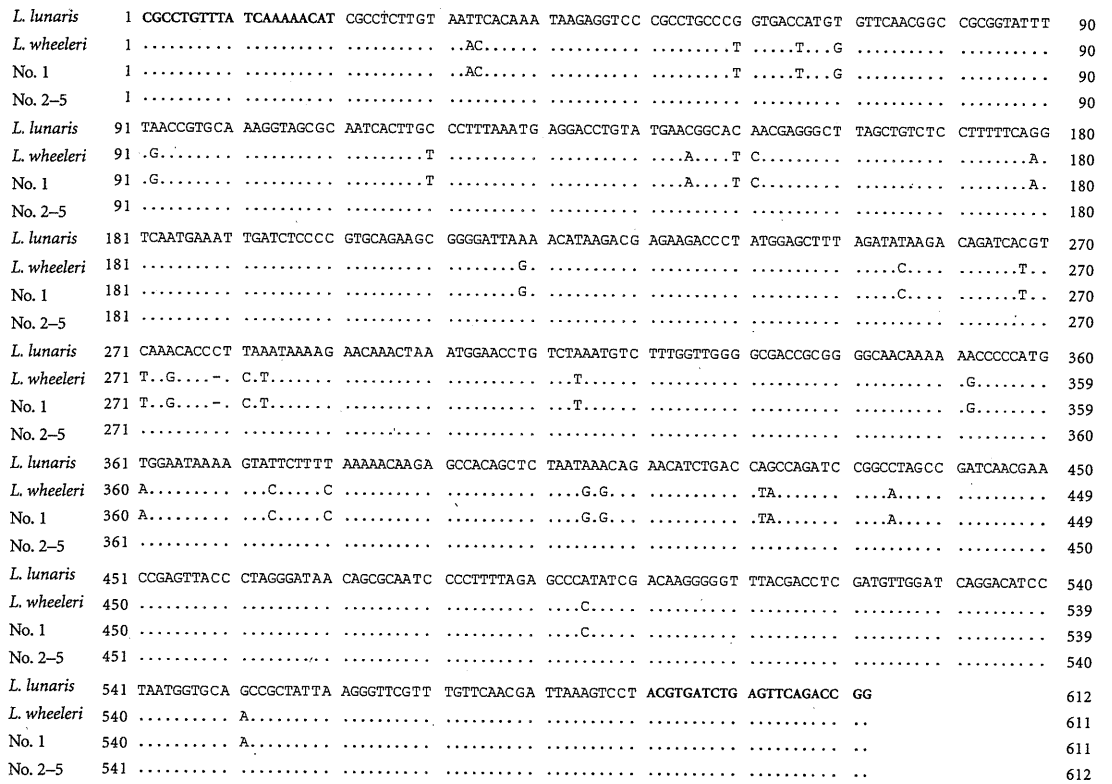

FIGURE 3: Aligned DNA sequences of the amplified 16S rRNA region of mitochondrial DNA from puffer fish. The positions of the primers 16SarL and 16SbrH used for PCR amplification and sequencing are indicated in bold typeface. A dot (.) indicates identity with *L. lunaris* sequence. A gap introduced into the sequences to optimize the alignment is represented by a dash (-).

muscle and liver as strongly toxic, and skin and intestine as weakly toxic. It should be noted that two muscle samples (nos. 2 and 4) of five tested showed the strongly toxic level exceeding 100 MU/g, comparing to those in the East China Sea (261 MU/g), Taiwan (140 MU/g), and Thailand (243 MU/g) [8, 10, 11]. These results indicate that the green toadfish obtained in the Kyushu coast was also enough toxic to cause puffer fish poisoning by ingesting muscle and that it is hazardous for human consumption as well as that in tropical and subtropical waters. Another concern is whether the toxic green toadfish accidentally migrates to coastal regions of Japan or inhabits them throughout the year.

Figure 2 illustrates LC/ESI-MS of the muscle extract of sample no. 2. The mass chromatograms were scanned at m/z 320 for TTX ($C_{11}H_{17}O_8N_3$, 319.27 Da), m/z 304 for deoxyTTX ($C_{11}H_{17}O_7N_3$, 303.27 Da), m/z 302 for anhydroTTX ($C_{11}H_{15}O_7N_3$, 301.26 Da), and m/z 290 for norTTX ($C_{10}H_{15}O_7N_3$, 289.25 Da). In the selected ion mass chromatogram at m/z 320, the peak at a retention time of 7.98 min was consistent with that of TTX standard at a retention time of 8.01 min (Figure 2, the top). The peaks at a retention time of 9.72 min at m/z 304 and that of 9.90 min at m/z 302 were estimated to be deoxyTTX and anhydroTTX; respectively, although they were not identified in detail because of lack of the standard of TTX analogues. Total ion current mass chromatogram demonstrated that TTX was a major toxic principle in the muscle extract (Figure 2, the bottom). The liver extract of sample no. 2 showed the same toxin profile as that of the muscle extract (data not shown). Toxin amounts of the sample extracts estimated by LC-ESI/MS were well related to toxicity scores assessed by bioassay. Brillantes et al. detected TTX along with 4-epi-TTX and 4,9-anhydroTTX in muscle and liver extracts from *L. lunaris* in Thailand by HPLC analysis [11]. Moreover, Ngy et al. determined TTX as the major toxin and anhydroTTX as the minor in the Cambodian *L. lunaris* by LC/ESI-MS [12]. In addition, they reported no detectable paralytic shellfish toxins in *L. lunaris* specimens from Thailand and Cambodia. It is likely that the green toadfish *L. lunaris* preferably accumulates TTX and its analogues.

Species identification of the puffer fish specimens was carried out by a direct DNA sequencing analysis according to the method of Ishizaki et al. [17]. Total DNA was isolated from the muscle of green toadfish specimens used for toxicity test and subjected to mitochondrial 16S rRNA gene specific PCR using 16SarL and 16SbrH primers. Figure 3 shows aligned DNA sequences of the amplified partial 16S rRNA region from the samples, along with the authentic DNA sequences of green toadfish *L. lunaris* and brown-backed toadfish *L. wheeleri*. The PCR products of *L. lunaris* and *L. wheeleri* had a length of 612 bp and 611 bp, respectively. The latter deleted a nucleotide at a site of 279th in

L. lunaris sequence and had thirty substitutions in nucleotide sequences of *L. lunaris*.

The partial sequences of the PCR products from four samples (nos. 2–5) were identical with that of *L. lunaris*. However, the sequence of sample no. 1 with no toxicity except for ovary did not agree with that of *L. lunaris*, but *L. wheeleri*, nontoxic species, indicating that sample no. 1 was not regarded as green toadfish by molecular identification, despite broad extension of spines in the back. It is possible that sample no. 1 could be a hybrid of *L. wheeleri* and other, since natural hybrids within the genus of puffer fish have been frequently found [18, 19]. Further study is needed to identify the species in detail by microsatellite DNA analysis and by morphological characterization.

Food poisoning from a dried dressed fish fillet and an adulterated dried mullet roe occurred in Taiwan. Hwang and coworkers identified the causative fish as *L. lunaris* by nucleotide sequence analysis and PCR-restriction fragment length polymorphism using the sequence of 376-nucleotide region in cytochrome *b* gene of mitochondrial DNA [1, 3]. In this study, we analyzed a 16S rRNA gene fragment of mitochondrial DNA and confirmed that 16S rRNA markers are useful and applicable to identify puffer fish species.

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の活動を測定すると，涙を提示された被験者では，性的な情動に関わるとされる視床下部や紡錘状回において神経活動が低下することが明らかとなった．以上の結果より，女性が悲しいときに流す涙は男性の性的な情動を抑制することが示された．

筆者らの研究は，オスマウスの涙に含まれるESP1の生理作用機構を解析することで，哺乳類の性フェロモンの分子実体と作用機構を世界で初めて明らかにした（図1-A）．また，Gelsteinらの研究によって，ヒトの涙にも化学シグナルとして機能するフェロモン様物質が含まれることが示唆された．さらに，両者の研究を比較すると，涙に含まれる化学シグナルは，マウスにおいてもヒトにおいても，脳の視床下部領域に作用し，性行動に対する情動を調節するという興味深い結果が得られた（図1-B）．今後，マウスにおけるESP1の作用機構の知見をもとに，これまでほぼ未解明であったヒトにおけるフェロモン様化学シグナルの分子実体ならびに作用機構が明らかにされることが期待される．

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テトロドトキシンの薬物動態解析
トラフグ肝臓の毒化メカニズム

俗に「ふぐは喰いたし命は惜しし」といわれるほどフグは魅力的な魚である．食の安全・安心が求められる今日，命がけでフグを食べることはないが，毎年フグ中毒で死者が出ているのも事実である．フグの毒化については本誌でもときどき取り上げられているが，今なお不明な部分が多い．本稿では，フグの毒化機構解明の新しいアプローチとして，トラフグをモデルとしたフグ毒テトロドトキシン (tetrodotoxin, TTX) の薬物動態解析を紹介する．

その前に，フグの毒化に関するポイントを整理しておきたい．TTXはフグ以外の魚類をはじめ両生類のイモリとカエル，棘皮動物のヒトデ，節足動物のカニとカブトガニ，軟体動物のタコや巻貝，さらに扁形動物，紐形動物，環形動物などいろいろな動物から検出され，1980年代後半に海藻やフグなどTTX保有動物から単離した細菌にTTXが見いだされた．この頃フグの養殖技術が確立され，人工ふ化させて毒のない餌で飼育したトラフグやクサフグから毒性は認められず，フグ類の毒化経路は微生物を毒の起源とする生物濃縮を伴う食物連鎖が有力となった[1]．また，トラフグやクサフグはTTXをもたない魚類に比べて，TTX投与に対する耐性が数百倍も高いが，それでもある量以上の投与で死亡する．

