STUDIES ON TOXIN UPTAKE AND ACCUMULATION

PROFILES IN PUFFERFISH TISSUES

フグ類各組織の毒取り込み・蓄積プロファイルに関する研究

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CONTENTS

General introduction

Pufferfish have a long history in the Japanese food culture, and also have been an important aquatic resource in Asian countries. However, it is well known that pufferfish possess a potent neurotoxin, tetrodotoxin (TTX). TTX is an alkaloid with a low molecular weight of 319 and was firstly isolated in 1950 as a crystalline prism from toxic pufferfish (Yokoo, 1950). TTX is lethal to humans and causes muscle paralysis by specifically blocking voltage-gated sodium channels (Geffeney and Ruben, 2006; Narahashi, 2001). Therefore, food poisoning due to the consumption of these fish often occurs in Japan and some Southeast Asian countries such as Vietnam, Philippine, and Cambodia (Toda et al., 2012). TTX was considered only distributed in pufferfish. However, since Mosher et al. (1964) detected TTX in the eggs of the California newt *Taricha torosa*, TTX was found in various other marine organisms including certain species of gobies *Gobius criniger* (Noguchi and Hashimoto, 1973), octopuses *Hapalochlaena maculosa* (Sheumack et al., 1978), gastropods *Charonia sauliae* (Noguchi et al., 1982), starfish *Astropecten polyacanthus* (Noguchi et al., 1982), crabs *Atergatis floridas* (Noguchi et al., 1983), flatworms (Miyazawa et al., 1986), and ribbon worms (Miyazawa et al., 1988). Since Noguchi et al. (1986) detected TTX in a marine bacteria *Vibrio* sp. isolated from intestines of xanthid crab *Atergatis*

floridus, and Yasumoto et al. (1986) found another TTX-containing bacteria *Alteromonas* sp. from herbivorous fish *Ypsiscarus ovifrons* and xanthid crab *Zosimus aeneus*, TTX-containing bacteria which distributes over a broad range in marine environment was reported and considered as origin of TTX and cause the toxification of those marine organisms (Noguchi and Arakawa, 2008). Pufferfish such as *Takifugu rubripes* and *Takifugu alboplumbeus* (formerly known as *Takifugu niphobles* (Jordan and Snyder, 1901)) become non-toxic when artificially reared with non-toxic diets after hatching (Matsui et al., 1982; Noguchi et al., 2006) and such non-toxic pufferfish become toxic when orally administered TTX (Honda et al., 2005; Yamamori et al., 2004). These findings indicate that the toxification of pufferfish is exogenous and derived from a food chain that begins with marine bacteria, although the transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via the food organisms remain to be elucidated.

The toxin content and distribution in the pufferfish fluctuate according to species. In the natural environment, *Takifugu flavipterus* (formerly known as *poecilonotus*) has high level of TTX in the skin, while adult *T. rubripes* accumulates little TTX in the skin. Several pufferfish species which possess high level of toxicity in the skin have secretory glands or secretory cells (sacciform cells) in the skin (Kodama et al., 1986), but in *T. rubripes* no glandular structure is observed, which is considered as the

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possible reason for the difference of toxicity in the skin among those species (Ikeda et al., 2009).

On the other hand, freshwater pufferfish contain paralytic shellfish poison (PSP) as the major toxic component. PSP is a group of neurotoxins produced by toxic dinoflagellates of the genera *Alexandrium, Gymnodinium,* and *Pyrodinium* in marine environments and by toxic cyanobacteria of the genera *Anabaena, Cylindrospermopsis, Aphanizomenon, Planktothrix*, and *Lyngbya* in freshwater environments (Cusick et al., 2013). The main component of PSP, saxitoxin (STX), has an almost equivalent molecular size and mode of action to TTX (Cusick et al., 2013). Marine *Sphoeroides* pufferfish in Florida (Landsberg et al., 2006) and marine *Arothron* pufferfish in the Philippines (Sato et al., 2000) and Japanese coastal waters (Nakashima et al., 2004) are also highly toxified by PSP. In addition, small amounts of PSP are detected in edible marine pufferfish of the genus *Takifugu* (Kodama et al., 1983; Nakamura et al., 1984; Jang et al., 2006), raising a food hygiene issue in Japan. Considering the similarity of the molecular size, toxicity and the poisoning mechanism between TTX and STX, why freshwater pufferfish specifically accumulate PSP still remains unclear.

To understand the TTX kinetics after TTX is absorbed into the pufferfish body through the intestine, various TTX administration experiments were conducted using artificially reared pufferfish specimens.

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Nontoxic specimens of *T. rubripes* and *T. alboplumbeus* were reared with TTX-containing diets on a long-term basis, and as results, they accumulate TTX efficiently into specific tissues, such as the skin, liver, and ovary (Matsui et al., 1981; Yamamori et al., 2004; Honda et al., 2005; Kono et al., 2008). Also it was suggested that after aborting the TTX-containing diet, the TTX content in the liver gradually decreased, while it increased in the skin and ovary. Intramuscular or intravenous administration of TTX to the nontoxic specimens to observe short-term toxin behavior just after administration revealed that the administered TTX is transferred first to the liver and then to the skin via blood circulation, but toxin transfer to the gonads differs greatly between males and females with the toxin transferring to and accumulating in only the ovary (Ikeda et al., 2009; Matsumoto et al., 2008b; Wang et al., 2011, 2012). According to these researches, the TTX kinetics inside the pufferfish body has been partly clarified. However, Nagashima et al. (2003) and Matsumoto et al. (2005, 2007) demonstrated that the ability to take up TTX is TTX-specific by *in vitro* experiments using *T. rubripes* liver tissue slices, and that the ability is higher than that of the livers of other fish, and suggested the exhibition of a carrier-mediated transport system for TTX into the liver. In addition, the toxin-binding protein (puffer fish saxitoxin and tetrodotoxin binding protein; PSTBP) was separated from the blood plasma of the pufferfish *T. alboplumbeus* and *Takifugu pardalis* (Matsui et al., 2000; Yotsu-Yamashita

et al., 2001), and genes homologous to that of PSTBP were also found in *T. rubripes* and other *Takifugu* pufferfishes (Tatsuno et al., 2013b; Hashiguchi et al., 2015). Yotsu-Yamashita et al. (2013) suggested that these TTX-binding proteins could be involved in TTX transfer via blood circulation to the skin. These studies indicated that the molecular mechanisms involved in the transfer/accumulation of TTX in the skin and liver could be different.

The distribution of TTX inside the pufferfish body is also affected by the growth of the individuals even in the same species. In wild adult *T. rubripes*, the liver and ovary are generally strongly toxic, and the skin, muscle, and testis are non-toxic (Noguchi and Arakawa, 2008), but in wild young fish, the skin is the main toxin-accumulating tissue. In TTX administration experiments using non-toxic cultured young *T. rubripes*, much of the TTX is transferred to the skin (Honda et al., 2005; Ikeda et al., 2009). Tatsuno et al. (2013) conducted an *in vivo* oral gavage TTX administration experiment in *T. rubripes* of different ages, and found that the administered TTX was mainly transferred to the skin in young fish (6 months old), whereas most of it was transferred to and accumulated in the liver in adult fish (15 months old). They speculated that because the liver is undeveloped and has low TTX-accumulating ability in young fish, the TTX mainly accumulates in the skin for elimination, but as the liver develops TTX accumulates and is stored in the liver. The distribution of

TTX in the pufferfish body also changes with the maturation of individuals. In the natural environment, *T. flavipterus*, and *T. alboplumbeus* typically have high concentrations of TTX in the liver and skin, but during maturation females accumulate TTX mainly in the ovary and skin, and males accumulate TTX mainly in the skin and liver, with the total TTX amount being higher in females (Ikeda et al., 2010; Itoi et al., 2016). Wang et al. (2011) reported that TTX administered intramuscularly to hybrid specimens produced by crossbreeding *T. rubripes* with *T. alboplumbeus*, which matures earlier than *T. rubripes*, is first taken up in the liver and then transferred to and accumulates in the ovary in females and the skin in males. It is possible that the organs of pufferfish have different TTX uptake/accumulation pattern depending on the species, and growth/maturation stage. However, there still are some unclear points on how the development of the liver, skin, and ovary affect the TTX distribution inside the pufferfish body.

In the present study, to further clarify the TTX transfer/accumulation mechanism in pufferfish, focusing on those main accumulation organs including the liver, skin, ovary, and the first barrier when TTX is absorbed into the pufferfish body, we investigated the toxin transfer/accumulation profiles of each tissue based on *in vivo* toxin administration experiments and *in vitro* tissue incubation experiments on nontoxic artificially reared pufferfish, and the survey on wild pufferfish.

In Chapter I, to clarify the differences of the toxin selectivity between marine and freshwater pufferfish, *in vivo* administration experiments of TTX and PSP were conducted using artificially reared nontoxic specimens of *T. pardalis* (marine) and *Pao suvattii* (freshwater). *T. pardalis* specimens were administered feed homogenate containing TTX or dicarbamoylSTX (dcSTX) (dose of toxin, 55.2 nmol/fish) and *P. suvattii* specimens were administered feed homogenate containing TTX + STX (dose of each toxin, 19.2 nmol/fish) by oral gavage, and then collected after 24 and 48 or 72 h. In *T. pardalis*, TTX administered into the intestine was absorbed into the body and transferred and retained mainly in the skin and liver, while dcSTX was hardly retained in the body, although it partly remained in the intestine. In strong contrast, in *P. suvattii,* little TTX remained in the body, whereas STX was absorbed into the body and was transferred and retained in the ovary and skin. The findings revealed that TTX/PSP selectivity differs between the marine species *T. pardalis* and the freshwater species *P. suvattii. T. pardalis*, which naturally harbors TTX, selectively accumulates TTX, and *P. suvattii*, which naturally harbors PSP, selectively accumulates PSP.

In Chapter II, the TTX uptake ability of the tissues in *T. rubripes* was investigated using *in vitro* tissue incubation experiment. The tissue slices prepared from the liver, skin, and intestine of the nontoxic cultured *T. rubripes*, were incubated in a 25 µg/mL TTX-containing buffer for 1-48 h,

and then the TTX content of each tissue was quantified. As a result, the TTX content temporally increased in all tissues. The TTX content in the skin and intestine slices was comparable to or slightly higher than that in the liver slices, with a similar transition pattern between the three tissue types. Therefore, we consider that the tissue slice incubation method can be applied for evaluating the TTX uptake ability of the skin and intestine. Same experiments were conducted using adult and young *T. rubripes* specimens. There is no significant difference of the TTX uptake ability of the liver and intestine between them, however, the skin slices of young fish took up approximately twice as much TTX as that of adult fish, suggesting that the TTX uptake ability of the skin is involved in the growth-dependent changes in the toxin distribution inside the body in *T. rubripes*. According to the observation on the TTX micro-distribution in the tissue slices using immunohistochemical staining, TTX is transferred and accumulates from pancreatic exocrine cells to hepatic parenchymal cells in the liver, from connective tissues to basal cells in the skin, and from villi epithelial cells via the lamina propria to the muscle layer in the intestine.

In Chapter III, TTX (dose of toxin, 30 (Low dose; LD), 100 (Medium dose; MD), and 300 (High dose; HD) µg/individual) was intramuscularly administered to cultured *T. rubripes*, and then fish were collected 24 h after toxin administration to elucidate the differences in TTX transfer/accumulation profiles between the liver and skin. As a result,

although the TTX concentration of both the liver and skin significantly increased with an increase in the dose, the slope coefficients differed significantly. At LD, the TTX concentration of the liver and skin is almost at the same level (0.43-0.48 µg/g), but at HD, the TTX concentration of the liver (6.0 µg/g) is approximately as 3.5 times higher than the skin (1.7 µg/g). Relationships between the dose and the TTX accumulation ratio (ratio (%) of accumulated TTX in each tissue (mg/tissue) to the administered dose (mg/individual)) in the skin and liver also significantly differed. The TTX accumulation ratio of the skin (11.1-38.6%) declined when the dose of administered TTX increased, while that of the liver (18.4-21.3%) almost remained constant. Therefore, it is possible that the TTX accumulation mechanisms in these two organs differ, and the higher the intake of TTX, the higher TTX accumulation ratio in the liver.

In Chapter IV, the maturation-associated changes in the TTX transfer/accumulation profiles of the ovary in wild *T. pardalis* was investigated. Twenty-two female individuals were collected at a specific location in Omura Bay, Nagasaki Prefecture, from November through December 2014, and then the maturation stage of those were separated to yolk vesicle stage (Yv), yolk globule stage (Yg)-1, -2, and -3 based on the ovary section. The TTX concentration in the tissues at each maturation stage was quantified. The TTX concentration in the ovary increased markedly from Yv to Yg-1, so was the TTX content in the ovary from Yg-

1 to Yg-3. On the other hand, 20 female individuals (February and March designed as the pre-spawning stage, and April and May as the post-spawning stage base on gonadosomatic index (GSI)) collected at the same place from February through May 2015 were investigated as well. In the post-spawning stage, the TTX concentration in the ovary were similar to that in the pre-spawning stage, but the TTX content of the ovary declined significantly while the TTX concentration of the liver increased rapidly. The TTX distribution in the oocytes differed among those maturation stages, positive signals of TTX were observed in the nucleus and yolk vesicles in the oocytes at the yolk vesicle stage, mainly in the yolk globules at the yolk globule stage and in the egg membrane at the pre-spawning stage.

In Chapter V, the above results were comprehensively discussed.

Chapter I

Contrasting Toxin Selectivity between the Marine Pufferfish *Takifugu pardalis* and the Freshwater Pufferfish *Pao suvattii*

Marine pufferfish of the genus *Takifugu*, including *Takifugu pardalis*, contain the potent neurotoxin TTX as a main toxic component. On the other hand, small-sized freshwater pufferfish, including *Pao suvattii* (formerly known as *Tetraodon suvattii*), contain PSP as the major toxic component in their skin and ovary, and consumption of these fish occasionally causes food poisoning in Southeast Asian countries such as Thailand and Cambodia (Kungsuwan et al., 1997; Ngy et al., 2008). Marine pufferfish are thought to accumulate PSP via the food chain starting from PSP-producing dinoflagellates (Landsberg et al., 2006; Sato et al., 2000), while freshwater pufferfish are thought to accumulate PSP from PSP-producing cyanobacteria (Zaman et al., 1997). It is unclear, however, whether the marine *Takifugu* pufferfish can accumulate PSP equally to freshwater pufferfish or whether freshwater pufferfish can accumulate TTX equally to marine *Takifugu* pufferfish if the toxin supply in the food is sufficient. In this chapter, we administered TTX and/or PSP to artificially reared nontoxic specimens of *T. pardalis* and *P. suvattii* to clarify the differences in toxin selectivity between marine and freshwater pufferfish.

