings in Taiwan were highly toxic. *L. lunaris* from Thailand and Cambodia (Brillantes et al., 2003; Ngy et al., 2008a), and *T. oblongus* from Bangladesh and Cambodia (Mahmud et al., 1999c; Ngy et al., 2009) are also highly toxic, and considered as a potential causative species of pufferfish poisonings in these countries.

Small pufferfish inhabiting brackish water (Mahmud et al., 1999a, b) or freshwater (Kungsuwan et al., 1997; Sato et al., 1997; Ngy et al., 2008b) in Southeast Asia are also toxic. Toxicity of the skin is usually higher than that of the viscera in these pufferfish. The toxin of brackish water species was identified as TTX (Mahmud et al., 1999a, b), but in the freshwater species, saxitoxins (STXs), toxins that belong to the paralytic shellfish poison (PSP) family (Deeds et al., 2008), were detected as the main toxic principles (Kungsuwan et al., 1997; Sato et al., 1997; Ngy et al., 2008b). In general, pufferfish shows large individual, regional, and seasonal variations in toxicity, and a fish of highly toxic species is not necessarily toxic. Thus the general public are often unaware of the danger, which has contributed to the frequent occurrence of pufferfish poisoning.

TTX was long believed to be present only in pufferfish. Since Mosher et al. (1965) identified a toxin from the eggs of the California newt *Taricha torosa* as TTX, however, TTX has been detected in a wide variety of animals, for example the goby *Yongeichthys criniger*, atelopid frogs, the blue-ringed octopus *Hapalochlaena maculosa*, the carnivorous gastropod *Charonia sauliae*, starfish of genus *Astropecten*, xanthid crabs, the horseshoe crab *Carcinoscorpius rotundicauda*, flatworms, and ribbon worms (Miyazawa and Noguchi, 2001; Hwang and Noguchi, 2007; Noguchi and Arakawa 2008). It is quite unlikely that these TTX-bearing organisms that belong to particular species in different phyla possess a common gene that codes for TTX production. Since the trumpet shell *C. sauliae* was found to accumulate TTX by ingesting toxic starfish (Noguchi et al., 1982), the TTX of pufferfish has also been considered to be not endogenous, but to come from toxic food organisms via the food chain. In the 1980s, several studies were carried out to seek the primary origin of TTX in the food chain, and TTX productivity was found in certain species of marine bacteria including *Vibrio alginolyticus*, *Shewanella alga*, and *Alteromonas tetraodonis* that had been isolated from TTX-bearing organisms such as pufferfish, toxic starfish, the xanthid crab *Atergatis floridus*, and the red alga *Jania* sp. (Noguchi et al., 1986; Yasumoto et al., 1986; Narita et al., 1987; Simidu et al., 1987; Hashimoto et al., 1990).

4. ACCUMULATION OF TTX IN PUFFERFISH

Many years of studies on TTX have revealed that (1) pufferfish toxicity shows remarkable individual and regional variations, (2) TTX is distributed over various organisms, including food animals of pufferfish, (3) the trumpet shell accumulates TTX by ingesting toxic starfish, (4) marine bacteria primarily produce TTX, (5) pufferfish become non-toxic when they are fed TTX-free diets in an environment in which the invasion of TTX-bearing organisms is completely prevented (Matsui et al., 1982; Saito et al., 1984; Noguchi et al., 2004, 2006b) (Fig. 2), and (6) such non-toxic pufferfish efficiently accumulate TTX when orally administered TTX (Matsui et al., 1981; Yamamori et al., 2004; Honda et al., 2005a; Noguchi et al., 2006a; Kono