TTXがフグに対して外来性異物であるなら，フグはどのような仕組みでTTXを体内に取り込み，特定の部位（特に肝臓）に蓄積するのだろうか？　筆者らは，TTXの蓄積メカニズムに，人体における薬剤の挙動や体内分布を速度論的に評価する薬物動態解析の概念を導入し，トラフグが経口的に摂取したTTXを肝臓に蓄積するまでを4つのステップ，①消化管における吸収，②血液循環による体内分布，③毒化部位への取り込み，④体外への排泄，に整理した．そして，これを検証するため，麻酔下の養殖トラフグにTTX溶液を投与して同一個体から経時的に採血する *in vivo* 実験系を構築した．

まず，トラフグが死亡しない程度のTTX（0.25～1.00 mg/kg体重）を消化管内に直接投与すると，血中TTX濃度は典型的な一次吸収曲線を示し，TTXはトラフグの消化管から速やかに吸収されて循環血液中に移行することが初めて確認された[2]．次に，TTXの体内動態（組織分布の経時変化）を調べるため，肝静脈内にTTXを投与して血液および各組織中のTTX濃度を経時的に測定したところ，血中TTX濃度は静脈投与直後に最大値を示してその後漸減し，腎臓のTTX濃度は血中濃度に比例して低下した[3]．興味深いことに，血液から直接TTXが分布すると考えられる筋肉と皮膚ではTTX濃度に有意な上昇は認められず，TTX濃度は両組織ともに低いレベルで推移した．一方，肝臓のTTX濃度は血

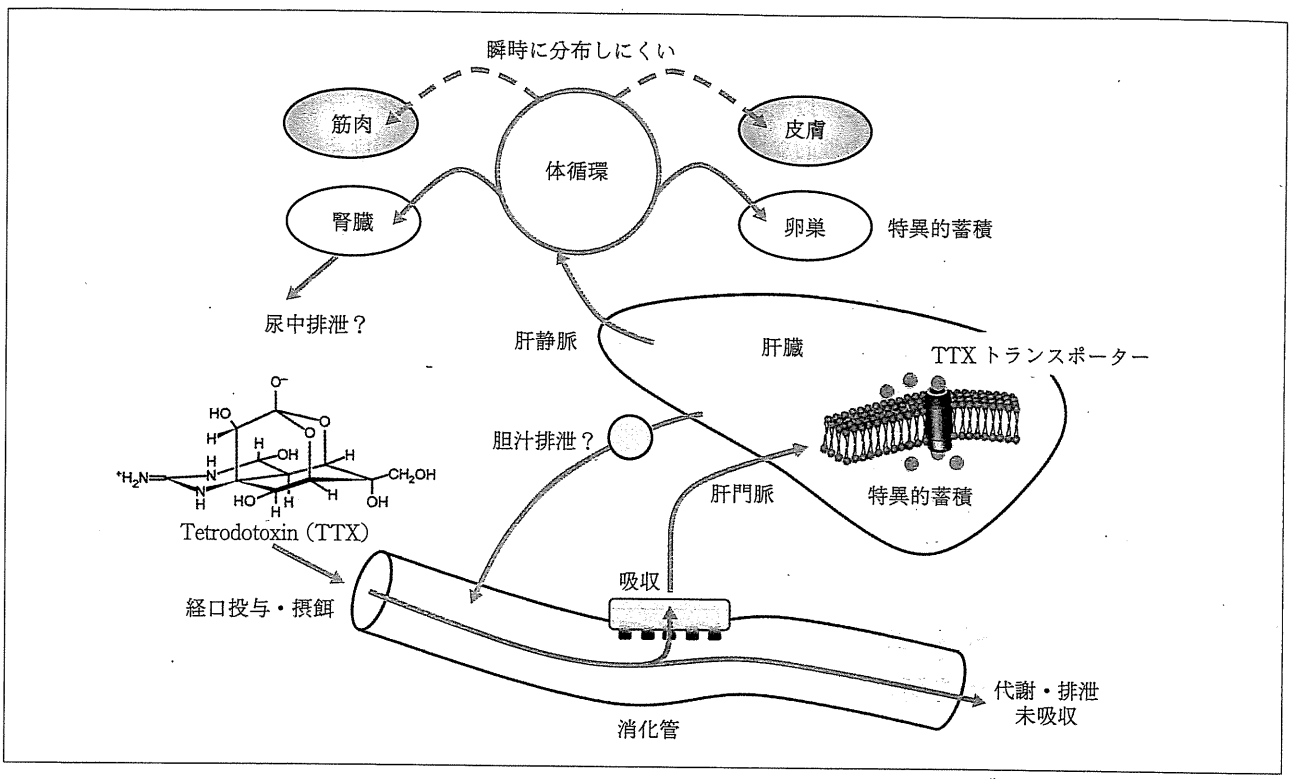

図1■トラフグにおけるテトロドトキシン体内動態の予想図

中濃度の低下に反して経時的に増加し，投与60分後にはTTX投与量の6割以上が肝臓に蓄積した．これらの結果をコンパートメントモデル（薬物濃度の推移が同じ挙動を示す組織を1つの区画“コンパートメント”として扱う）に当てはめると，TTXの分布は，①血中濃度と比例して同じ挙動を示す中央コンパートメント（腎臓），②血中からTTXを取り込んで濃縮する末梢コンパートメント（肝臓），③血中TTX濃度と瞬間的な分布平衡が成立しない末梢コンパートメント（筋肉，皮膚）の3つに分類できる（図1）．

トラフグの肝臓はTTXを能動的に取り込むことが確かめられたので，血液中から肝臓への物質の移行効率を評価する肝臓取り込みクリアランスを調べた[3]．クリアランスは血液から単位時間あたりに消失する物質の量を血液体積として表現したもので，肝臓での取り込みが物質の消失に関わる場合は「肝臓取り込みクリアランス」という．結論だけ述べると，血液から肝臓への取り込み効率を表わす肝抽出率（肝門脈の血液流量に対する肝臓取り込みクリアランスの割合）は9%と小さく，トラフグは血液から肝臓への取り込み効率が低いという意外な結果になった．これは，TTXが肝細胞膜を自由に透過しているわけではなく，輸送担体（トランスポーター）を介した分子選択的な膜透過機構によって肝臓内に取り込まれることを示唆している．この点については，肝組織切片を使った *in vitro* 培養実験の結果も支持している[4]．トラフグ肝組織切片のTTX取り込み速度は，インキュベーション溶液中のTTX濃度の増加に伴い上昇し，担体輸送の特徴であるMichaelis-Menten kinetics様の飽和性が確認された．さらに，TTX取り込み速度は溶液中の Na^+ をコリンに置換した場合では置換前の6割に，インキュベーション温度を20℃から5℃にすると4割に低下した．以上のことから，トラフグ肝臓にはTTXを取り込む Na^+ 依存性のトランスポーターの関与が示唆された．TTXの代わりに麻痺性貝毒サキシトキシン（saxitoxin, STX）群を用いてインキュベートした場合，トラフグ肝組織切片のSTX群蓄積量はTTXの1/10程度であったことから，トランスポーターは少なくともTTXと麻痺性貝毒を区別しており，トラフグ肝臓とTTXの間には特別な関係があるようだ．モデル実験では，性成熟による影響を排除するため生殖巣が発

達していない時期のトラフグを用いたが，卵巣も高濃度にTTXを蓄積するので，能動的なTTX取り込み機構が働いているのだろうか．

別の研究グループの実験によると，トラフグの幼魚に対するTTXの筋肉内投与試験では，投与したTTXが肝臓ではなく皮膚へ移行することが報告されており[5]，トラフグ肝臓の毒蓄積能力は成長とともに獲得されると推測される．TTXトランスポーターはいつ，どのような刺激で発現するのか興味がもたれる．ヒガンフグなどトラフグ属魚類の血液中にはSTXおよびTTXと結合するタンパク質 (puffer fish STX and TTX binding protein ; PSTBP) が存在することも報告されており，TTXの体内動態への関与が疑われている[6]．しかし，TTXはウシ血清アルブミンやウシ酸性糖タンパク質と非特異的に結合するため[7]，フグ類におけるTTXの体内動態にはトランスポーターや輸送タンパク質などが協調して機能し，有毒部位への輸送や蓄積に関与しているものと予測される．これらの機能が解明され，フグはなぜ毒をもつのかという永年の疑問が解決されることを期待したい．

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プロフィル

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Ⅲ-1. フグ毒の体内動態

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III-1. Pharmacokinetics of tetrodotoxin in tiger puffer fish *Takifugu rubripes*

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はじめに

フグの主な毒化経路は，微生物を毒の起源とする生物濃縮を伴う食物連鎖によると考えられている。給餌飼育実験において，フグ毒テトロドトキシン（TTX）をもたない養殖トラフグとクサフグにTTX含有餌料を投与すると，TTXは肝臓などに蓄積した。すなわち，フグは経口的に摂取したTTXを腸管で吸収し，血液による運搬を経て，肝臓など特定の組織に蓄積するという一連の体内動態が成立していることになる。そこで，これを検証するため，麻酔下の養殖トラフグにTTX溶液を単回投与して，同一個体から経時的に採血する*in vivo*実験モデルを構築し，TTXの体内動態を薬物動態学的に評価した。

1. TTX単回投与後のトラフグにおけるTTX体内動態解析

トラフグの消化管にTTX溶液（0.25 mg TTX/kg体重）を単回投与すると，投与3分後には血中からTTXが検出され，投与30分後に血中TTX濃度は最大（0.46±0.10 ng TTX/μL）となり，その後漸減した（図1）。TTX投与量0.50および1.0 mg TTX/kg体重でも同様の吸収曲線を示したが，最大血中濃度は投与150分後に遅延した（図1）。各TTX投与時の血中TTX濃度曲線下面積（AUC）と肝静脈投与時のAUCから，TTXの経口投与バイオアベイラビリティーはそれぞれ62, 84, 42%と見積もられ，TTX投与量0.50 mg/kg体重以上で消化管におけるTTX吸収に飽和が示唆された。また，腸管内に投与されたTTXの過半は投与300分後に肝臓から検出された。これらの結果から，TTXは消化管で吸収され，血液で運搬され，比較的短時間で肝臓に移行することが定量的に評価された。