1. Materials and methods

1.1. Pufferfish specimens

Artificially reared nontoxic 12-month-old *T. pardalis* (body length, 10.2 ± 0.5 cm; body weight, 36.7 ± 6.2 g; n = 30 (11 females and 19 males)) and *P. suvattii* (body length, 8.3 ± 0.4 cm; body weight, 28.0 ± 3.1 g; n = 8 (5 females and 3 males)) were used for the toxin administration experiments, as described below. The GSI (100 x gonad weight/body weight) in *T. pardalis* was 0.29 ± 0.06 in females and 0.35 ± 0.16 in males, and that in *P. suvattii* was 9.6 ± 2.1 in females and 0.45 ± 0.16 in males.

1.2. Preparation of toxic feed homogenate

TTX extracted from the ovaries of wild *T. rubripes* and PSP extracted from the xanthid crab *Zosimus aeneus* were purified by solvent partitioning, activated charcoal treatment, and Bio-Gel P-2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Bio-Rex 70 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) column chromatography according to the previously reported method (Arakawa et al, 1994), affording TTX (purity >60%), and dcSTX and STX (purity >80%) as essentially single components (content of 4-*epi*TTX in the TTX preparation, <2%). We administered dcSTX to *T. pardalis* and STX to *P. suvattii* because the availability of STX was insufficient. An aqueous solution of TTX or dcSTX was mixed with artificial feed for marine juvenile fish (Otohime C2, Marubeni Nisshin

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Feed Co., Ltd., Tokyo, Japan) at a ratio of 2/1 (v/w) and homogenized, and then feed homogenates containing either TTX or dcSTX (552 nmol/mL) were administered to *T. pardalis*. For *P. suvattii*, the feed homogenate was similarly prepared to contain a mixture of TTX and STX (192 nmol/mL each).

1.3. Toxin administration to *T. pardalis*

T. pardalis specimens were divided into three groups of 10 individuals—two TTX administration groups and one dcSTX administration group—and then maintained separately in three aerated 90-L tanks with artificial seawater at 25 °C. Each specimen was administered TTX- or dcSTX-containing feed homogenate (dose of toxin, 55.2 nmol/fish) by oral gavage and immediately returned to the tank. One TTX administration group and the dcSTX administration group were collected at 72 h. As the amount of TTX available was sufficient, a 24 h TTX administration group was also used to confirm the transition of the toxin distribution inside the body.

1.4. Toxin administration to *P. suvattii*

P. suvattii specimens were maintained in an aerated 60 L tank with dechlorinated tap water at 25 °C. Each specimen was administered the feed homogenate containing TTX and STX (dose of each toxin, 19.2 nmol/fish)

by oral gavage and immediately returned to the tank. Four specimens were randomly collected at 24 or 48 h after toxin administration, and toxin quantification was performed as described below to compare the distribution of each toxin.

1.5. Toxin quantification

The intestine, muscle, skin, liver, and gonads (testes/ovaries) were removed an extracted with 0.1 M HCl, passed through an HLC-DISK membrane filter (0.45 µm, Kanto Chemical Co., Inc., Tokyo, Japan), and submitted to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for TTX and/or high-performance liquid chromatography with post-column fluorescence derivatization (HPLC-FLD) for PSP.

In the LC-MS/MS analysis, chromatography was carried out using an Alliance 2690 Separations Module (Waters, Milford, MA, USA) with a Mightysil RP-18 GP column (2.0 x 250 mm, particle size 5 µm, Kanto Chemical Co., Inc., Japan) and mobile phase comprising 30 mM heptafluorobutyric acid in 1 mM ammonium acetate buffer (pH 5.0) at a flow rate of 0.2 ml/min. The eluate was introduced into a Quattro micro™ API detector (Waters) in which the TTX was ionized by positive-mode electrospray ionization with a desolvation temperature of 350 °C, source block temperature of 120 °C, and cone voltage of 50 V, and monitored at m/z 162 (for quantitative) and 302 (for qualitative) as product ions

(collision voltage 38 V) with *m/z* 320 as a precursor ion through a MassLynx^{TM} NT operating system (Waters). The limit of detection (LOD) and limit of quantification (LOQ) of TTX was 0.0009 nmol/mL (0.003 nmol/g tissue; $S/N = 3$) and 0.003 nmol/mL (0.009 nmol/g tissue; $S/N = 10$), respectively.

HPLC-FLD was performed using an Alliance 2690 Separations Module with a LiChroCART Superspher RP18(e) column (4.6 x 250 mm, particle size 4 μm, Merck, Germany) and a mobile phase comprising 2 mM heptanesulfonic acid in 4% acetonitrile-30 mM ammonium phosphate buffer (pH 7.3) at a flow rate of 0.8 ml/min. The eluate was continuously mixed with 50 mM periodic acid and 0.2 M KOH containing 1 M ammonium formate and 50% formamide, and heated at 65 °C. The formation of fluorophores was monitored at 392 nm with 336 nm excitation. The LOD of both dcSTX and STX was 0.007 nmol/mL (0.02 nmol/g tissue; $S/N = 3$) and the LOQ of both dcSTX and STX was 0.02 nmol/mL (0.06 nmol/g tissue; $S/N = 10$).

2. Results and discussion

The toxin concentration in the examined organs of *T. pardalis* and *P. suvattii* at 24 and 48 or 72 h after TTX and/or PSP administration is shown in **Fig. 1** and **Tables 1** and **2**. In *T. pardalis*, TTX administered into the intestine by oral gavage was taken up into the body and transferred mainly
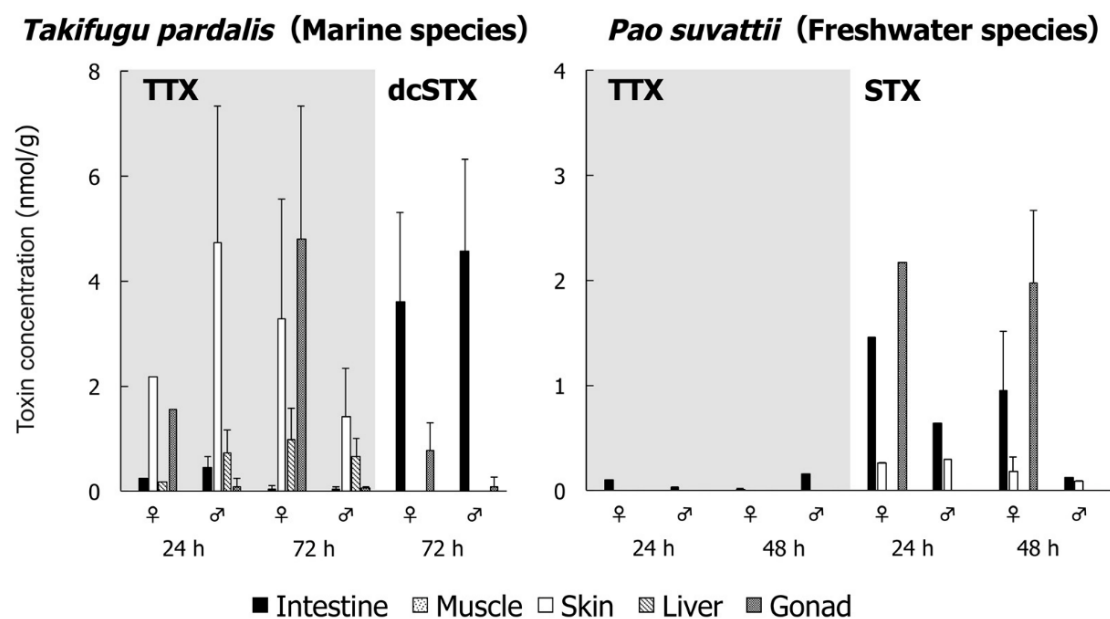
Fig. 1. Toxin concentration in different organs of *T. pardalis* (left) and *P. suvattii* (right) at 24 and 48 or 72 h after TTX and/or PSP (dcSTX or STX) administration. Data are shown as means (columns) and SDs (error bars).

Table 1. Toxin concentration (mean value) in different organs of *T. pardalis* at 24 and/or 72 h after TTX or dcSTX administration.

Toxin	Rearing Time	Sex	*n*	Toxin Concentration (nmol/g) *				
				Intestine	Muscle	Skin	Liver	Gonad
TTX	24 h	Female	2	0.25	0	2.18	0.17	1.56
		Male	8	0.45	0	4.72	0.72	0.09
	72 h	Female	5	0.05	0	3.28	0.98	4.80
		Male	5	0.04	0	1.42	0.65	0.05
dcSTX	72 h	Female	4	3.60	0	0	0	0.78
		Male	6	4.57	0	0	0	0.08

* Below the limit of quantification (LOQ) was considered to be zero (0).

Table 2. Toxin concentration (mean value) in different organs of *P. suvattii* at 24 and 48 h after TTX + STX administration.

Toxin	Rearing Time	Sex	n	Toxin Concentration (nmol/g) *				
				Intestine	Muscle	Skin	Liver	Gonad
TTX	24 h	Female	2	0.10	0	0	0	0
		Male	2	0.03	0	0	0	0
	48 h	Female	3	0.01	0	0	0	0
		Male	1	0.15	0	0	0	0
STX	24 h	Female	2	1.45	0	0.26	0	2.16
		Male	2	0.64	0	0.29	0	0
	48 h	Female	3	0.95	0	0.18	0	1.97
		Male	1	0.12	0	0.09	0	0

* Below the LOQ was considered to be zero (0).

to the skin, ovary, and liver. The TTX concentration was the highest in the skin at 24 h and in the ovary at 72 h, and the TTX concentration in the liver was generally lower than that in the ovary and skin. At 24 h, a low concentration of TTX remained in the intestine, and at 72 h, the concentration in the intestine further decreased to a trace level. On the other hand, the dcSTX concentration in the intestine remained high even at 72 h and was detected at low concentrations only in the gonads among the other organs examined. In *P. suvattii*, in strong contrast to *T. pardalis*, no TTX was detected in any organs other than the intestine at 24 and 48 h, while STX was taken up into the body and transferred only to the ovary and skin. Interestingly, a much higher concentration of STX than TTX remained in the intestine, similar to *T. pardalis*.

The relative toxin amount (% of the administered toxin) in each organ of *T. pardalis* and *P. suvattii* is shown in **Fig. 2** and **Tables 3** and **4**. In *T. pardalis*, 16.6–55.1% of the administered TTX was absorbed and retained in the body at 24 and 72 h, with most (82.7–95.1% of the "Total") distributed in the skin and the rest distributed mainly in the liver. At 72 h, little toxin (~0.1% of the administered toxin) remained in the intestine. On the other hand, dcSTX was not retained in the rest of the body except for in the gonads, in which little toxin accumulated (0.2% in females and 0.01% in males), and 8.4–10.3% of the dose remained in the intestine. In strong contrast, in *P. suvattii*, little TTX (0.03–0.5%) remained in the body
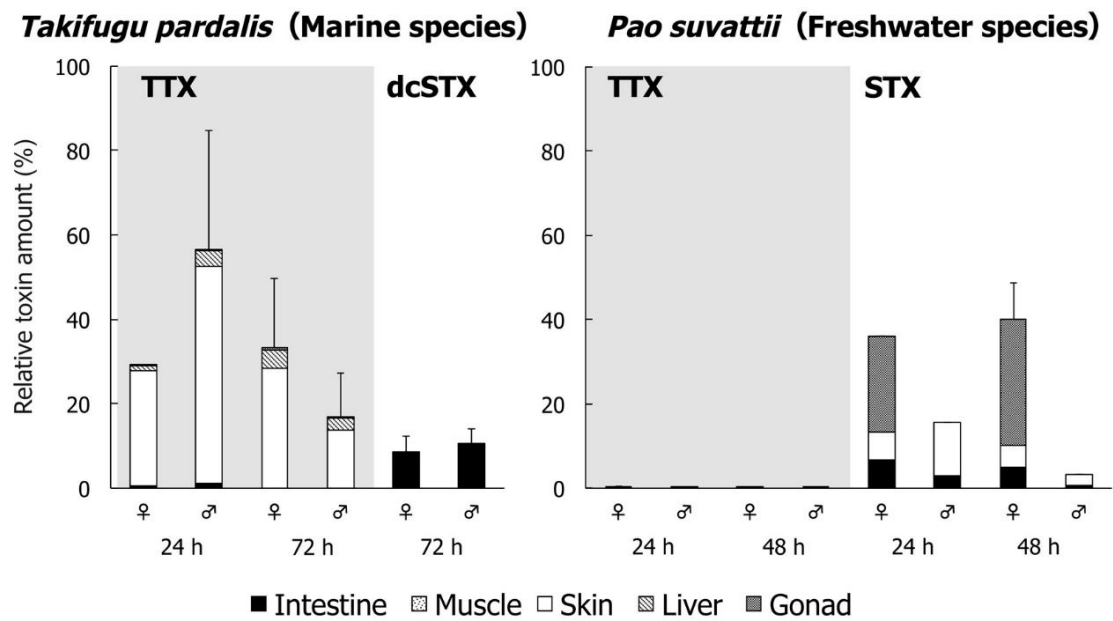
Fig. 2. Relative toxin amount (% of the administered toxin) in different organs of *T. pardalis* (left) and *P. suvattii* (right) at 24 and 48 or 72 h after TTX and/or PSP administration. Data are shown as means (columns) and SDs (error bars).

Table. 3. Relative toxin amount (% of the administered toxin, mean value) in different organs of *T. pardalis* at 24 and/or 72 h after TTX or dcSTX administration.

Toxin	Rearing Time	Sex	*n*	Relative Toxin Amount (%)					
				Intestine	Muscle	Skin	Liver	Gonad	Total *
TTX	24 h	Female	2	0.6	0	27.2	1.2	0.2	28.6
		Male	8	1.0	0	51.5	3.7	0.01	55.1
	72 h	Female	5	0.1	0	28.4	4.1	0.7	33.2
		Male	5	0.1	0	13.7	2.9	0.01	16.6
dcSTX	72 h	Female	4	8.4	0	0	0	0.2	0.2
		Male	6	10.3	0	0	0	0.01	0.01

* Total of the organs other than intestine.

Table. 4. Relative toxin amount (% of the administered toxin, mean value) in different organs of *P. suvattii* at 24 and 48 h after TTX + STX administration.

Toxin	Rearing Time	Sex	*n*	Relative Toxin Amount (%)					
				Intestine	Muscle	Skin	Liver	Gonad	Total *
TTX	24 h	Female	2	0.5	0	0	0	0	0
		Male	2	0.03	0	0	0	0	0
	48 h	Female	3	0.04	0	0	0	0	0
		Male	1	0.1	0	0	0	0	0
STX	24 h	Female	2	6.8	0	6.5	0	22.8	29.3
		Male	2	3.0	0	12.6	0	0	12.6
	48 h	Female	3	5.1	0	5.2	0	29.8	35.0
		Male	1	0.6	0	2.7	0	0	2.7

* Total of the organs other than intestine.

at 24 and 48 h, whereas STX (29.3–35.0% in females and 2.7–12.6% in males) was absorbed by the body and retained in the ovary and skin. The intestine contained 0.6–6.8% of the administered dose.

It is strongly indicated that the marine species *T. pardalis* and the freshwater species *P. suvattii* are endowed with contrasting TTX/PSP selectivity. Due to the scarcity of PSP preparations and of artificially reared pufferfish specimens, or as a matter of practical convenience, the two toxin administration experiments conducted in the present study differed with respect to the experimental conditions, such as the PSP components, dose, administration method (TTX and PSP administered alone or in combination), number of test fish, and rearing period, thereby preventing a quantitative comparison of the selectivity between species. Nevertheless, our findings clearly demonstrate for the first time that *T. pardalis*, which naturally harbors TTX, selectively accumulates TTX, and *P. suvattii*, which naturally harbors PSP, selectively accumulates PSP. Ngy et al. (2008) intramuscularly administered TTX and PSP into artificially reared specimens of the freshwater pufferfish *Leiodon* (formerly known as *Tetraodon*) *tergidus* and found that PSP rapidly transferred from the muscle to the skin, where it accumulated. On the other hand, all the fish administered TTX died within 3–4 h, and approximately half the TTX dose remained in the muscles of the dead fish. Although the toxin administration method differed from that in the present study, *L. tergidus* is considered to

have similar toxin selectivity as *P. suvattii*. In addition, this finding indicates that freshwater pufferfish are not very resistant to TTX. Marine pufferfish have much stronger resistance to TTX than do general fish, but their resistance to PSP is not very strong (Saito et al, 1984; Saito et al, 1985; Saito et al, 1989). Thus, toxin selectivity could be closely related to toxin resistance.

Chapter II

Evaluation of the tetrodotoxin uptake ability of pufferfish *Takifugu rubripes* tissues according to age using an *in vitro* tissue slice incubation method

Distribution of TTX inside the pufferfish body varies depending on the developmental stage. To understand the mechanism of the age-dependent changes, most of the previous studies have focused on the TTX uptake ability of the liver. Nagashima et al. (2003) and Matsumoto et al. (2005, 2007), using an *in vitro* tissue slice incubation method, demonstrated that liver tissues of marine *Takifugu* pufferfish, unlike those of general marine fish, take up a considerable amount of TTX. They did not consider the TTX uptake ability of the intestine, however, which serves as the first barrier when TTX in the food organisms is absorbed into the pufferfish body, or of the skin, which, like the liver, is a main transfer destination of TTX absorbed into the body. In this chapter, to clarify the mechanisms involved in the unique kinetics of TTX in the pufferfish body and growth-associated changes, firstly whether the *in vitro* tissue slice incubation method is applicable for evaluating the TTX uptake ability of not only the liver but also the skin and intestine, was investigated, and then the TTX uptake ability of these tissues between young (8 months old) and adult (20 months old) *Takifugu rubripes* was compared. Moreover, to estimate the TTX

uptake pathway in each tissue, an immunohistochemical technique (Tanu et al., 2002) was used to observe temporal changes in the intra-tissue micro-distribution of TTX during incubation.

Section 1

TTX accumulation profiles of tissue slices after *in vitro* incubation

To investigate whether the *in vitro* tissue slice incubation method is applicable for evaluating the TTX uptake ability of various tissues, I applied the liver, skin and intestine of artificial adult *T. rubripes* for the *in vitro* incubation experiment, and then similar experiments were conducted for young individuals and additional adults.

1. Materials and methods

1.1. Pufferfish specimens

Non-toxic cultured young (8 months old; body length, 13.5 ± 0.5 cm; body weight, 80.0 ± 6.1 g; *n*=3) and adult (20 months old; body length, 28.4 ± 0.8 cm; body weight, 743 ± 61 g; *n*=3) *T. rubripes* were used for the tissue slice incubation experiments described below.

1.2. TTX preparation

TTX extracted from the ovaries and livers of *T. pardalis*, and purified by solvent partitioning, activated charcoal treatment, and Bio-Gel P-2 (Bio-Rad Laboratories, Hercules, CA, USA) column chromatography according to a previously reported method (Arakawa et al., 1994) was used for the tissue slice incubation experiments. Crystalline TTX (Nacalai

Tesque, Inc., Kyoto, Japan) was used as a standard for the TTX quantification analysis described below.

1.3. Tissue slice incubation experiments

The liver, skin and intestine were collected from one of the adult fish, and an incubation experiment was conducted according to the method of Matsumoto et al. (2007). Briefly, 12 tissue slices (8 mm in diameter, ~1 mm in thickness) were prepared from the liver, dorsal skin, and intestine, which was first sliced longitudinally to form a sheet. Each slice was incubated with a 1.5 ml of incubation buffer (160 mM NaCl, 4.8 mM KCl, 23.8 mM $NaHCO_3$, 0.96 mM KH_2PO_4, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 12.5 mM HEPES, and 5.0 mM D-glucose; adjusted to pH 7.4 with NaOH solution) containing 25 µg/mL TTX in a 15-mL plastic tube aerated with O_2 and CO_2 at a 9:1 ratio at 20 °C for a maximum of 48 h. During the incubation, 3 slices of each tissue were collected at 1, 8, 24, and 48 h, washed with neutral phosphate buffer (0.15 M NaCl and 0.01 M Na_2HPO_4; adjusted to pH 7.0 with 0.15 M NaCl and 0.01 M NaH_2PO_4), and weighed. To investigate whether the TTX uptake ability of each tissue differed according to the age of the fish, three tissue slices were similarly prepared from the liver, skin, and intestine of the three young fish and the remaining two adult fish, and an incubation experiment was conducted for 8 h.

1.4. TTX quantification

TTX was quantified by LC-MS/MS analysis according to the method described in Chapter I 1.5. Toxin quantification.

1.5. Statistical analysis

Statistical analysis was performed by combining the data from the 8-h incubation in the first incubation experiment and the data from the second incubation experiment. Considering that it is difficult to compare the toxin uptake ability between different tissues in this experiment, Student's *t*-test was performed for each tissue between young fish and adult fish using the mean TTX content of each individual (mean TTX content of the 3 slices).

2. Results and discussion

Changes in the TTX content of liver, skin, and intestine slices of adult *T. rubripes* during the incubation are shown in **Fig. 3**. The TTX content temporally increased in all tissues. The TTX content of the liver was 4.7 ± 1.7, 9.3 ± 2.5, 10.7 ± 0.5, and 13.6 ± 1.2 µg/g at 1, 8, 24, and 48 h of incubation, respectively, and was highest among the three tissues at 1 h, but the content of the skin and intestine exceeded that of the liver at 8 h and thereafter. The TTX content of the intestine was highest at 8 and 24 h (12.1 ± 1.7 and 17.3 ± 1.3 µg/g, respectively), and that of the skin was highest at 48 h (18.8 ± 1.4 µg/g).
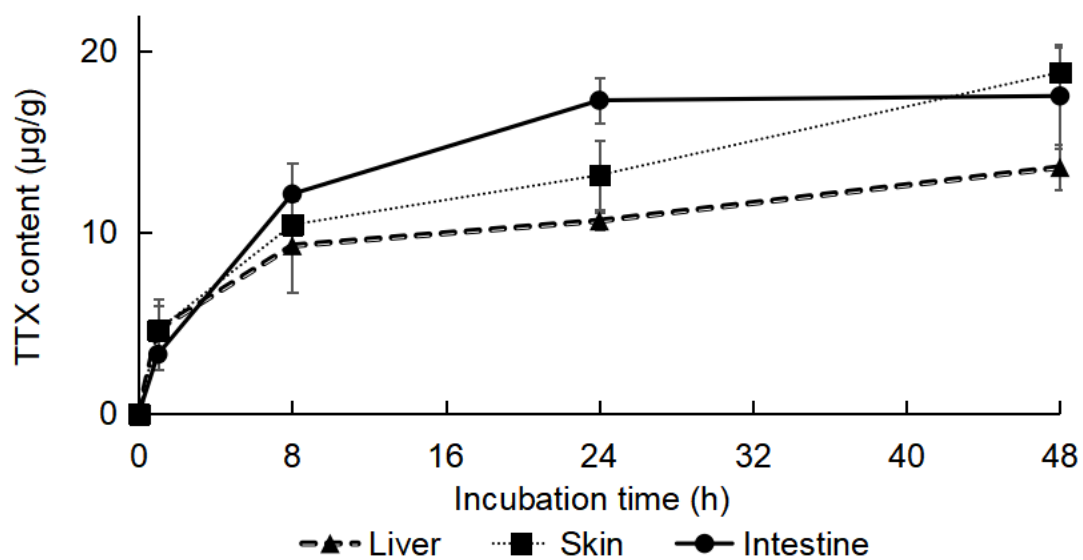
Fig. 3. Change in TTX content of the liver, skin, and intestine tissue slices

(n=4) from one adult *T. rubripes* individual during incubation for 48 h. The

bars indicate the standard deviation.

The TTX content of the tissue slices of the young and adult *T. rubripes* after 8 h of incubation is shown in **Figs. 4**. In the young fish and adult fish, the TTX content was 12.6 ± 1.2 and 11.0 ± 1.6 µg/g in the liver, 26.9 ± 2.7 and 11.6 ± 1.7 µg/g in the skin, and 15.5 ± 3.0 µg/g and 12.7 ± 4.8 µg/g in the intestine, respectively. The TTX content in the liver and intestine did not differ significantly between the young fish and adult fish, while it was significantly higher in the skin of the young fish compared with the adult fish ($p<0.05$).

Nagashima et al. (2003) reported that when the liver slices of several general fish species were incubated in the same TTX concentration as used in the present study (25 µg/mL), ~3-4 µg/g TTX was detected at 0.5 h, but the amount changed little thereafter. In contrast, in the liver slices of *T. rubripes*, the TTX content increased over time, and reached up to ~12 µg/g at 24 h and ~15 µg/g at 48 h. After that, the TTX content did not decrease even when incubated in incubation buffer containing no TTX. These findings led them to conclude that the liver tissue of *T. rubripes* is endowed with high TTX uptake ability. In the present study, TTX was taken up into the liver slices at nearly the same level at the same incubation times (~11 µg/g at 24 h, ~14 µg/g at 48 h), confirming the high reproducibility of this experimental system. The TTX content in the skin and intestine slices was comparable to or slightly higher than that in the liver slices, with a similar transition pattern between the three tissue types. The tissue structures and
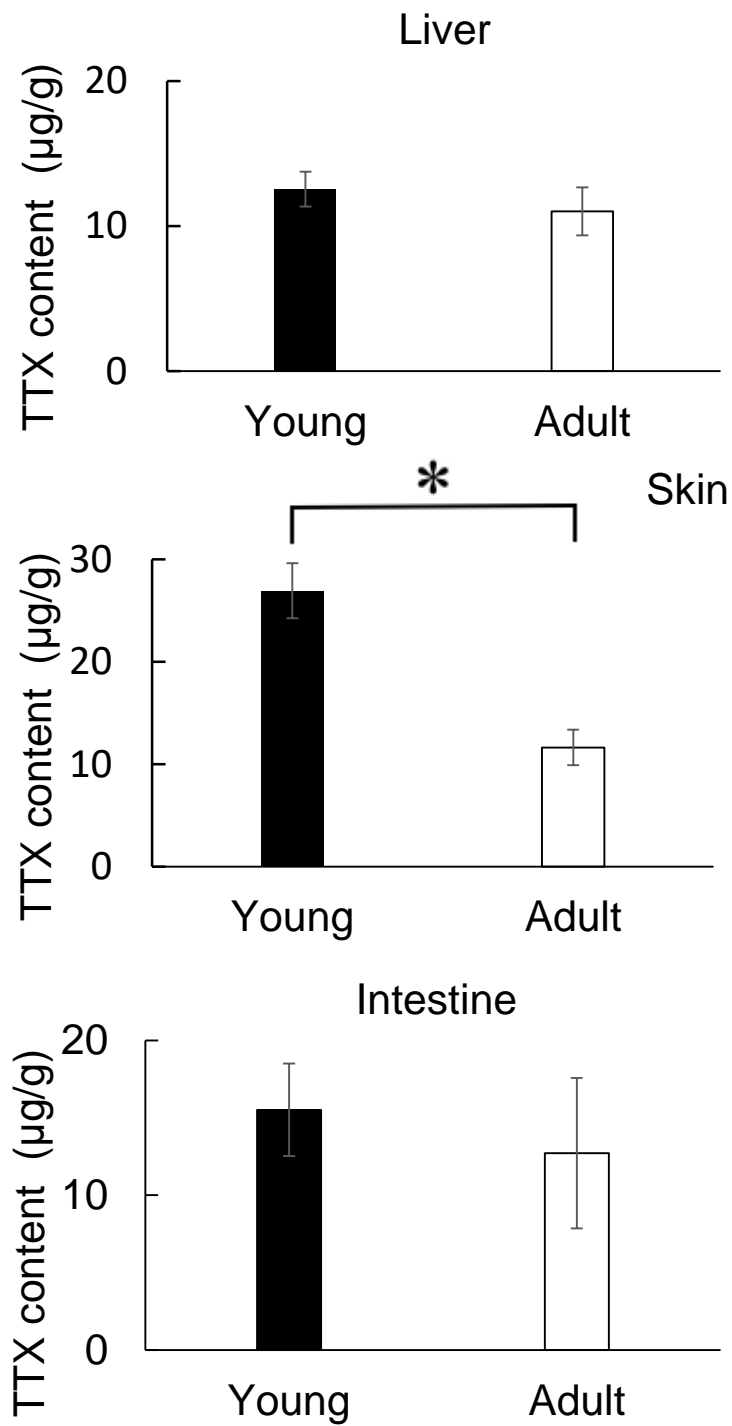
Fig. 4. Difference in TTX content of the liver (upper), skin (middle), and intestine (lower) tissue slices between young and adult *T. rubripes* after incubation for 8 h. Asterisk indicates significant difference (t-test, p < 0.05)

properties differ between the liver and skin/intestine, and it is unlikely that such a liver-like TTX uptake profile was caused by mere physical diffusion of TTX. Therefore, we concluded that the tissue slice incubation method can be applied for evaluating the TTX uptake ability of the skin and intestine, and that the TTX uptake ability of the skin and intestine of *T. rubripes* is similar to that of the liver. In future studies, the TTX uptake ability of the skin and intestine should be evaluated in non-toxic pufferfish and in general fish as well.