次に，TTX溶液（0.25 mg TTX/kg体重）を肝静脈内に単回投与し，組織中TTX濃度を測定した。血中TTX濃度は経時的に減少し，腎臓および脾臓中TTX濃度は血中TTX濃度に平行して低下した。筋肉および皮膚では60分間の観察中，TTX濃度に有意な増減は認められず，両組織ともに低いレベルで推移した。一方，肝臓中TTX濃度は，血中TTX濃度の減少に反して経時的に増加する傾向を示し，投与60分後の肝臓TTX量は投与量の63±5%を示した。この結果から，TTXの体内動態に関してトラフグの組織を3つのコンパートメントに分けることができる。①血中濃度と同じ挙動を示す体循環コンパートメント（腎臓，脾臓），②血中からTTXを濃縮して蓄積する抹消コンパートメント（肝臓），③血中TTX濃度と瞬間的な分布平衡が成立しない抹消コンパートメント（筋肉，皮）である。

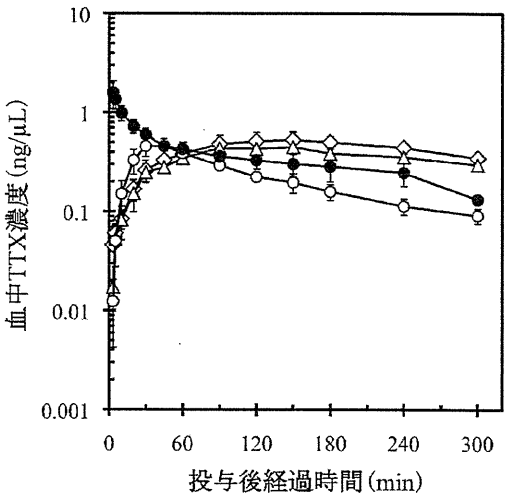

図1　トラフグ消化管投与後の血中TTX濃度変化
○；消化管投与0.25 mg TTX/kg体重，△；消化管投与0.50 mg TTX/kg体重，◇；消化管投与1.0 mg TTX/kg体重，●；肝静脈投与0.25 mg TTX/kg体重（n=3，平均値±標準誤差）

2. トラフグ肝臓におけるTTX取込みクリアランス

トラフグはTTXを比較的短時間で肝臓へ優先的に蓄積されることが確認されたので，組織への薬物移行効率評価に有効なパラメーターであるTTXの肝臓取込みクリアランス（CL_{uptake}）を測定した。積分プロットの傾きから，CL_{uptake}は3.1 mL/min/kg体重と見積もられた。超音波血流計で測定したトラフグ肝門脈の血液流量は36.0±0.9 mL/min/体重だったので，CL_{uptake}は血液流量の9%に相当し，血液流量と肝臓取込みクリアランスCL_{uptake}の比で表されるTTXの肝抽出率は著しく低いという予想外の結果を得た。このことから，トラフグ肝臓におけるTTXの取込み過程は，血液流量には依存せず（血流律速でない），肝臓の細胞膜透過過程が律速であることが示唆された。

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Transfer profile of intramuscularly administered tetrodotoxin to artificial hybrid specimens of pufferfish, *Takifugu rubripes* and *Takifugu niphobles*

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ABSTRACT

Tetrodotoxin (TTX) was intramuscularly administered to artificially hybridized specimens of the pufferfish *Takifugu rubripes* and *Takifugu niphobles* to investigate toxin accumulation in hybrids, and TTX transfer/accumulation profiles in the pufferfish body. In the test fish administered 146 MU TTX in physiologic saline, TTX rapidly transferred from the muscle via the blood to other organs. Toxin transfer to the ovary rapidly increased to 53.5 MU/g tissue at the end of the 72-h test period. The TTX content in the liver and skin was, at most, around 4–6 MU/g tissue, and in the testis it was less than 0.01 MU/g tissue. On the other hand, based on the total amount of toxin per individual (% of the administered toxin), the skin and the liver contained higher amounts (20–54% and 2–24%, respectively), but the amount in the liver rapidly decreased after 8–12 h, and fell below the level in the ovary after 48 h. These findings suggest that part of the TTX is first taken up in the liver and then transferred/accumulated in the skin in male specimens and in the ovary in female specimens.

1. Introduction

Marine pufferfish of the genus *Takifugu* possess a potent neurotoxin, tetrodotoxin (TTX). Although TTX is exogenous in pufferfish and is derived from the food chain (Noguchi and Arakawa, 2008), the transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. TTX administered intramuscularly to non-toxic cultured specimens of *Takifugu rubripes* is first transferred to the liver and then to the skin via the blood (Ikeda et al., 2009). Kono et al. (2008b) conducted a similar toxin administration experiment using cultured *Takifugu niphobles* specimens. Unlike general non-toxic fish, the liver tissue of *T. rubripes* has a specific TTX-uptake mechanism (Nagashima et al., 2003; Matsumoto et al., 2005, 2007). TTX introduced into the pufferfish body is rapidly taken up into the liver via the blood (Matsumoto et al., 2008a, 2008b).

In wild pufferfish, 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. We recently investigated seasonal changes in tissue toxicity, as well as the amount and forms of TTX in the blood plasma using wild specimens of the pufferfish *Takifugu poecilonotus*, and demonstrated that maturation greatly affects the inter-tissue transfer and/or accumulation of TTX via the bloodstream in nature (Ikeda et al., 2010). In the above-mentioned toxin administration experiments or the pharmacokinetic studies, however, juveniles or non-mature

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young fish were used, and the influence of aging or maturation was not considered.

Among *Takifugu* pufferfish, natural hybrids occasionally appear. Although some morphologic or genetic studies of these hybrids have been conducted (Masuda et al., 1991; Yokogawa and Urayama, 2000), little information is available on their toxicity. In our studies, to clarify the toxicity, toxin accumulation ability, and the inherited characteristics of hybrid pufferfish, as well as the transfer/accumulation/elimination mechanisms of TTX in the pufferfish body, we administered TTX intramuscularly to artificially hybridized *T. rubripes* and *T. niphobles* offspring, and investigated the toxin transfer/accumulation profile of the hybrid fish. In a previous toxin administration experiment (Ikeda et al., 2009), we used young and small *T. rubripes* specimens, which are relatively easy to rear and handle, and are most suitable for this type of experiment. The maturation of *T. rubripes*, however, is very slow, and the sexual differences in the toxin transfer/accumulation profile could not be clarified with young specimens. In the present study, we attempted to elucidate this point using the hybrid specimens produced by crossbreeding *T. rubripes* with *T. niphobles*, which matures earlier than *T. rubripes*.

2. Materials and methods

2.1. Pufferfish specimens

A female specimen of 'torafugu' *T. rubripes* (3 years old; body weight, 2010 g) that had been cultured in Nagasaki Prefectural Institute of Fisheries, and a wild male specimen of 'kusafugu' *T. niphobles* (unknown age; body weight, 58 g) collected from Omura Bay, Nagasaki Prefecture, were used as the parent fish. After long-day treatment (14L10D), the female fish was intramuscularly administered a luteinizing hormone-releasing hormone analog (400 μg/kg body weight), and ovulated eggs were artificially fertilized by the dry method (Takushima et al., 2003) with sperm obtained from the male fish. Larvae that hatched from the fertilized eggs were reared in the institute for approximately 10 months with rotifers, brine shrimp, or an artificial diet depending on the growth stage. Thirty-one specimens (body weight, 71.5 ± 15.1 g; body length, 12.7 ± 0.6 cm) of the artificial hybrid pufferfish were obtained (designated 'torakusa', whose morphologic and genetic characterization is currently in progress) and transported to the laboratory at Nagasaki University, and acclimatized in aerated tanks for several days. TTX levels were quantified in 4 of the 31 specimens as described below for the non-administration (NA) group, and the remaining 27 specimens were subjected to the toxin administration experiment.

2.2. Preparation of toxin solution

The toxicity of TTX standard purchased from Wako (purity >90%) was calibrated using 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.

The TTX standard was dissolved in a physiologic saline solution containing 1.35% NaCl, 0.06% KCl, 0.025% $CaCl_2$, 0.035% $MgCl_2$, and 0.02% $NaHCO_3$ at a concentration of 1460 MU/ml and used in the following toxin administration experiments.

2.3. Toxin administration experiment

Each fish was intramuscularly administered 0.1 ml (146 MU) of TTX solution [a dose (approximately 40 MU/20 g body weight) equal to about 1/10 of the minimum lethal dose of TTX to *T. rubripes* (Noguchi and Arakawa, 2008)], and immediately returned to the tank (total handing time < 30 s to minimize stress to the fish). Subsequently, 3 to 4 specimens were randomly collected at 1, 4, 8, 12, 24, 48, and 72 h after toxin administration, and toxin quantification was performed as described below.