Wild adult *T. rubripes* accumulate high levels of TTX in the liver and ovary, but the skin, muscle, and testis are generally non-toxic (Noguchi and Arakawa, 2008). According to studies by Ikeda (2009) and Tatsuno (2012), however, the TTX content in the skin accounts for more than 90% of the total TTX content in wild young *T. rubripes* (small-sized fish with a body weight of 20.9 ± 3.9 g). Medium-sized fish (body weight 261 ± 66 g) have a lower TTX ratio in the skin than small sized fish, and the TTX content in the liver accounts for 15%-86%. Therefore, it is presumed that the skin is rather the main toxin accumulation tissue in young *T. rubripes*, but the liver becomes the main toxin repository as the fish grows. Similarly, in a rearing experiment in which cultured young (under 1 year old) and adult (under 2 years old) *T. rubripes* were fed a TTX-containing diet for 60 days, the TTX accumulation rate in the skin was higher in the young fish than in the adult fish (Honda et al., 2005). When TTX was administered intramuscularly to

cultured young *T. rubripes*, most of the toxin was transported to the skin where it accumulated (Ikeda et al., 2009). In a previous study (Tatsuno et al., 2013a), the TTX administered to *T. rubripes* by oral gavage was transferred mainly to the skin in 6- month-old fish, and to the liver in 15-month-old fish, which have a more developed liver. In *T. rubripes*, the TTX taken up into the body is transferred mainly to the skin in younger fish, but as the age increases and the liver matures, the ratio of toxin transferred/accumulated in the liver gradually increases. Recently, Kiriake et al. (2016) conducted a TTX uptake experiment using liver tissue slices from 4-month-old and 18-month-old nontoxic cultured *T. rubripes*, and claimed that there was no difference between TTX uptake amounts between the two ages. In the present study, the TTX uptake ability is higher in the skin of young fish than in the skin of adult fish. it is highly possible that the TTX uptake ability of the skin is involved with the growth-associated change of the TTX distribution in the *T. rubripes.*

Section 2

Temporal change in the microdistribution of TTX in tissue slices

after *in vitro* incubation

To estimate the TTX uptake pathway in the liver, skin, and intestine of *T. rupripes*, the tissue slices after *in vitro* incubation were used for immunohistochemical staining.

1. Materials and methods

1.1. Pufferfish specimens

A non-toxic cultured young *T. rubripes* (8 months old; body length, 13.6 cm; body weight, 82.4 g) and was used for the tissue slice incubation experiments described below.

1.2. Tissue slice incubation experiment

By the method described in Section 1, 6 tissue slices were prepared from the liver, skin, and intestine of the young *T. rubripes*, respectively, and incubated with incubation buffer containing 25 µg/mL TTX for a maximum of 8 h. During the incubation, 2 slices of each tissue were collected at 0.5, 2, and 8 h, and submitted to immunohistochemistry as described below.

1.3. Immunohistochemical observation

Tissue sections (6-µm thick) were prepared from the incubated tissue slices by conventional histologic procedures, and immunostained according to the previously reported method (Gao et al., 2018; Tanu et al., 2002). Briefly, the sections were successively treated with 10% H_2O_2 in water and 25% goat serum in 0.01 M phosphate-buffered saline (Iatron Lab. Inc., South Bend, IN, USA), and then incubated with a monoclonal anti-TTX antibody (Kawatsu et al., 1997), followed by a polymer, EnVision+ (Dako North America Inc., Carpinteria, CA, USA) for 60 min. For a negative control, mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA) was used instead of the anti-TTX antibody. After treating the sections with 0.017% 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries Ltd., Tokyo, Japan) substrate solution in 0.01 M phosphate-buffered saline, they were counter-stained with Mayer's hematoxylin (Merck, Darmstadt, Germany), and observed under an optical microscope (BZ-X700, Keyence Corp., Osaka, Japan). TTX-positive signals were indicated by a brown color.

2. Results and discussion

Changes in the micro-distribution of TTX in the liver, skin, and intestine tissue slices during the incubation are shown in **Figs. 5-7**. In the liver, weak TTX-positive signals were observed at the pancreatic exocrine
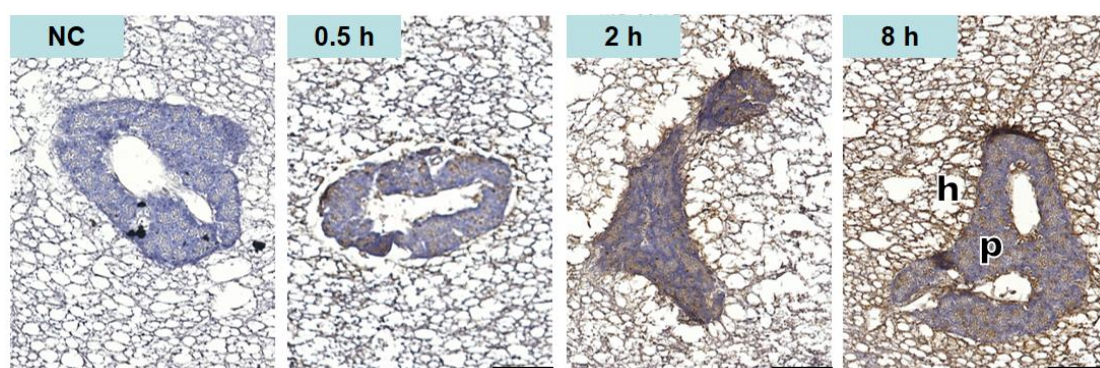
Fig. 5. Immunohistologic observation of the liver tissue slices after *in vitro* incubation. Positive immunoreaction was visualized as a brown color. Alphabetical letters h and p indicate hepatic parenchymal cell, and pancreatic exocrine cell, respectively. Scale bars indicate 50 µm.
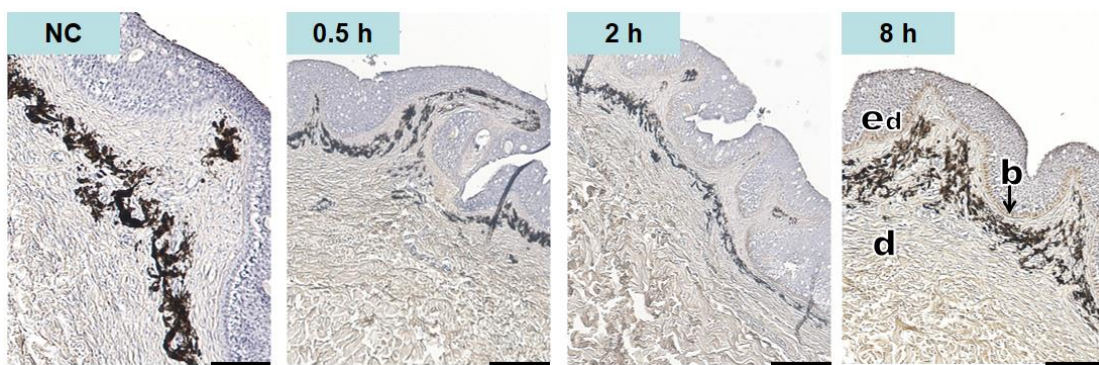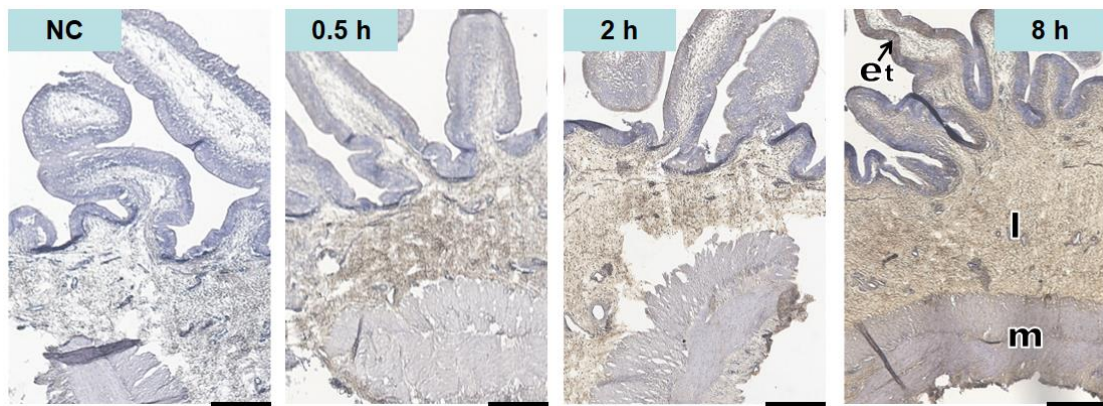
Fig. 6. Immunohistologic observation of the skin tissue slices after *in vitro* incubation. Positive immunoreaction was visualized as a brown color. Alphabetical letters e_d, d, and b indicates epidermis layer, dermis layer, and basal cells. Scale bars indicate 50 µm.

Fig. 7. Immunohistologic observation of the intestine tissue slices after *in vitro* incubation. Positive immunoreaction was visualized as a brown color. Alphabetical letters e_t, l, and m indicate epithelial cells, lamina proprial and muscular layer, respectively. Scale bars indicate 50 µm.

cells at 0.5 h of incubation, and the signal became stronger in the pancreatic exocrine cells and spread to surrounding hepatic parenchymal cells at 2 h. At 8 h, the whole section was stained brown. In the skin, weak positive signals were observed at the connective tissue on the muscle side (data not shown), but the epidermis and dermis layer were not stained at 0.5 h. Although there was no obvious change between 0.5 h and 2 h, strong TTX-positive signals were confirmed at basal cells between the epidermis and dermis at 8 h. In the intestine, weak positive signals were observed at the epithelial cells and lamina propria of the intestinal villi at 0.5 h, then the signals became stronger at 2 h and extended to the muscular layer at 8 h.

From the temporal change in the micro-distribution of TTX in each tissue slice, the TTX uptake pathway in each tissue can be estimated to some extent. In the liver, it seems that TTX is first taken up into pancreatic exocrine cells, and then spreads to hepatic parenchymal cells. In the skin, TTX seems to be first taken up into the connective tissues, and is then transferred to and accumulates in the basal cells. Many pufferfish have secretory glands or secretory cells (sacciform cells) in the skin (Itoi et al., 2012; Kodama et al., 1986; Mahmud et al., 2003a,b; Tanu et al., 2002) and release TTX from the skin in response to external stimuli (Kodama et al., 1985; Saito et al., 1985), but in *T. rubripes*, no glandular structure is observed in the skin, and TTX-positive signals are found only in the basal cells (Ikeda et al., 2009; Okita et al., 2013). Therefore, it is highly likely

that the basal cell properties are involved in the difference in the TTX uptake ability of the skin between young and adult fish, consistent with the findings of the present study. In the intestine, TTX was assumed to be taken up from the epithelial cells of the villi into the lamina propria, and gradually transferred to the muscle layer. It is unclear, however, how such uptake of TTX by the intestine slices is involved in intestinal TTX absorption *in vivo*. This point requires further clarification to apply the TTX uptake ability of the intestine slices as an index of TTX absorption ability or TTX selectivity in the intestine.

Chapter III

Profile differences in tetrodotoxin transfer to skin and liver in the pufferfish *Takifugu rubripes*

Tetrodotoxin accumulates mainly in the skin, liver, and ovary, and the muscle and testis are nontoxic in many species of marine pufferfish of the genus *Takifugu* (Tani, 1945). To clarify the transfer/accumulation mechanisms, various TTX administration experiments have been conducted using nontoxic specimens reared from eggs using nontoxic feeds. Those results revealed that the administered TTX is transferred first to the liver and then to the skin via blood circulation, but toxin transfer to the gonads differs greatly between males and females with the toxin transferring to and accumulating in only the ovary. According to the results from Chapter II, it is possible that the molecular mechanisms involved in the transfer/accumulation of TTX differ between the two main TTX-accumulating tissues, the skin and liver, but few studies have focused on this mechanism. In this chapter, to provide more insight on this mechanism, TTX was intramuscularly administered at different doses to nontoxic *T. rubripes* specimens, and the transfer profiles of TTX to the skin and liver were investigated.

Section 1

Transfer profiles of TTX to the skin and liver

To investigate the differences in accumulation mechanism between the skin and liver, TTX was intramuscularly administered at different doses to nontoxic *T. rubripes* specimens, then the TTX content of the skin and liver were quantified.

1. Materials and methods

1.1. Pufferfish specimens

Nontoxic cultured specimens of *T. rubripes* (8-month-old, body length 16.0 ± 1.2 cm, body weight 142.4 ± 23.7 g, GSI 0.06-0.23, $n = 17$), which had been reared with TTX-free diet since hatching, were used as test fish for the TTX administration experiment as described below.

1.2. Preparation of TTX solution

A TTX standard (Wako, purity >90%) was dissolved in 1.35% NaCl solution at different concentrations (0.3, 1.0, 3.0 mg/mL), and were used in the following experiments.