2.4. Toxin quantification

Using a syringe precoated with sodium heparin, all of the blood of each specimen was withdrawn from the portal vein and centrifuged at 4200g for 10 min. The supernatant (blood plasma) obtained was subjected to enzyme-linked immunosorbent assay (ELISA) for TTX (Kawatsu et al., 1997; Ngy et al., 2008). After blood collection, all specimens were dissected into different anatomic tissues (muscle, skin, liver, and gonads), and extracted with 0.1% acetic acid (Japan Food Hygiene Association, 2005). Each extract of the muscle, liver, and gonads was filtered through a USY-1 membrane (0.45 μm; Toyo Roshi Co., Ltd., Japan) and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis (Nakashima et al., 2004), while that of the skin was subjected to ELISA. The amount of TTX (in ng) determined by LC/MS or ELISA was converted to MU based on the specific toxicity of TTX (220 ng/MU). In a preliminary experiment, a significant and positive correlation (Pearson's test; $r = 0.9641$, $p < 0.01$) was observed between the TTX amounts determined by ELISA and LC/MS, with the regression line of $y = 0.9874x + 7.301$ ($r^2 = 0.9295$), as described previously (Ikeda et al., 2009).

2.5. Assessment of gonadosomatic index (GSI)

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

3. Results

The GSI of the female 'torakusa' specimens used in the present study was 0.40 ± 0.06% and that of the male specimens was 5.96 ± 2.41%. TTX was not detected in any tissues of the four specimens of the NA group.

Changes in the toxin content (MU/g or MU/ml) of each tissue, except the gonads, of the TTX-administered 'torakusa' specimens during the rearing period are shown in Fig. 1 (refer to Fig. 3 for the number of specimens tested at each rearing period). In both females and males, little toxin remained in the muscle, which was the site of toxin administration, even 1 h after toxin administration, and the

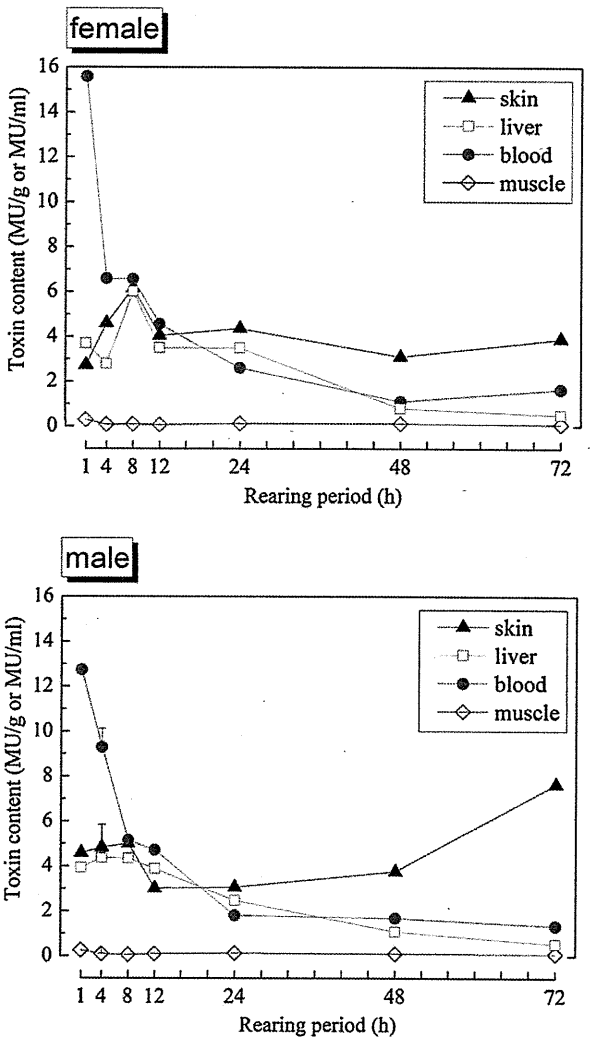

Fig. 1. Changes in the TTX (MU/g or MU/ml) content retained in each tissue, except for gonads, of the 'torakusa' specimens during the rearing period after toxin administration. The TTX content was determined for each individual, and the symbols indicate the mean value (refer to Fig. 3 for specimen numbers for each rearing period).

toxin content of the muscle became lower than the detection limit (0.01 MU/g) after 4 h. In contrast, the toxin content of both skin and liver reached around 4 MU/g at 1 h, and further increased to a maximum at 4–8 h. The toxin content of the liver significantly decreased thereafter. The regression lines of females and males during 12–72 h were expressed as $y = -0.0581x + 4.3393$ ($n = 8$, $r^2 = 0.7534$, $p < 0.05$) and $y = -0.0500x + 3.8610$ ($n = 7$, $r^2 = 0.8668$, $p < 0.05$), respectively, with no significant difference between the two lines. On the other hand, the toxin content of the skin decreased once, but remained at the same level in females, and had a significant increasing trend ($y = 0.0807x + 1.1054$, $n = 7$, $r^2 = 0.7341$, $p < 0.05$) again in males after 12 h. The toxin content of the blood plasma was highest at 1 h, and rapidly decreased thereafter [female: $y = -3.271\ln(x) + 13.631$ ($n = 13$, $r^2 = 0.876$, $p < 0.05$); male: $y = -2.874\ln(x) + 12.443$ ($n = 14$, $r^2 = 0.915$, $p < 0.05$); no significant difference between the two regression lines].

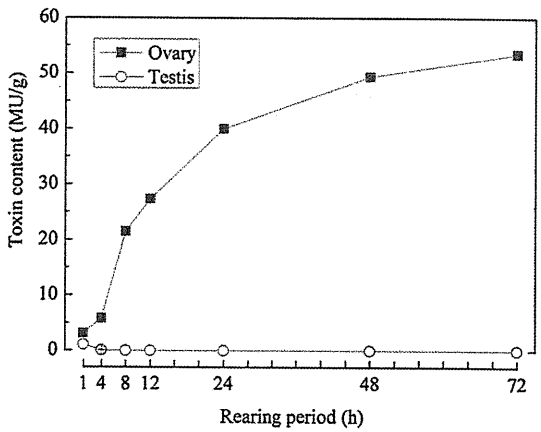

Fig. 2. Changes in the content (MU/g) of TTX in the gonads of 'torakusa' specimens during the rearing period after toxin administration. The TTX content was determined for each individual, and the symbols indicate the mean value (refer to Fig. 3 for specimen numbers for each rearing period).

Changes in the toxin content of the gonads are shown in Fig. 2. The toxin content of the ovary was strikingly higher compared with the other tissues, and continued to rapidly increase ($y = 12.716\ln(x) - 2.0188$, $n = 13$, $r^2 = 0.9148$, $p < 0.05$) throughout the rearing period to reach 53.5 MU/g at the end of the period (72 h). In contrast, little toxin transferred to the testis, whose toxin content was lower than the detection limit after 4 h.

Changes in the anatomic distribution of TTX, demonstrated by the relative amount of toxin retained in each tissue [% of the administered toxin (146 MU/individual)], are shown in Fig. 3. In both females and males, the total amount of toxin remaining in the whole body was 60–80% of the administered toxin up to 8 h, and decreased a little to 45–65% thereafter. The amount of toxin in the skin, followed by the liver, was generally high (20–54% and 2–24%, respectively), but the amount in the liver rapidly decreased after 8–12 h. During this period, the toxin transfer profile was different between females and males; in females, the amount of toxin in the ovary gradually increased, exceeding that in the liver after 48 h, and then reached 9.8% at 72 h, whereas in males the toxin did not transfer to the testis, but instead the toxin amount in the skin increased gradually.

4. Discussion

The GSI of the male 'torakusa' specimens used in the present study was much higher than that of the female specimens. In wild *T. niphobles* and *T. poecilonotus*, the GSI of both females and males is generally less than 2% prior to maturation, but rapidly rises when maturation begins and reaches as high as 10–20% (Yu and Yu, 2002; Ikeda et al., 2010), indicating that maturation had begun to occur, at least in the male specimens. It takes more than 2 years for cultured *T. rubripes* to mature (unpublished data), whereas *T. niphobles* usually mature within 1 year (Honma et al., 1980). Therefore, in terms of maturation, the hybrid specimens (10 months old) seem to be closer to *T. niphobles*. The GSI of females normally begins to increase about 1 month later than that of males (Yu and Yu, 2002; Ikeda et al., 2010),

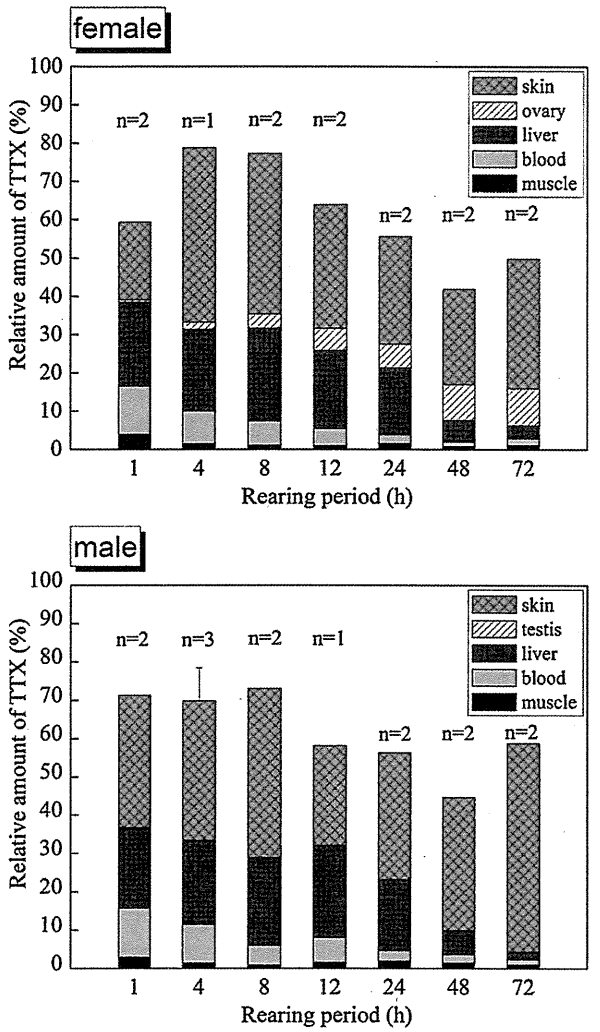

Fig. 3. Changes in the relative amount of TTX [% of administered amount (146 MU/individual)] retained in each tissue of the 'torakusa' specimens during the rearing period after toxin administration. The values over each column indicate the number of tested specimen for each rearing period.

suggesting that the present female specimens were in a very early stage of maturation, just before the GSI began to increase. Although histologic observation of the ovary is necessary to clarify this point, histology was not performed because the ovaries were still very small and the whole ovary was used for toxin quantification.

TTX was not detected in the four specimens of the NA group. TTX is exogenous in pufferfish, and cultured *T. rubripes* or *T. niphobles* do not have detectable levels of TTX (Noguchi and Arakawa, 2008). It was confirmed that 'torakusa' also become non-toxic when they are reared with non-toxic food under conditions in which toxic food organisms are completely excluded.

Intramuscularly administered TTX in the 'torakusa' specimens was rapidly transferred to other body tissues, and the toxin content of the skin, liver, and ovary exceeded that of the muscle within as little as 1 h after administration. At 1 h after intramuscular administration, a high concentration of TTX was present in the blood plasma, indicating that TTX was transferred mainly via the bloodstream. The fact that muscles in toxic wild specimens of *T. rubripes* are not toxic, and those of *T. niphobles*, whose toxicity is usually higher than *T. rubripes*, are also non-toxic or weakly toxic (Noguchi and Arakawa, 2008) suggests that the muscles of their hybrid have little ability to retain and accumulate TTX.

In both females and males, the toxin content of the skin and liver reached a maximum at 4–8 h after TTX-administration. Although the toxin content of the liver gradually decreased thereafter, the toxin content of the skin remained at the same level in females, and tended to increase in males after 12 h. This suggested that the toxin transferred to the skin is retained there for a long period, whereas the toxin that transferred to the liver is then transferred to other organs within a relatively short period. We observed a similar phenomenon when a TTX standard (purified TTX) was intramuscularly administered to *T. rubripes* (Ikeda et al., 2009). When the crude extract of toxic pufferfish ovary (crude TTX) was administered, however, the toxin was retained in the liver at a higher concentration for a longer period of time than the purified TTX. Moreover, in previous oral TTX-administration experiments (Honda et al., 2005; Kono et al., 2008a), pufferfish retained TTX in the liver for a long period, even after stopping the feeding of a TTX-containing diet, indicating that the form and/or uptake route of TTX affects its transfer/accumulation to the liver, though the mechanism remains to be elucidated.

On the other hand, the toxin continued to transfer to the ovary throughout the rearing period, and accumulated there at an extraordinarily higher concentration than in the skin and liver. The amount of toxin transferred to the gonads could not be examined because the tissues were undeveloped in the experiment with *T. rubripes*; therefore, the present study is the first study to demonstrate that the intramuscularly administered toxin transfers rapidly and in large quantities to the ovary. We recently investigated seasonal changes in tissue toxicity using wild specimens of the pufferfish *T. poecilonotus*, and determined that the TTX absorbed from toxic food organisms into the female pufferfish body is actively transported and accumulated in the ovary during the maturation period, as liver toxicity was high prior to maturation, and that of the ovary was high during the maturation period (Ikeda et al., 2010). The female 'torakusa' specimens used in the present study seem to be in the very early stage of maturation, as expected, in which such a toxin transportation mechanism would have begun to function. To further clarify the effect of maturation on the transfer and accumulation of TTX, comparative studies of completely non-matured and/or fully matured 'torakusa' specimens are needed.

In the males, most of the toxin remaining in the body, including the toxin that was first in the liver, was eventually transferred/accumulated in the skin; a toxin transfer profile that is essentially very similar to that of the immature *T. rubripes* specimens (Ikeda et al., 2009). Wild adult specimens of *T. rubripes* generally possess no toxin in the skin, but toxicity of several tens of mouse units is occasionally detected in juveniles (unpublished data). Most of the toxin was also transferred/accumulated in the skin during the toxin administration experiment. Therefore, the species to which the toxin accumulating ability of the 'torakusa' is

closer is unclear; *T. rubripes* or *T. niphobles*, which have highly toxic skin. The testis, like muscle, seems to have little ability to retain or accumulate toxin, as little toxin transferred to the testis despite the fact that the GSI of males was much higher than that of the females. In contrast, the amount of toxin in the ovary of females gradually increased, and exceeded that of the liver after 48 h. Although statistically not significant, the sum of the toxin amount in the skin and ovary in female specimens seemed to correspond to the change to the toxin amount in the skin in the male specimens. In addition, the toxin content of the skin remained at the same level in females, but tended to increase in males after 12 h, suggesting that a part of the TTX that should be, after first being taken up into the liver, transferred/accumulated into the skin in male specimens is transferred to the ovary in female specimens.

TTX-binding proteins are found in the blood plasma of toxic marine pufferfish (Matsui et al., 2000; Yotsu-Yamashita et al., 2001) and may be involved in toxin transportation. Detailed inter-tissue transfer mechanisms of TTX, especially those involved in the specific and powerful uptake of the ovary during the maturation period, however, remain to be elucidated. Further studies are in progress.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Review Article

Tetrodotoxin Poisoning Due to Pufferfish and Gastropods, and Their Intoxication Mechanism

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Marine pufferfish generally contain a large amount of tetrodotoxin (TTX) in their skin and viscera, and have caused many incidences of food poisoning, especially in Japan. Edible species and body tissues of pufferfish, as well as their allowable fishing areas, are therefore clearly stipulated in Japan, but still 2 to 3 people die every year due to pufferfish poisoning. TTX is originally produced by marine bacteria, and pufferfish are intoxicated through the food chain that starts with the bacteria. Pufferfish become nontoxic when fed TTX-free diets in a closed environment in which there is no possible invasion of TTX-bearing organisms. On the other hand, TTX poisoning due to marine snails has recently spread through Japan, China, Taiwan, and Europe. In addition, TTX poisoning of dogs due to the ingestion of sea slugs was recently reported in New Zealand. TTX in these gastropods also seems to be exogenous; carnivorous large snails are intoxicated by eating toxic starfish, and necrophagous small-to-medium snails, the viscera of dead pufferfish after spawning. Close attention must be paid to the geographic expansion and/or diversification of TTX-bearing organisms, and to the sudden occurrence of other forms of TTX poisoning due to their ingestion.

1. Introduction

In Japan, tetrodotoxin (TTX) is the most common natural marine toxin to cause food poisoning, and it poses a serious hazard to public health. This toxin ($C_{11}H_{17}N_3O_8$; Figure 1) is a potent neurotoxin with a molecular weight of 319, whose various derivatives have been separated from pufferfish, newts, frogs, and other TTX-bearing organisms [1]. When ingested by humans, TTX acts to block the sodium channels in the nerve cells and skeletal muscles [2], and to thereby block excitatory conduction, resulting in the occurrence of typical symptoms and signs (Table 1) and even death in severe cases [3]. 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-gram male mouse within 30 min after intraperitoneal administration), and the minimum lethal dose for humans is estimated to be approximately 10,000 MU (≈2 mg).

Since 1964 [4], the distribution of TTX has spread to animals other than pufferfish, including newts, gobies, frogs, octopuses, gastropods, starfish, crabs, flatworms, and ribbon worms (Table 2) [5, 6]. Pufferfish are thought to accumulate TTX through several steps of the food chain, starting from TTX production by marine bacteria (Figure 2) [6, 7]. TTX poisoning due to marine gastropods occurs not only in Japan [5], but also in China [3], Taiwan [3], Europe [8], and New Zeeland [9], suggesting further diversification of TTX-bearing organisms and therefore geographic expansion of TTX poisoning. In the present paper, we review TTX poisoning cases due to the ingestion of pufferfish and gastropods, and discuss the TTX intoxication mechanism of these organisms in an effort to contribute to the development of an effective means of protecting humans against TTX poisoning.

2. TTX Poisoning due to Pufferfish

Marine pufferfish of the family Tetraodontidae generally contain a large amount of TTX in their skin and viscera,

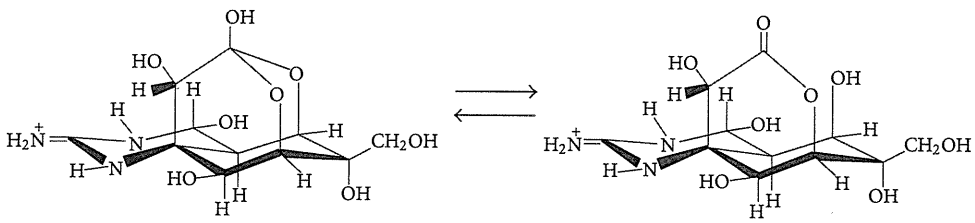

FIGURE 1: Chemical structure of TTX.