1.3. TTX administration experiment

The test fish were divided into three groups, Low dose (LD; $n = 6$),

Medium dose (MD; $n = 6$), and High dose (HD; $n = 5$), and each group was reared in a 200-L aerated tank. The TTX dose for each group was set at 30, 100, and 300 μg/individual (administration concentration 0.3, 1.0, and 3.0 mg/mL), respectively, referring to the minimum lethal intraperitoneal-dose of TTX to cultured *T. rubripes*, 3.3-5.5 μg/g body weight, (~500-800 μg/fish) (Saito et al., 1984, 1985a).

The absorption rate of TTX in the pufferfish digestive tract changes according to the administration concentration (Matsumoto et al., 2008a). To avoid this influence, intramuscular administration was used rather than oral gavage administration. The test fish of each group were injected with 0.1 mL TTX solution (30, 100, or 300 μg/individual) into the dorsal muscle, returned to the tank, and then collected 24 h after toxin administration. The skin, liver, and muscle were dissected from each fish, and toxin quantification each tissue were performed as described below.

1.4. TTX quantification

TTX from each tissue was extracted with 0.1% acetic acid in a 100 °C water bath (Japan Food Hygiene Association, 2005), and the extract, after passing through an HLC-DISK membrane filter (0.45 mm, Kanto Chemical Co., Inc., Japan) and appropriately diluting with pure water, was subjected to a liquid chromatography/mass spectrometry (LC/MS) analysis according to the method of Nakashima et al. (2004). Namely, LC/MS was

carried out on an alliance LC/MS system (Waters) equipped with a Zspray™ MS 2000 detector. A Mightysil RP-18 GP column (2.0 x 250 mm, Kanto Chemical) was used with 30 mmol/l heptafluorobutyric acid in 1 mmol/l ammonium acetate buffer (pH 5.0) for the mobile phase.

1.5. Statistical analysis

General linearized models were used to determine how the dose and its interaction with the tissue (i.e., liver or skin) influenced the TTX concentration and the TTX accumulation ratio. A Gaussian distribution and an identity link function were applied in the TTX concentration analysis and a binomial distribution with a logit function was used to model the TTX accumulation ratio. Models were fitted with R version 3.3.0 (R Development Core Team, 2016), and a likelihood ratio test was used to determine the statistical significance of the slopes among the liver and skin.

2. Results and discussion

The test fish in HD showed temporary paralysis just after TTX injection, and one died. The remaining 4 fish, however, recovered, and we observed no other abnormal signs thereafter. In all groups, TTX was detected in the skin and liver, but not in the muscle. Relationships between the dose and TTX concentration in the skin and liver are shown in **Fig. 8**. In both tissues, the TTX concentration significantly increased with an
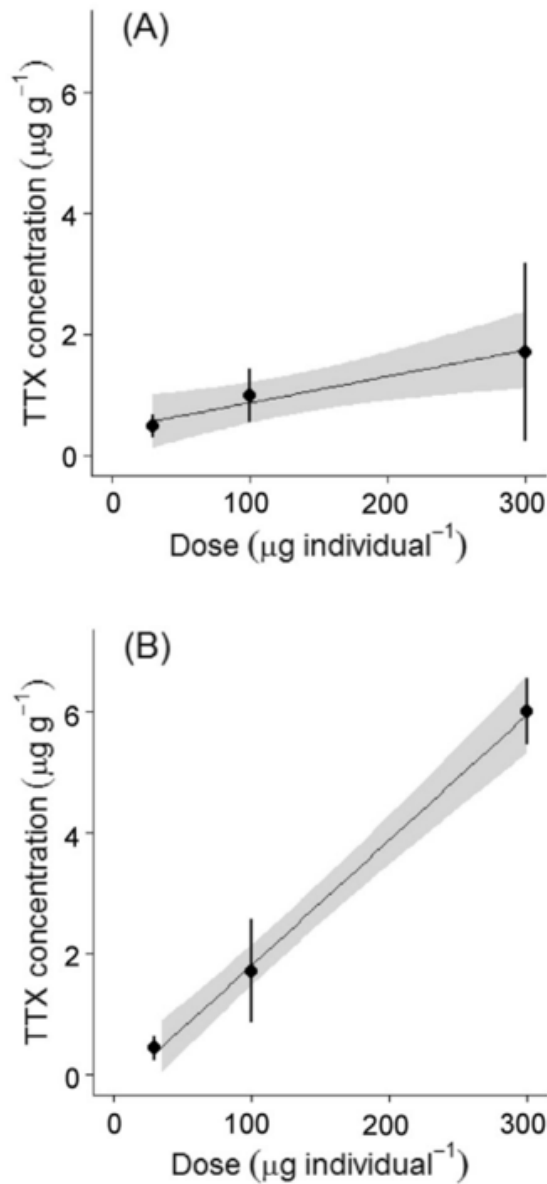
Fig. 8. TTX concentration (μg/g) of skin (A) and liver (B) as a function of administered dose; 30 ($n = 6$), 100 ($n = 6$), and 300 ($n = 4$) μg/individual. Data are shown as the mean (point) and SD (error bar) for each dose. Line and gray zone indicate the expected values of the Gaussian generalized linear model and the 95% confidence interval, respectively.

increase in the dose, but the slope coefficients differed significantly (skin, 0.0044 ± 0.002 (estimate ± 1 standard error); liver, 0.0208 ± 0.002, $p <$ 0.0001). Thus, the TTX concentration in the liver increased much more quickly than that in the skin as dose increased.

Relationships between the dose and the TTX accumulation ratio in the skin and liver are shown in **Fig. 9**. Binomial general linearized models revealed significant differences in the slope coefficient (skin, -0.0063 ±0.0007; liver, 0.0005 ± 0.006, $p <$ 0.0001). A slope coefficient of essentially 0 for the liver indicates that TTX dose had no effect on the TTX accumulation ratio in the liver.

As described above, when TTX was administered intramuscularly to immature specimens of *T. rubripes* at doses of 30, 100, or 300 µg/individual, the toxin transfer profile after 24 h differed greatly between the skin and liver. The toxin concentration of the liver was almost equivalent to that of the skin at the low dose (30 µg/ individual), but reached 3-fold that of the skin at the high dose (300 µg/individual). Therefore, it is presumed that the more TTX ingested by *T. rubripes*, the higher the accumulated toxin ratio in the liver. Although TTX in the present study was administered through a route that does not naturally occur, Honda et al. (2005) reported that when cultured *T. rubripes* are reared with TTX-containing diets for 60 days, they accumulate a small amount of toxin in the skin and liver at low doses, and a large amount of toxin in the liver
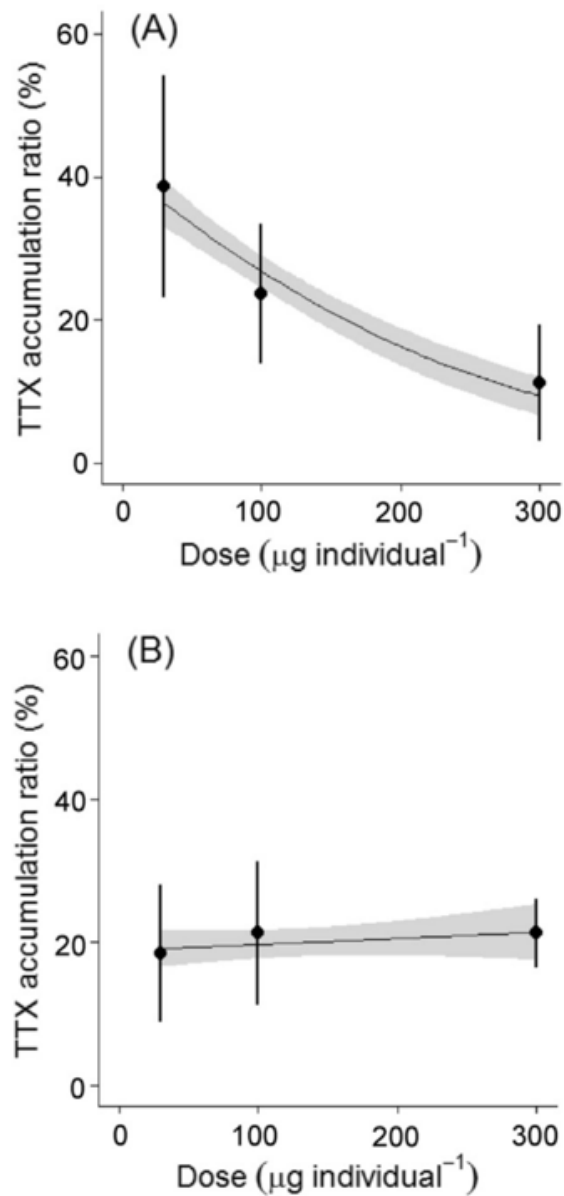
Fig. 9. TTX accumulation ratio (%) of skin (A) and liver (B) as a function of administered dose; 30 ($n = 6$), 100 ($n = 6$), and 300 ($n = 4$) μg/individual. Data are shown as the mean (point) and SD (error bar) for each dose. Line and gray zone indicate the expected values of the binomial generalized linear model and the 95% confidence interval, respectively.

at high doses, suggesting that a similar phenomenon occurs in the natural

environment.

Section 2

Immunohistochemical observation of the skin and liver

To observe the micro-distribution of TTX in the skin and liver after administration at different doses from Section 1, immunohistochemical staining was conducted.

1. Materials and methods

1.1. Materials

A part of the skin and liver of each test fish in Section 1 was submitted to the following immunohistochemical observation.

1.2. Immunohistochemical observation

Representative parts of the skin and liver were fixed in 10% neutrally buffered formalin for 7 days, embedded in paraffin, and then sectioned at 3-mm thickness using a microtome. The obtained sections were immunostained as described in 1.3. Immunohistochemical observation, Section 2, Chapter II, and observed under a light microscope.

1.3. Results and discussion

Positive immunoreactions for TTX (brown color) were observed in both the skin and liver. TTX localization in representative skin sections from each group are shown in **Fig. 10**. In LD, TTX-positive signals were
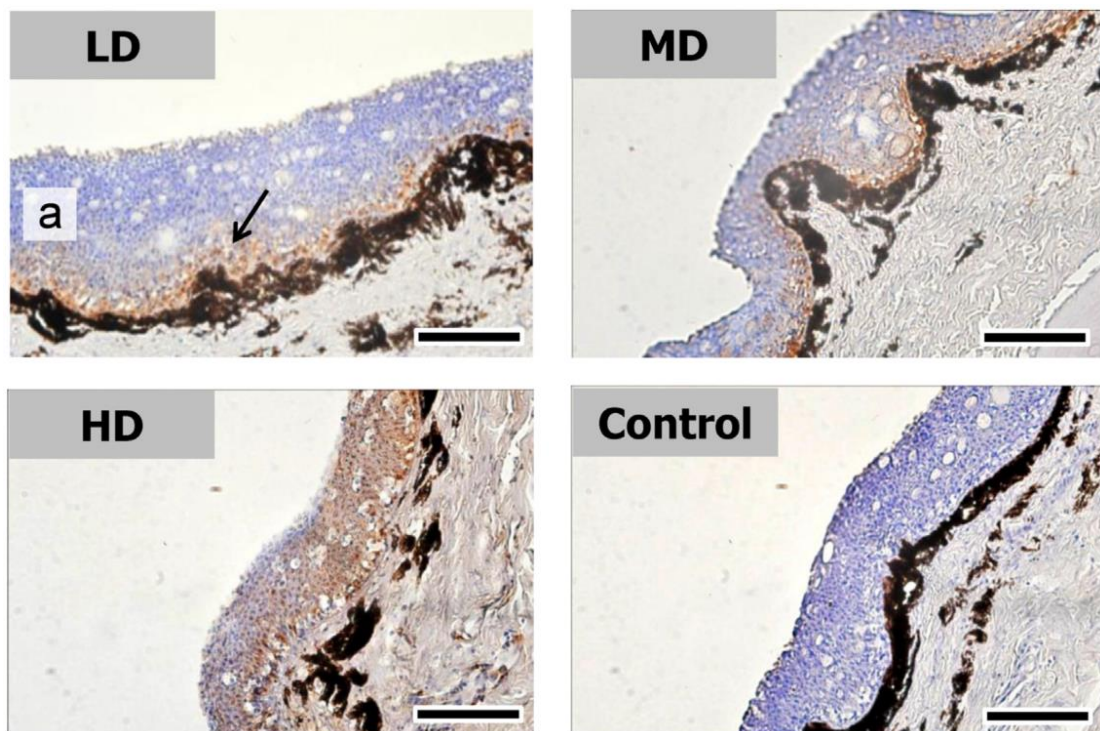
Fig. 10. Immunohistologic observation of the skin in each group. TTX was visualized as a brown color. Arrow and (a) indicate basal cell and epidermis layer, respectively. Scale bars, 25 μm.

observed in basal cells, as described in previous studies (Ikeda et al., 2009; Okita et al., 2013). As the dose increased, however, TTX-positive signals were also observed in the epidermis layer. TTX localization in the representative liver sections from each group are shown in **Fig. 11**. TTX-positive signals were rarely observed in LD and MD, but they were distributed over the whole liver tissue in HD, particularly at the pancreatic exocrine cells as stronger immunoreactions.