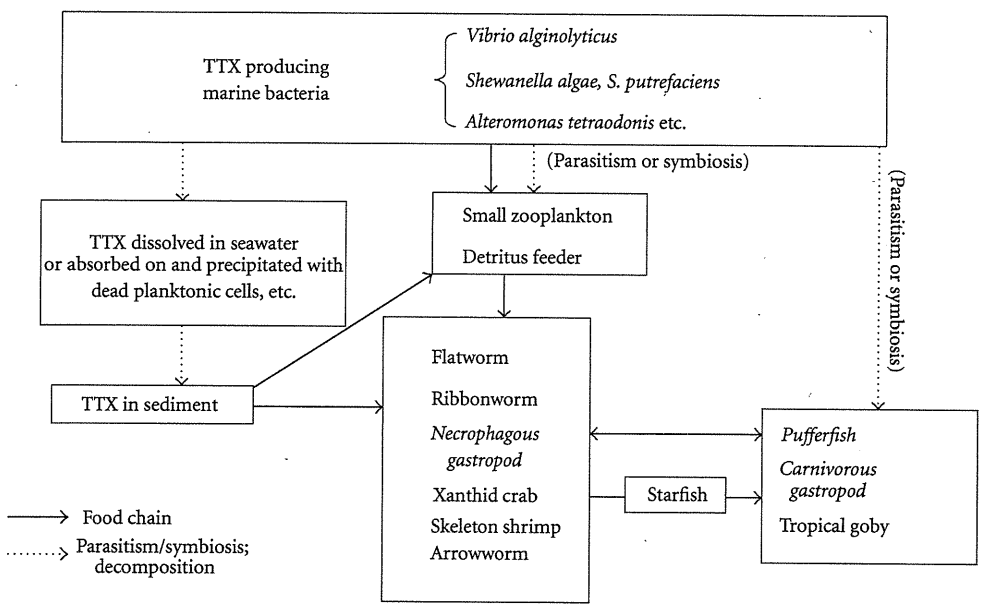

FIGURE 2: Proposed mechanism of TTX intoxication in marine animals.

especially the liver and ovary [6]. Accordingly, edible species and their body tissues, and the allowable pufferfish fishing areas have been clearly stipulated in Japan since 1983, but still several tens of people are poisoned by pufferfish annually, and 2 to 3 people die as a result of pufferfish poisoning (Table 3). The incidence in specializing restaurants is rare, and most cases of poisoning result when people with little knowledge of pufferfish toxicity cook a pufferfish that they caught or received from someone else and mistakenly eating strongly toxic parts such as liver and ovary. Some pufferfish fans dare to ingest the liver, believing that the toxin can be eliminated by their own special detoxification methods.

In October 2008, a 69-year-old male died at a hospital in Isahaya, Nagasaki Prefecture [10]. He stated that he cooked a "usubahagi" (a sort of thread-sail filefish "kawahagi") that he caught by himself and ate its raw meat (sashimi) after dipping in a mixture of the liver and soy sauce. Approximately 30 minutes after ingestion, he felt numbness in his limbs, and 30 minutes later, he vomited and became comatose before being transported by an ambulance to the hospital. The doctor confirmed his death approximately 4 hours after ingestion, with an initial diagnosis of "ciguatera due to the ingestion of "kawahagi" liver, the possibility of TTX is not denied". Thereafter, it was determined that the patient cooked a "kinfugu" (local name of pufferfish) with the "usubahagi," but the liver was missing among the leftovers. We investigated the leftovers, and revealed that the "usubahagi" was nontoxic, but the "kinfugu" was actually a highly toxic species, "komonfugu" *Takifugu poecilonotus*, and 600 MU/g of TTX was detected in the skin. Furthermore, 0.7 MU/mL, 2 MU/mL, and 45 MU/g of TTX was detected in the blood, urine, and vomit of the patient, respectively, leading to the conclusion that this was a case of TTX intoxication due to the mistaken ingestion of *T. poecilonotus* liver.

Recently, the nonedible pufferfish *Lagocephalus lunaris*, which usually inhabits tropical or subtropical waters, has been frequently mixed up with edible species in Japanese coastal waters, posing a serious food hygiene problem. This pufferfish, which bears a very similar appearance to the almost nontoxic species *L. wheeleri*, also possesses high levels of TTX in their muscles [6, 11], caused 5 poisoning incidents in 11 patients due to mistaken ingestion in Kyushu

Table 1: Symptoms of TTX poisoning.

Degree	Characteristic symptoms
First	Neuromuscular symptoms (paresthesia of lips, tongue, and pharynx; taste disturbance; dizziness; headache; diaphoresis; pupillary constriction); gastrointestinal symptoms (salivation, hypersalivation, nausea, vomiting, hyperemesis, hematenesis, hypermotility, diarrhea, abdominal pain)
Second	Additional neuromuscular symptoms (advanced general paresthesia; paralysis of phalanges and extremities; pupillary dilatation, reflex changes)
Third	Increased neuromuscular symptoms (dysarthria; dysphagia, aphagia; lethargy; incoordination, ataxia; floating sensation; cranial nerve palsies; muscular fasciculations); cardiovascular/pulmonary symptoms (hypotension or hypertension; vasomotor blockade; cardiac arrhythmias including sinus bradycardia, asystole, tachycardia, and atrioventricular node conduction abnormalities; cyanosis; pallor; dyspnea); dermatologic symptoms (exfoliative dermatitis, betechiae, blistering)
Fourth	Respiratory failure, impaired mental faculties, extreme hypotension, seizures, loss of deep tendon and spinal reflexes

Table 2: Distribution of TTX in animals other than pufferfish.

Animals			Toxic parts	Maximal toxicity
Platyhelminthes	Turbellaria, Flatworms	*Planocera spp.*	Whole body	>1000 MU/g
Nemertinea	Ribbonworms	*Lineus fuscoviridis*	Whole body	>1000 MU/g
		Tubulanus punctatus	Whole body	100–1000 MU/g
		Cephalothrix linearis	Whole body	>1000 MU/g
Mollusca	Gastropoda	*Charonia sauliae*	Digestive gland	>1000 MU/g
		Charonia lampas lampas	Digestive gland	10–100 MU/g
		Babylonia japonica	Digestive gland	100–1000 MU/g
		Tutufa lissostoma	Digestive gland	>1000 MU/g
		Zeuxis siquijorensis	Whole body	>1000 MU/g
		Niotha clathrata	Whole body	>1000 MU/g
		Niotha lineata	Whole body	10–100 MU/g
		Cymatium echo	Digestive gland	10–100 MU/g
		Pugilina ternotoma	Digestive gland	
		Pleurobranchaea maculata	skin	
	Cephalopoda	*Hapalochlaena maculosa*	Posterior salivary gland (adult), Whole body (semi-adult)	>1000 MU/g
Annelida	Polychaeta	*Pseudopolamilla occelata*	Whole body	10–100 MU/g
Arthropoda	Xanthidae crabs	*Atergatis floridus*	Whole body	10–100 MU/g
		Zosimus aeneus	Whole body	10–100 MU/g
	Horseshoe crab	*Carcinoscorpius rotundicauda*	Egg	10–100 MU/g
Chaetognatha	Arrowworms	*Parasagitta spp.*	Head	detected
		Flaccisagitta spp.	Head	detected
Echinodermata	Starfish	*Astropecten spp.*	Whole body	100–1000 MU/g
Vertebrata	Pisces, Goby, Amphibia	*Yongeichthys criniger*	Skin, viscera, gonad	100–1000 MU/g
	Newts	*Tarica spp.*	Skin, egg, ovary, muscle, blood	100–1000 MU/g
		Notophthalmus spp.	Skin, egg, ovary	10–100 MU/g
		Cynopsis spp.	Skin, egg, ovary, muscle, blood	10–100 MU/g
		Triturus spp.	Skin, egg, ovary, muscle, blood	detected
	Frogs	*Atelopus spp.*	Skin	>1000 MU/g
		Colostethus sp.	Skin	100–1000 MU/g
		Polypedates sp.	Skin	100–1000 MU/g
		Brachycephalus spp.	Skin, liver	100–1000 MU/g

Table 3: Pufferfish poisoning incidents in Japan.

Year	Number of incidents	Number of patients	Number of deaths	Mortality (%)
1965	106	152	88	57.9
1970	46	73	33	45.2
1975	52	75	30	40.0
1980	46	90	15	16.7
1985	30	41	9	22.0
1990	33	55	1	1.8
1995	30	42	2	4.8
1996	21	34	3	8.8
1997	28	44	6	13.6
1998	27	39	4	10.3
1999	20	34	2	5.9
2000	29	40	0	0.0
2001	31	52	3	5.8
2002	37	56	6	10.7
2003	28	35	3	8.6
2004	44	61	2	3.3
2005	40	49	2	4.1
2006	26	33	1	3.0
2007	29	44	3	6.8
2008	40	56	3	5.4
2009	24	50	0	0.0
2010	23	29	0	0.0

and Shikoku Islands during 2008-2009. Though not as frequent as in Japan, many food poisoning cases due to ingestion of wild pufferfish have also occurred in China and Taiwan [3, 6].

3. TTX Poisoning due to Gastropods

TTX-bearing gastropods and the food poisoning incidents due to their ingestion are summarized in Tables 4 and 5, and Figure 3.

3.1. Large Marine Snails. Although the trumpet shell *Charonia sauliae* is not usually sold on the market, it is sometimes eaten locally in Japan. In December 1979, a man in Shimizu, Shizuoka Prefecture, Japan, ingested the digestive gland of *C. sauliae* and was seriously poisoned. He showed paralysis of his lips and mouth, and respiration failure, which are the typical symptoms and signs of pufferfish poisoning. TTX was detected for the first time in a marine snail, that is, the leftovers of *C. sauliae*, and the causative agent was therefore concluded to be TTX [12]. Similar poisonings occurred in 1 patient in the Wakayama Prefecture in December 1982, and in 2 patients in the Miyazaki Prefecture in January 1987.

In *C. sauliae*, TTX localizes in the digestive gland, and other organs, including the muscle, are nearly nontoxic [12]. The digestive gland toxicity of *C. sauliae* collected from Shimizu Bay in 1981 ranged from 77 to 350 MU/g. A subsequent toxicity survey based on a total of 1406 digestive glands of *C. sauliae* from 7 prefectures indicated that the frequency of toxic specimens in each prefecture ranged from 19% to 87%. TTX or its derivative been also detected in closely related species, such as the frog shell *Tutufa lissostoma* [13] and the European trumpet shell *Charonia lampas lampus* [8], the latter of which caused TTX poisoning in Spain in 2007.

3.2. Medium Marine Snails. The ivory shell *Babylonia japonica* is usually ingested as a side dish with sake. In June 1957, 5 persons were poisoned due to ingestion of the shellfish in Teradomari, Niigata Prefecture, and 3 of them died [14]. The causative substance was estimated to be TTX based on the facts that the symptoms and signs of the victims were similar to those of the pufferfish poisoning, and that TTX was later detected in *B. Japonica* collected from Kawajiri Bay, Fukui Prefecture in May 1980 [15].

In April 2004, a food poisoning incident resulting from the ingestion of the necrophagous marine snail *Nassarius (Alectricon) glans* occurred in Tungsa Island located in the South China Sea, Taiwan. Five patients were involved, and there were 2 deaths. The causative agent was identified as TTX by instrumental analyses [16, 17]. In a toxicity survey of 20 *N. glans* specimens collected from the same sea area, high toxicity was observed not only in the digestive gland, but also in the muscle (average of 538 and 1167 MU/g, resp.).

TTX poisonings due to *N. glans* have also occurred in Japan recently [10]. In July 2007 in Nagasaki, Nagasaki

Table 4: TTX-bearing gastropods and food poisoning cases due to them in Japan.

Name of gastropod	Poisoning Year	Place	Number of patient	Number of death	Predatory habit
Ivory shell, *Babylonia japonica*	1957, Jun.	Niigata	5	3	Necrophagous
Trumpet shell, *Charonia sauliae*	1979, Dec.	Shizuoka	1	0	Carnivorous
	1982, Dec.	Wakayama	1	0	Carnivorous
	1987, Jan.	Miyazaki	2	0	Carnivorous
"Kinshibai," *Alectricon glans*	2007, Jul.	Nagasaki	1	0	Necrophagous
	2008, Jul.	Kumamoto	1	0	Necrophagous
Frog shell, *Tutufa lissostoma*		Shizuoka			Carnivorous
"Hanamushirogai," *Zeuxis siquijorensis*		Shizuoka			Necrophagous
"Araregai," *Niotha clathrata*		Shizuoka			Necrophagous
Total			11	3	

Table 5: TTX-bearing gastropods and food poisoning cases due to them in other countries.

Taiwan and China					
Name of gastropod	Poisoning Year	Place	Number of patient	Number of death	Predatory habit
Zeuxis samiplicutus	1977–2001, Jun.	Zhoushan, China	310	16	Necrophagous
Niotha clathrata, *Zeuxis scalaris*	1994, May	Pingtung, Taiwan	26	0	Necrophagous
Z. sufflatus, *N. clathrata*	2001, Apr.	Taipei, Taiwan	5	0	Necrophagous
Z. siquijorensis	2002	Fujian, China	>20	>3	Necrophagous
	2004	China	55	1	
Zeuxis sp. and/or *Niotha* sp.	2002, Jul.	Tsingtao, China	3	0	Necrophagous
	2002-up to date	Fujian to Tsingtao, China	—	—	Necrophagous
Total			>419	>20	
New Zealand					
Sea slug, *Pleurobranchaea maculata*	2009, Jul.	Auckland		14 (dogs)	Herbivorous Carnivorous

Prefecture, a 60-year-old female developed a feverish feeling in the limbs, abdominal pain, and an active flush and edema in the face 15 minutes after ingesting the shellfish and was administered intravenous fluids at a clinic near her home. Thereafter, her condition worsened, and she developed dyspnea, whole-body paralysis, and mydriasis; she was finally transported to an emergency hospital. The patient required an artificial respirator for the first 3 days, but recovered enough to take breakfast on the 4th day. She unexpectedly relapsed after lunch, however, and developed respiratory arrest and was placed on the respirator again. She gradually recovered and was discharged from the hospital 3 weeks later.

Immediately after the incident, we investigated the left-over gastropods and detected a maximum of 4290 MU/g of TTX in the cooked muscles and digestive glands of *N. glans*. Moreover, during subsequent investigations, an extremely high concentration of TTX and a putative derivative of TTX, that is, a maximum of 10,200 MU/g (15,100 MU/individual) in the viscera and 2370 MU/g (9860 MU/individual) in the muscle, were detected in *N. glans* specimens collected from the same sea area as the ingested snail [18]. In this case, the once-recovered symptoms recurred after the patient began eating again. Although the reason is not clear, the recurrence might have been due to the digestion of a highly toxic, previously undigested tissue fragment of *N. glans* and absorption due to the resumption of meals, again exposing her respiratory center to a high concentration of TTX. In July 2008, another poisoning incident due to *N. glans* occurred in Amakusa, Kumamoto Prefecture.

3.3. Small Marine Snails. In association with the occurrence of TTX poisoning by *C. sauriae* in Shizuoka Prefecture in 1979, TTX screening was performed in several species of small marine snails in Japan. *Zeuxis siquijorensis* [19] and *Niotha clathrata* [20] were found to possess TTX or a TTX-like substance. There have been, however, no poisoning cases in Japan, as Japanese people do not typically feed on these species. On the other hand, inhabitants along the coast of the East China Sea in China and Taiwan have a long history of eating small marine snails, and *Zeuxis* spp. *N. clathrata*, and *Natica* spp. are generally sold at the supermarket or fish markets in these areas. From 1977 to 2004, more than 419 people were poisoned by ingesting these snails, and

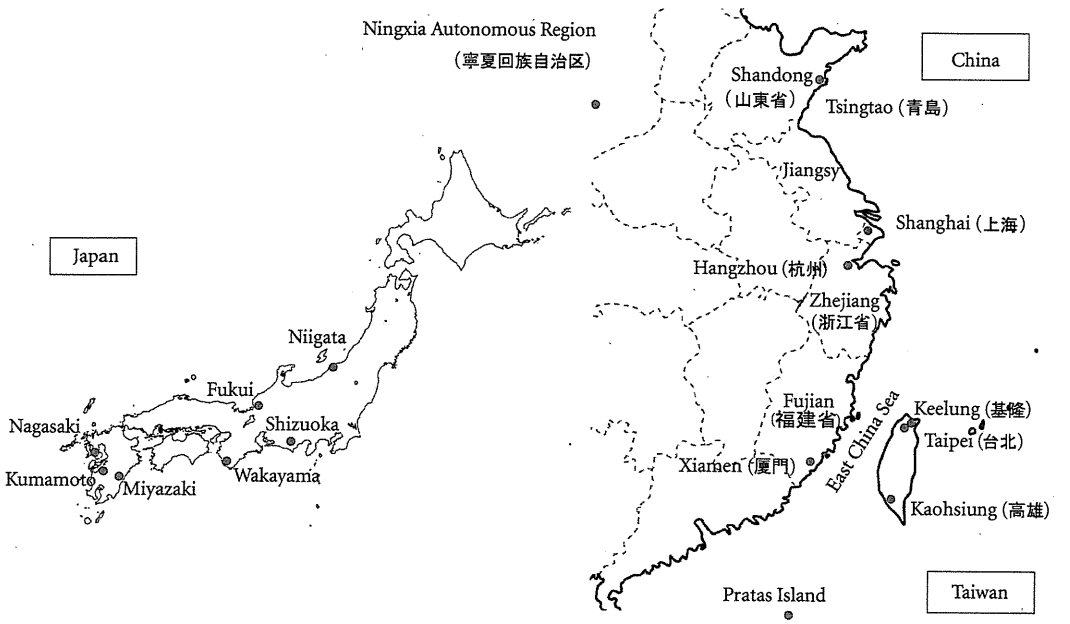

FIGURE 3: Maps showing the locations where TTX poisoning due to marine snails occurred in Japan, China, and Taiwan.

over 19 people died in Zhoushan, Fujian, and the Ninxia Hui Automous Region in China [3, 21–23]. Furthermore, poisoning cases have spread along the coasts from Fujian to Tsuingtao. In 1994 and 2001, similar poisonings occurred in the southern and northern parts of Taiwan, respectively, and the main causative substance was identified as TTX [23–25].

3.4. Sea Slugs. From July to November 2009, 15 dogs were suddenly poisoned at the beaches adjacent to Hauraki Gulf, Auckland, New Zealand, all exhibiting similar symptoms, and 5 of them died. McNabb et al. [9] detected a very high level of TTX in the grey side-gilled sea slug *Pleurobranchaea maculate* found in tide pools near the beach and claimed that the dogs were poisoned with TTX by contact with the sea slugs. TTX was found in the eggs and larvae and distributed over the whole body with increasing concentrations toward the outer tissues in the adult sea slugs.

4. TTX Intoxication Mechanism of Pufferfish

Marked individual and regional variations are observed in pufferfish toxicity. In addition, the facts that the TTX of *C. sauliae* and *B. Japonica* comes from the food chain as described below and that several shell fragments of *Z. siquijorensis* are detected in the digestive tract of the toxic pufferfish *Takifugu pardalis* suggest that TTX contained by pufferfish is exogenous via the food chain [6, 7]. Moreover, many studies of TTX have revealed that (1) TTX is distributed over various organisms other than pufferfish, (2) marine bacteria primarily produce TTX (Table 6), (3) pufferfish become nontoxic when they are fed TTX-free diets in a closed environment in which there has been no invasion of TTX-bearing organisms, (4) such nontoxic pufferfish efficiently accumulate TTX when TTX is orally administered, and (5) pufferfish are equipped with high resistance to TTX, supporting the exogenous intoxication theory—a hypothesis that TTX is originally produced by marine bacteria, and pufferfish accumulate TTX through the food chain that starts with the bacteria [6, 7].

TABLE 6: Primary TTX producers.

Marine bacteria	Source
Vibrio alginolyticus	From starfish
Vibrio VIII	From crab
Shewanella algae	From *Jania* sp.
Alteromonas tetraodonis	
S. putrefaciens	From pufferfish
Other marine bacteria	

To test (3), we investigated the toxicity of more than 8700 individual pufferfish that had been reared in an environment in which the invasion of TTX-bearers was prevented and were provided nontoxic diets in netcages in the sea, or in tanks with an open or closed circulation system on land, and confirmed that all the livers remained nontoxic (Table 7) [6, 25]. Production of nontoxic pufferfish can reduce the risk of food poisoning from eating toxic pufferfish and reduce the mortality rate. Moreover, this method might

Table 7: Toxicity of cultured pufferfish liver (1982–2009).