These results revealed differences in TTX localization according to the administered dose. Many pufferfish have secretory glands or secretory cells (sacciform cells) in the skin (Kodama et al., 1986; Tanu et al., 2002; Mahmud et al., 2003a, b; Itoi et al., 2012), and external stimuli induce the release TTX from the skin (Kodama et al., 1985; Saito et al., 1985b). In *T. rubripes,* no secretory glands or gland-like structures have been observed, and the positive immunoreactions for TTX are localized only at the basal cells along the basal membrane (Ikeda et al., 2009; Okita et al., 2013). However, cultured *T. rubripes* fed with toxic pufferfish livers released a considerable amount of TTX from the skin with a mild external stimulus (Saito et al., 1985b). In addition, Itoi et al. (2014) observed juveniles of generally nontoxic fish that ingest pufferfish larvae (*T. rubripes* as well as *T. niphobles*) and then promptly spit them out, and presumed that the TTX transferred from the mother works to repel predators, based on the findings that TTX was primarily localized on the body surface of the larvae.
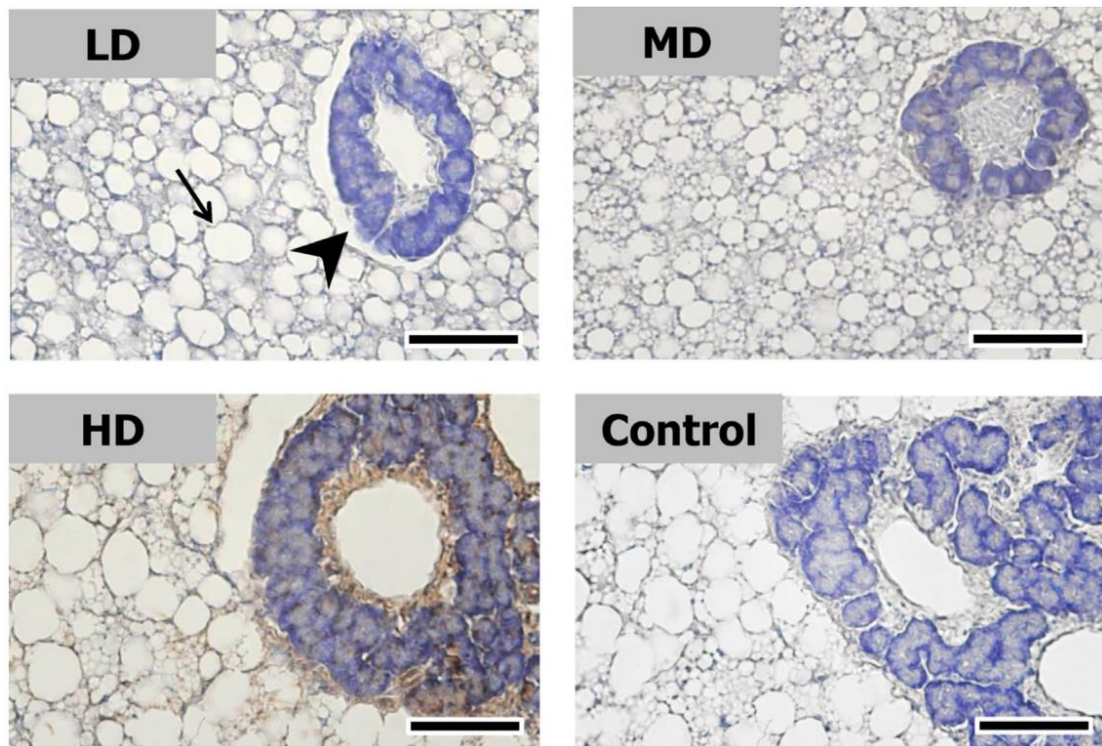
Fig. 11. Immunohistologic observation of the liver in each group. Arrow and arrow head indicate hepatic parenchymal cell, and pancreatic exocrine cell, respectively. Scale bars, 25 mm.

Although the mechanism remains to be clarified, *T. ruburipes* are considered to release TTX from the skin, despite that they have no gland structure in the skin. In the skin of LD, TTX-positive signals were observed in basal cells as previously described (Ikeda et al., 2009; Okita et al., 2013). In contrast, in the MD and HD the TTX-positive signals were not only observed in basal cells but were also spread over the epidermis layer. This may because the time from TTX injection to fish collection was shorter, and the dose was higher in the present study than in the previous reports. Pufferfish are endowed with high resistance to TTX, but can die when a large amount of TTX is administered (Saito et al., 1984, 1985a). In fact, paralysis occurred after administration and one fish died in the HD group. Therefore, it is possible that pufferfish eliminate part of the TTX taken up into the body via the skin to reduce the TTX load to the body just after administration.

Chapter 4

Maturation-associated changes in internal distribution and intra-ovarian microdistribution of tetrodotoxin in the pufferfish *Takifugu pardalis*

Maturation has been proved to be a significant factor to the TTX distribution inside the pufferfish body. Ikeda et al. (2010) investigated seasonal changes in tissue toxicity using wild specimens of the pufferfish *Takifugu flavipterus*, and found that liver and skin toxicity is high during the "ordinary period" (i.e., the months outside of the maturation and spawning periods), but in the maturation period during which the GSI increases, liver toxicity declines and ovary toxicity increases. A similar phenomenon is observed in *Takifugu alboplumbeus* (Itoi et al. 2016), suggesting that the TTX absorbed from toxic food organisms into the female pufferfish body is actively transported and accumulates in the ovary through its development during the maturation period. On the other hand, an immunohistochemical technique suggested that the micro-distribution of TTX in the pufferfish ovary changes with ovarian maturation (Itoi et al. 2012; Mahmud et al. 2003). In this chapter, to clarify these points, we collected wild specimens of *Takifugu pardalis* during the maturation period, and investigated changes in the internal distribution and intra-ovarian micro-distribution of TTX following oocyte maturation or spawning.

Section 1

Changes in the toxin distribution with ovary maturation

To clarify the maturation-associated accumulation profile of TTX in the pufferfish *T. pardalis*, female specimens were separated into each maturation stage based on the histology of ovarian sections, and then TTX quantification in each tissue in each maturation stage, as well as observation of the micro-distribution of TTX in the ovary was conducted.

1. Materials and methods

1.1. Pufferfish specimens

From November through December 2014, 22 female and 9 male specimens (designated Group I) of *T. pardalis* (**Table 5**) were collected in Omura Bay, Nagasaki Prefecture, Japan (**Fig. 12**). They were transported to the laboratory at Nagasaki University, and the skin, muscle, liver, and gonads were dissected out. A part of each ovary was used for histologic and immunohistochemical observations as described below, and the remainder of the ovary and the other tissues were frozen and kept below -30 ^{0}C until TTX quantification.

1.2. Gonadosomatic index (GSI)

The GSI of each specimen was calculated from its gonad weight (GW)

Table. 5. Specification of *T. pardalis* specimens.

	Collection month	Sex	Number	Body length (cm)	Body weight (g)
Group I	November–December, 2014	♀	22	13.0 ± 2.77	92.3 ± 53.0
		♂	9	11.8 ± 2.88	78.8 ± 70.6
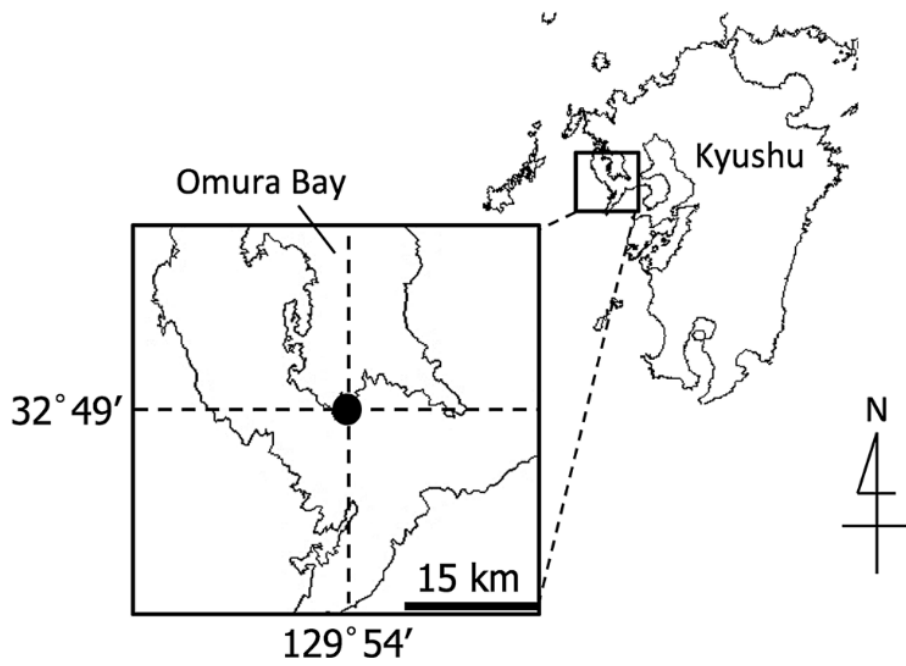
Fig. 12. Map showing the collection site of *T. pardalis* specimens.

and body weight (BW) using the following equation: $GSI = 100 \times GW/BW$.

1.3. Histologic observation of the ovary

The Ovary sections were prepared according to conventional histologic procedures. Briefly, a tiny block from the ovary of each specimen fixed in 10% neutrally buffered formalin for 1 week and decalcified in a 5% ethylenediaminetet-raacetic acid solution for 3 days was dehydrated through an ascending series of ethanol (70–100%), cleared in xylene, and embedded in paraffin. The embedded ovary was sectioned with a microtome at a thickness of 3 µm, and each section was deparaffinized in xylene, rehydrated through a descending ethanol series (100–70%), and then rinsed with water. The sections were stained with hematoxylin-eosin, and observed under a light microscope.

1.4. Immunohistochemical observation

A representative section prepared for each ovary as described above was immunostained using the same method as described in 1.3. Immunohistochemical observation, Section 2, Chapter II, and observed under a light microscope.

1.5. TTX quantification

The method described in Chapter I was used.

1.6. Statistical analysis

Statistical analyses were performed using R version 3.2.1 (R Development Core Team 2015). Analysis of variance (ANOVA) was used to compare GSI, as well as TTX concentration and TTX amount in each tissue among the different maturation stages.

2. Results and discussion

In the *T. pardalis* specimens, the oocytes had begun to mature (yolk accumulation) in all the ovaries, where not only immature oocytes in the perinucleolus stage, but also oocytes in the yolk vesicle stage and/or yolk globule stage were observed. The maturation stages of the ovaries, however, differed among individuals. Individuals in which the most mature oocytes in the ovary were at the yolk vesicle stage were designated as yolk vesicle stage (Yv; $n = 3$), and those in which the most mature oocytes were in the yolk globule stage were designated as yolk globule stage (Yg). Among those in the yolk globule stage, individuals in which the mean cell diameter of the largest 10 oocytes (the diameter of 20 oocytes per individual was measured, and the largest 10 values were selected and averaged) was less than 400 µm, 400–500 µm, and more than 500 µm were further designated as Yg-1 ($n = 3$), Yg-2 ($n = 10$), and Yg-3 ($n = 6$), respectively (**Fig. 13**).

The GSI of the female specimens at each maturation stage and of the male specimens is shown in **Fig.14**. The GSI of the females, which was
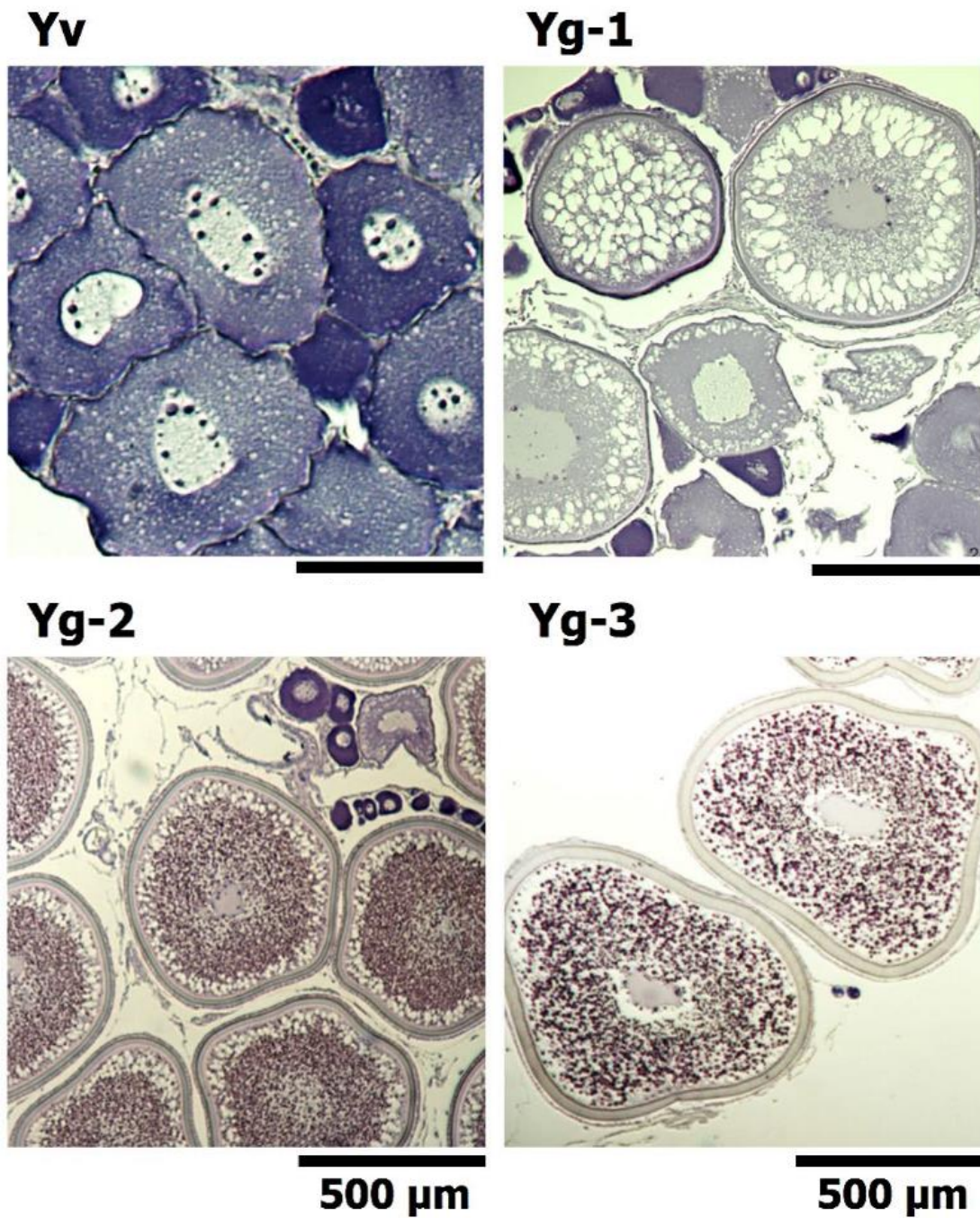
Fig. 13. Light micrographs of a representative ovarian section from *T. pardalis* specimens in different maturation stages; yolk vesicle stage (Yv), yolk globule stage (Yg)-1, Yg-2, and Yg-3.
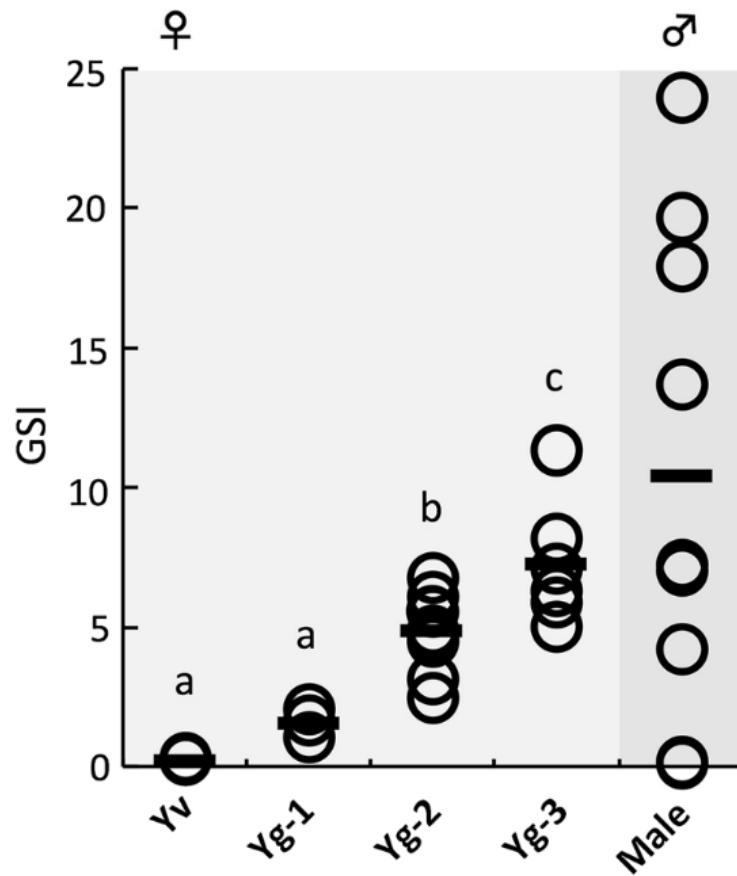
Fig. 14. GSI of females at each maturation stage, along with the GSI of males. Data are shown as individual values (circles) and mean of each maturation stage or males (cross bars). a < b < c, $p < 0.05$.

0.2–11, increased significantly with maturation from Yg-1 to Yg-3 ($p <$ 0.05). The GSI of the males (maturation stage was not evaluated) was 0.1–24 with large individual differences, and the maximum value reached approximately two-fold that of the female specimens.

TTX concentration in the tissues at each maturation stage is shown in **Fig. 15**. The TTX concentration in the ovary (61–555 MU/g on average) was highest among the four tissues tested at any maturation stage in females, followed by the skin (30–97 MU/g) and liver (5.7–87 MU/g). The TTX concentration in the ovary increased markedly from Yv to Yg-1 (the difference between Yv and Yg-1–Yg-3 was significant, $p <$ 0.05), and plateaued thereafter from Yg-1 to Yg-3. The TTX concentrations in the skin and liver showed no conspicuous change from Yv to Yg-2, although the concentration increased in Yg-3. TTX concentrations in the skin, muscle, and liver of male specimens were at almost the same level as those of female specimens from Yv to Yg-2, but little TTX was detected in the testis.

The TTX amount per tissue is shown in **Fig. 16**. In females, the TTX amount in the ovary largely increased with maturation from Yv (4.3 MU/individual on average) to Yg-3 (4330 MU/individual). The increase from Yv to Yg-2 was statistically significant ($p <$ 0.05). TTX amounts in the skin, muscle, and liver were also highest at Yg-3. The skin had the highest value (~ 270 MU/individual) in the male specimens, which was the
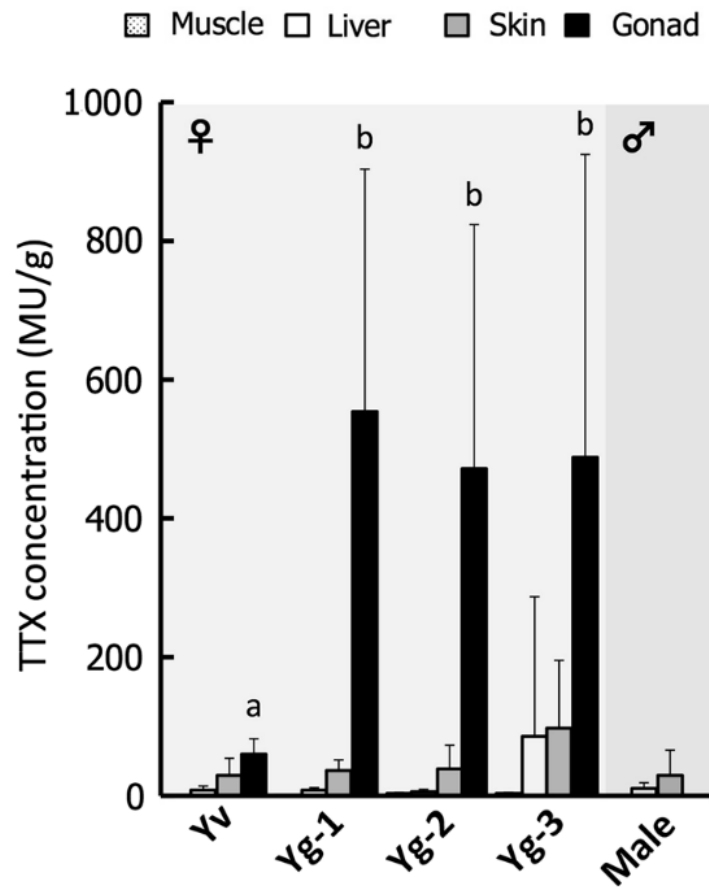
Fig. 15. TTX concentration (MU/g) in tissues from females at each maturation stage, along with that of males. Data are shown as means (columns) and SD (error bars). a < b, $p < 0.05$.
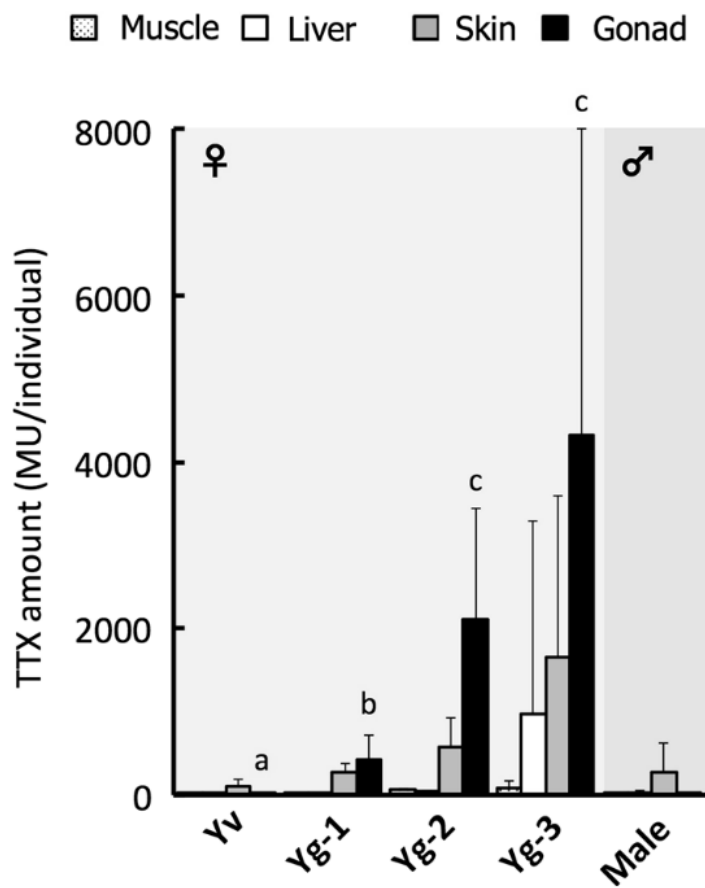
Fig. 16. TTX amount (MU/individual) in tissues from females at each maturation stage, along with that in the males. Data are shown as means (columns) and SD (error bars). a < b < c, $p < 0.05$.

same level as that in females at Yg-1. During this period, the relative TTX amount in the ovary gradually increased, and finally the TTX content in the ovary accounted for approximately 70–80% of the total toxin in the fish. Therefore, it is presumed that when maturation starts, most of the TTX taken up into the body (or that had previously accumulated in the liver) transfers to the ovary and accumulates in oocytes at the yolk vesicle stage. Oocytes progressing to the yolk globule stage actively take up TTX during the process, and the TTX concentration of the cell greatly increases. Furthermore, as the yolk globule oocytes grow (increase their weight) while continuing to actively take up TTX, the TTX amount increases with the TTX concentration remaining unchanged.

The relative TTX amount (ratio of TTX amount of each tissue to the total TTX amount of the individual) is shown in **Fig. 17**. In Yv females, 86 ± 3% (mean ± SD) of the toxin in the body was distributed in the skin, and the ratio of the ovary was 7.5 ± 7.7%. As maturation progressed, however, Changes the ratio of the skin decreased, and the ratio of the ovaries increased, and in Yg-2 and Yg-3, the content in the ovaries accounted for 76±9 and 69±19% of the total TTX amount, respectively. In the male specimens, the ratio of the skin (77±32%) was the highest, indicating a similar internal toxin distribution as in the Yv females.

Immunohistologic observation of a representative oocyte in each maturation stage is shown in **Fig. 18**. In Yv, positive TTX reactions (brown
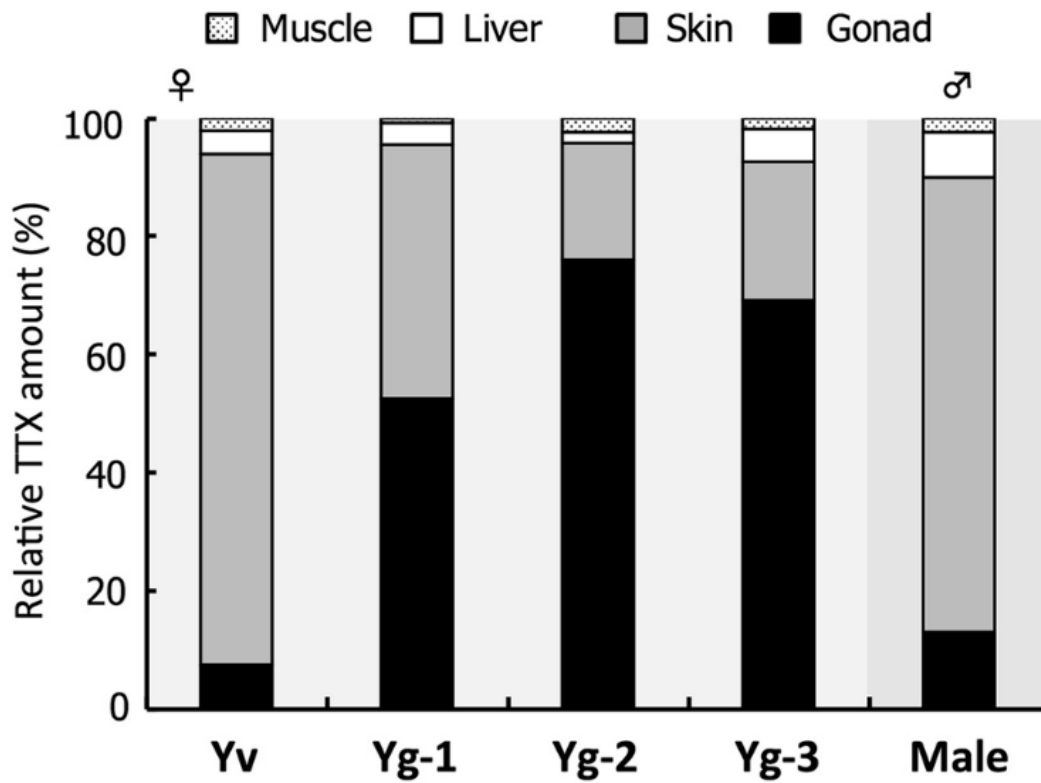
Fig. 17. Relative TTX amount (%) of tissues of females (n=22) in each maturation stage, along with that of the males (n=9).
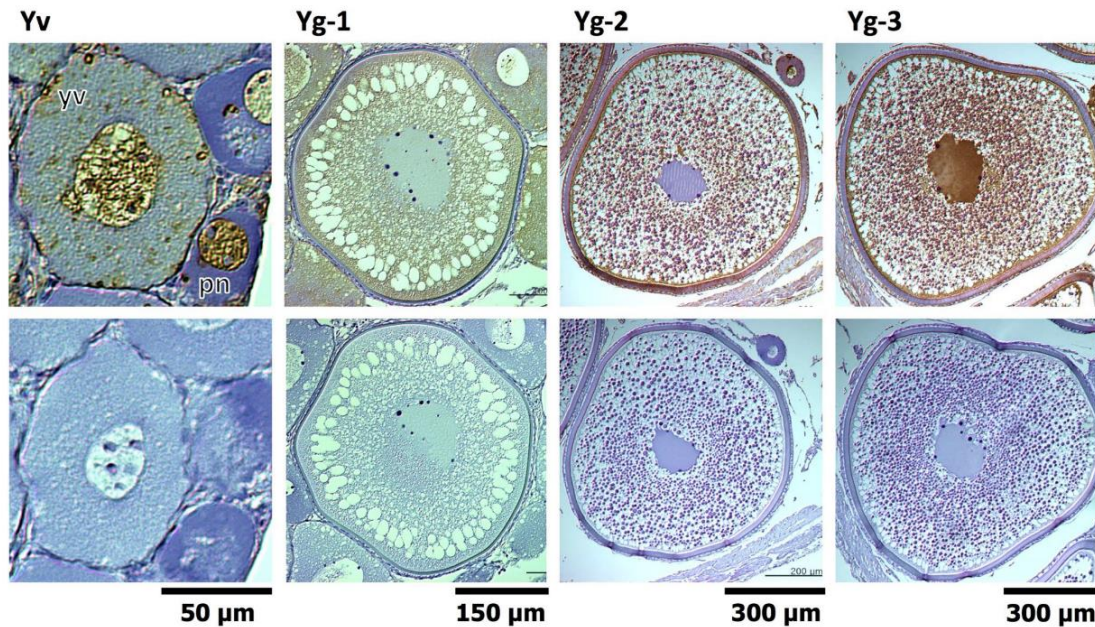
Fig. 18. Immunohistologic observation of a representative oocyte in each maturation stage, where TTX was visualized as a brown color (upper). No positive reaction of TTX was observed in the negative control sections (lower). In Yv, yv and pn indicate oocytes in the yolk vesicle stage and the perinucleolus stage, respectively.