Culture	Year of collection	Age	Number of collection	Toxicity (MU/g)
Sea	1981–2003	1–3	4258	<2–<10
Land			4504	<2–<8
open system	2001–2009	1-2	4173	
closed system	2008-	1-2	331	
Total			8762	

also contribute to maintain the Japanese food culture by reviving pufferfish liver dishes as a safe traditional food, which, although eaten previously, has been prohibited as a food since the regulation of 1983 in Japan. The transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. We recently found that TTX administered intramuscularly to nontoxic cultured specimens of the pufferfish *Takifugu rubripes* was transferred first to the liver and then to the skin via the blood [26]. Matsumoto/Nagashima et al. demonstrated that, unlike general nontoxic fish, the liver tissue of *T. rubripes* is equipped with a specific TTX-uptake mechanism [27–29], and using a pharmacokinetic model showed that TTX introduced into the pufferfish body is rapidly taken up into the liver via the blood [30, 31]. These findings indicate that marine pufferfish are endowed with a mechanism by which they transport TTX specifically and actively. TTX-binding proteins have been isolated from the blood plasma of marine pufferfish, and may be involved in the transportation mechanism [32, 33].

In wild pufferfish, the liver and ovary usually have strong toxicity, whereas the muscle and testis are weakly toxic or nontoxic [6]. In addition, the toxicity varies with the season, usually reaching the highest level during the spawning season (March to June in Japan), indicating sexual differences in pufferfish toxicity and that maturation may affect toxin kinetics in the pufferfish body. Recently, we investigated seasonal changes in tissue toxicity and the amount and forms of TTX in the blood plasma using wild specimens of the pufferfish *T. poecilonotus* and demonstrated that maturation greatly affects the intertissue transfer and/or accumulation of TTX via the bloodstream [34].

5. TTX Intoxication Mechanism of Gastropods

5.1. Large Marine Snails. The trumpet shell *C. sauliae* is a carnivorous marine snail, and fragments of the starfish *Astropecten polyacanthus* were detected in the digestive tract of the specimens collected from Shimizu Bay in association with the food poisoning in 1979. The starfish were toxic, and the toxic molecule was identified as TTX [35]. The closely related species *A. scoparius* [36] and *A. latespinosus* [37] also had TTX. Moreover, an experiment in which nontoxic *C. sauliae* were fed toxic starfish demonstrated that the TTX of *C. sauliae* is derived from these starfish, namely, their food source [35, 38]. The starfish of genus *Astropecten* are also carnivorous, and their toxin is also estimated to come from their food.

5.2. Medium Marine Snails. The ivory shell *B. japonica* is necrophagous and feeds on the muscles and viscera of dead fish. In the Hokuriku and Joetsu districts along the Japan Sea where Sakajiri Bay is located, and TTX intoxication of *B. japonica* was recognized in 1980 [15], fishermen are familiar with the feeding habits of *B. japonica* and catch them using the viscera of dead toxic pufferfish *Takifugu niphobles* as bait. We performed a similar experiment with *C. sauliae* and observed that *B. japonica* preferentially ate dead pufferfish viscera, thereby accumulating TTX. It is presumed that the *B. japonica* that caused poisoning in Teradomari of the Joetsu district were intoxicated with TTX by a similar mechanism.

Although the TTX intoxication mechanisms of *N. glans* in Tsungsa Island as well as Nagasaki and Kumamoto Prefectures are unclear, the necrophagous characteristics of the snail suggest that dead pufferfish viscera are one of the origins of TTX. The toxicity of the Nagasaki/Kumamoto specimens of *N. glans* collected from September to January was highest in September, and gradually decreased thereafter (Figure 4) [10, 18]. There are no data on the other months, but both poisoning incidents in Nagasaki and Kumamoto occurred in July, indicating that the *N. glans* had already accumulated a high concentration of TTX that month. In Japan, *T. niphobles* comes *en masse* to the seashore to spawn their eggs in June, and die shortly thereafter. The spawning season of *T. niphobles* almost corresponds to the intoxication season of *N. glans*, indicating a possibility that *N. glans* is intoxicated by feeding on the mass of dead *T. niphobles* at the sea bottom.

5.3. Small Marine Snails. The occurrence of food poisoning cases in China and Taiwan is concentrated from spring to early summer (Table 5), somewhat earlier than that of the Nagasaki/Kumamoto incidents. On the other hand, the season during which toxic pufferfish approach the seacoast in a group to spawn is earlier in China and Taiwan than in Japan, as the latitude of the area where the poisonings occur is lower than that of Japan proper (Figure 3). Therefore, the season when poisonings occur appears to correspond to the spawning season of toxic pufferfish. The small marine snails that have caused food poisonings in China and Taiwan are all necrophagous, having the same feeding habit as *B. japonica* and *N. glans*, and seem to be intoxicated by the same mechanism; they accumulate TTX by feeding on the viscera of toxic pufferfish that died after spawning.

In this context, TTX has been found to act as an attractant to toxic marine snails. In our experiment using 8 toxic and 2 nontoxic snail species to investigate the attracting

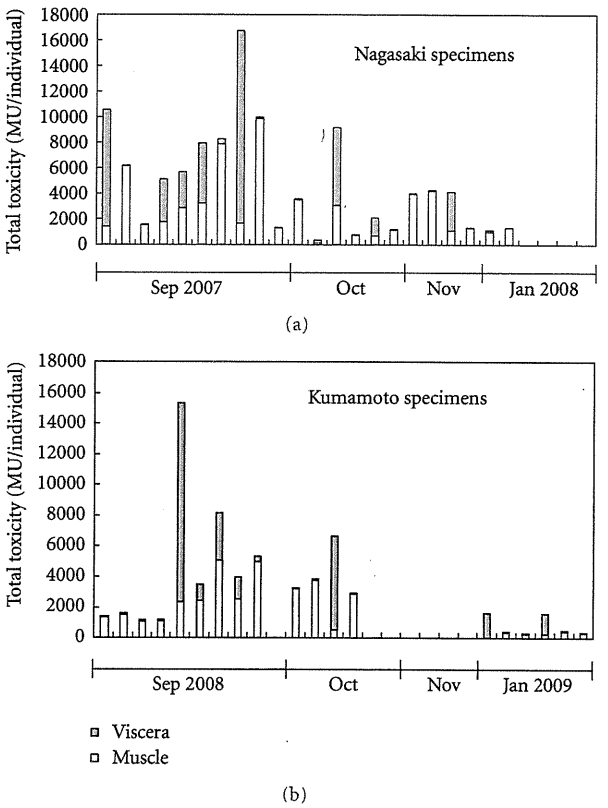

FIGURE 4: Toxicity of *N. glans* collected from Tachibana Bay, Nagasaki Prefectuire (a) and Miyanokawachi Bay, Kumamoto Prefecture (b).

effect of TTX, we observed a significantly positive correlation between toxicity and comparative attracting variations in toxic species, whereas nontoxic species showed a negative response to TTX [39]. Carnivorous or necrophagous marine snails generally live at the sea bottom, and their habitat, including their prey and food sources, is very limited. Under such conditions, the snails may be endowed with the ability to detect TTX-bearing foods and to ingest them selectively as a species-specific characteristic.

Although necrophagous small snails ingest TTX-containing foods selectively, they also have access to a diet contaminated with paralytic shellfish poison (PSP; i.e., a group of neurotoxins produced by certain species of dinoflagellates, and the main component, STX, has an almost equivalent molecular size and action mechanism to TTX [40]). In such cases, they accumulate not only TTX but also PSP, as seen in *Natica lineate* [41], *Niotha clathrata* [23, 24], and *Zeuxis scalaris* [23, 24] in Pingtung, Taiwan. This is also the case in the toxic crabs *Zosimus aeneus* in the Philippines [42] and Taiwan [43], and *Atergatis floridus* in Taiwan [44].

5.4. Sea Slugs. According to McNabb et al., sea slugs are carnivorous scavengers living in the shallow subtidal crustose turf/benthic algal communities [9]. The mechanisms of their TTX intoxication remain uncertain. Sea slugs are generally not used for human food, but the dog poisonings may be viewed as a warning to human public hygiene. Namely, if their intoxication is caused by a route other than the presently known food chain, this may suggest a novel original organism of TTX, and the food chain that begins with this organism may contaminate seafood previously thought to be safe with TTX.

6. Conclusion

TTX was originally named after the family name, Tetraodontidae, of pufferfish as their exclusive toxin, and TTX poisoning due to ingestion of pufferfish has long been recognized. TTX poisoning due to gastropods, however, has also begun to occur frequently, posing a serious food hygiene problem. TTX is exogenous to both pufferfish and gastropods, and they are thought to ingest it from toxic food organisms and to accumulate the TTX in specific organs. Interestingly, it is presumed that live pufferfish ingest/accumulate TTX from necrophagous small or medium marine snails, while on the other hand, these snails ingest/accumulate the toxin from dead pufferfish. Thus, it is possible that the TTX produced by bacteria not only transfers to higher organisms through the food chain, but that it also partly circulates between certain organisms (Figure 2).

As described above, the pufferfish *L. lunaris*, originally inhabiting tropical to subtropical sea areas, now frequently appear in the temperate coastal waters of Japan, and dog poisonings due to sea slugs have suddenly begun to occur in the Southern Hemisphere. Such facts indicate the possibility of further geographic expansion and/or diversification of TTX-bearing organisms, or of TTX contamination of seafood caused by a change in the marine environment, such as an increase in the water temperature due to global warming. Careful attention must be paid to this point from the food hygiene perspective for the future.

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