color) were observed in the nucleus in oocytes at the perinucleolus stage, and in the nucleus and yolk vesicles in the oocytes at the yolk vesicle stage. In oocytes at the yolk globule stage in Yg-1, however, the positive TTX reactions in the nucleus faded and reactions were observed inside the whole cell except for in the oil globules. When the accumulation of yolk globules progressed in the Yg-2 oocytes, positive TTX reactions were observed in the yolk globules and cytoplasm near the egg membrane (**Fig. 19**). In the Yg-3 oocytes, the TTX localization was almost the same as that in Yg-2 oocytes. In Yg-2 and Yg-3 oocytes, we observed both positive reactions and no reaction in the nucleus within the same ovary (the rates for oocytes with a positive reaction was about 6%).
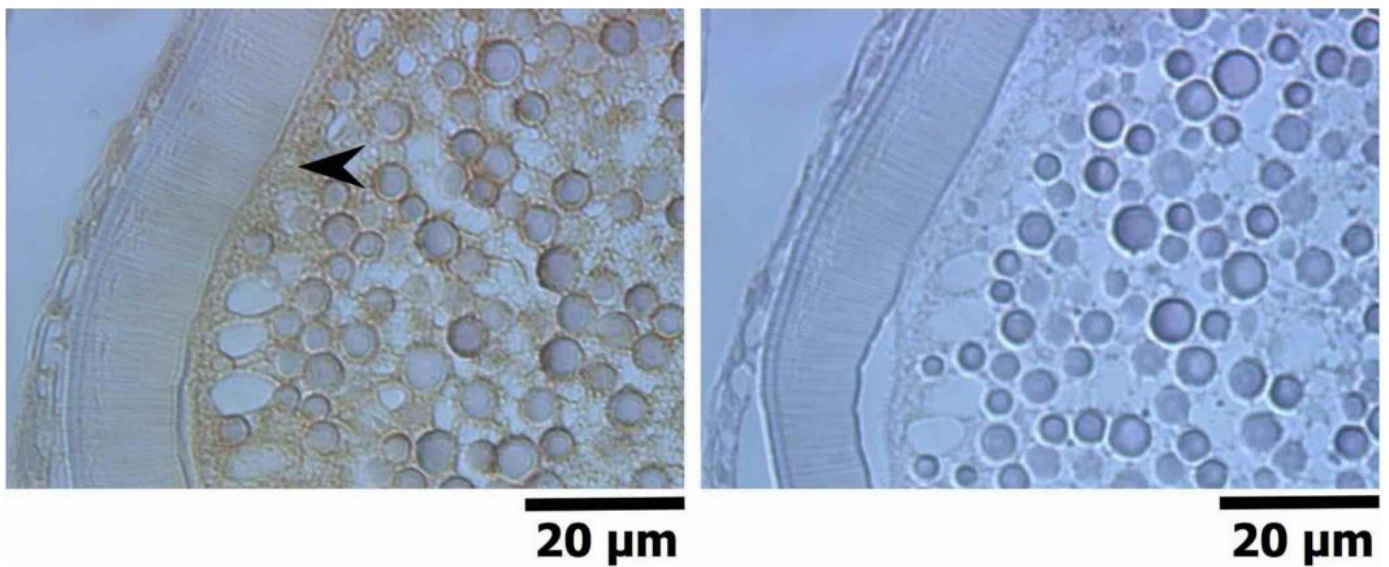
Fig. 19. Enlarged view of a representative oocyte in Yg-2 (left), where positive TTX reactions (brown color) were observed at the cytoplasm near the egg membrane (arrowhead). No positive reaction of TTX was observed in the negative control section (right).

Section 2

Changes in the toxin distribution following spawning

The spawning period was estimated based on the GSI of another group of *T. pardalis* specimens, and then the changes in the toxin distribution following spawning were investigated.

1. Materials and methods

1.1. Pufferfish specimens

From February through May 2015, 20 female specimens (designated Group II) of *T. pardalis* (**Table 6**) were collected at the same location as Section 1.

1.2. Immunohistochemical observation and TTX quantification

The *T. pardalis* speciemens were transported to the laboratory at Nagasaki University, and the skin, muscle, liver, and gonads were dissected out. A part of each ovary was used for histological and immunohistochemical observations, and the TTX contained in the remainder of the ovary and the other tissues were quantified, by the methods described in Section 1.

2. Results and discussion

In all specimens collected in February and March, the formation of

Table. 6. Specification of *T. pardalis* specimens.

	Collection month	Sex	Number	Body length (cm)	Body weight (g)
Group II	February, 2015	♀	4	14.1 ± 2.48	109 ± 62.3
	March	♀	5	14.8 ± 2.27	129 ± 63.5
	April	♀	6	13.8 ± 1.69	81.6 ± 28.9
	May	♀	5	15.3 ± 2.38	121 ± 45.7

oocytes was not remarkably different from that in Yg-3 of Group I in Section1. In contrast, in specimens collected in April and May, oocytes in the yolk vesicle stage that seemed to be left unspawned were sparsely scattered in the ovary (data not shown). GSI significantly increased from February through March, and then abruptly decreased from March through April (GSI of April and May specimens was significantly lower than that of the February or May specimens, $p < 0.01$), suggesting that the spawning period is from late March through early April (**Fig. 20**). Therefore, we designated February and March as the pre-spawning stage, and April and May as the post-spawning stage. Unlike in Group 1, no difference in the maturation stage was recognized among individuals collected within the same month. In Group 2, although the TTX concentration in the ovary was similar in the pre- and post-spawning stages, the TTX amount decreased markedly after spawning, and TTX appeared in the liver instead, suggesting that a part of the TTX that accumulated in the ovary during the maturation period remains at a certain concentration in oocytes that are insufficiently matured and left unspawned, but most of the accumulated TTX is released outside with the ovulated eggs.

The changes in the TTX concentration in each tissue are shown in **Fig. 21**. In the pre-spawning stage, the TTX concentration in the skin and ovary were similarly high, but little TTX was detected in the liver and muscle. In contrast, in the post-spawning stage, the TTX concentrations in the skin
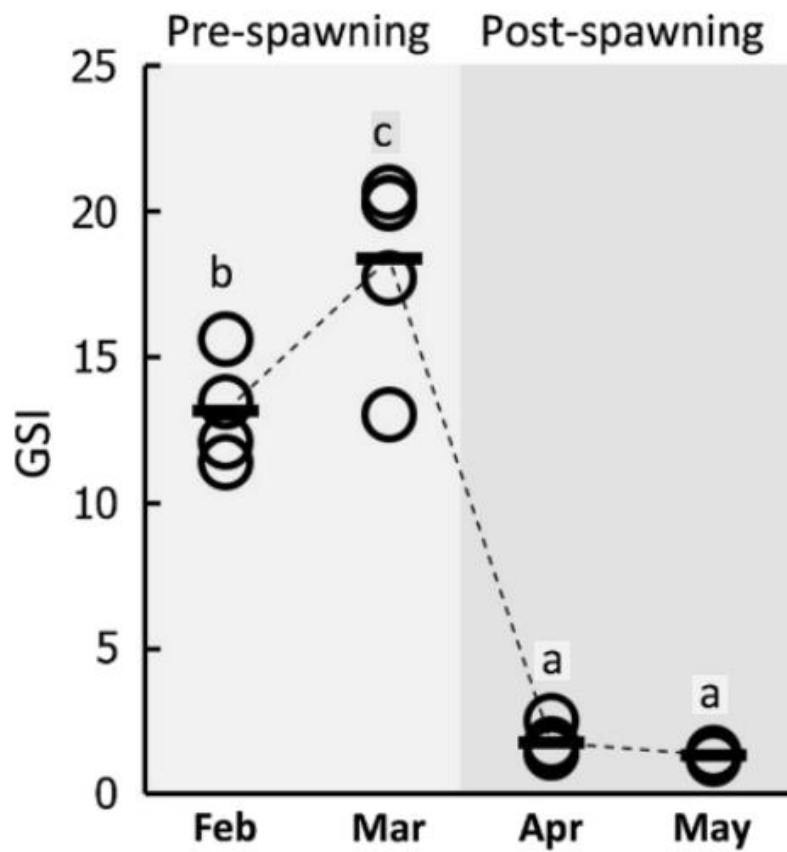
Fig. 20. Change in GSI of *T. pardalis* females before and after spawning. Data are shown as individual values (circles) and mean of each month (cross bars). a < b < c, $p < 0.01$.
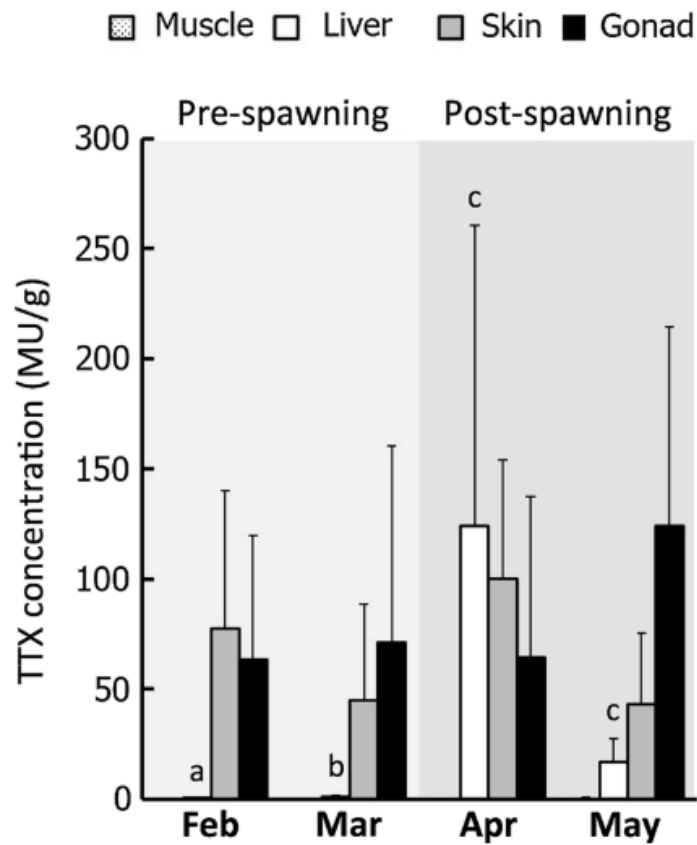
Fig. 21. Change in TTX concentration (MU/g) in each tissue before and after spawning. Data are shown as means (columns) and SD (error bars). a < b < c, $p < 0.05$.

and ovary were similar to those in the pre-spawning stage, but the TTX concentration in the liver was significantly higher ($p < 0.05$).

Changes in the TTX amount are shown in **Fig. 22**. The TTX amount in the skin did not change much between the pre- and post-spawning stages, whereas the TTX amount in the ovary, which was highest in the pre-spawning stage, significantly decreased ($p < 0.05$) following spawning. In particular, just after spawning (April), the TTX amount in the ovary decreased to levels below that in the liver. The relative TTX amount (**Fig. 23**) indicated that $55 \pm 29\%$ of the toxin in the body distributed in the ovary in the pre-spawning stage, but the skin content accounted for $72 \pm 16\%$ in the post-spawning stage, and the ratio of the ovary decreased to $11 \pm 9\%$, similar to that in the liver ($16 \pm 11\%$).

In oocytes at the yolk globule stage before spawning, positive TTX reactions were observed in the yolk globules and egg membrane (**Fig. 24a**). Like in Yg-2 and Yg-3 in Group I, oocytes showing a positive reaction and those not showing a positive reaction in the nucleus were intermingled during this period (the rates for oocytes with a positive reaction was about 23%). In oocytes at the yolk vesicle stage that were left unspawned after spawning, positive TTX reactions were observed throughout the cytoplasm (**Fig. 24b**).
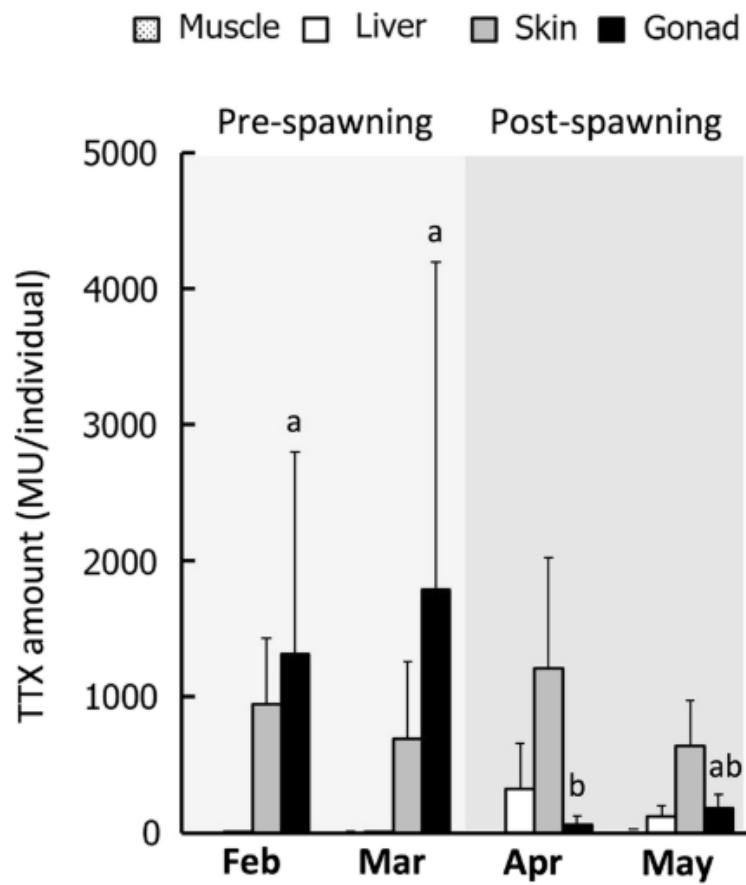
Fig. 22. Change in TTX amount (MU/individual) in each tissue before and after spawning. Data are shown as means (columns) and SD (error bars). a > b, $p < 0.05$.
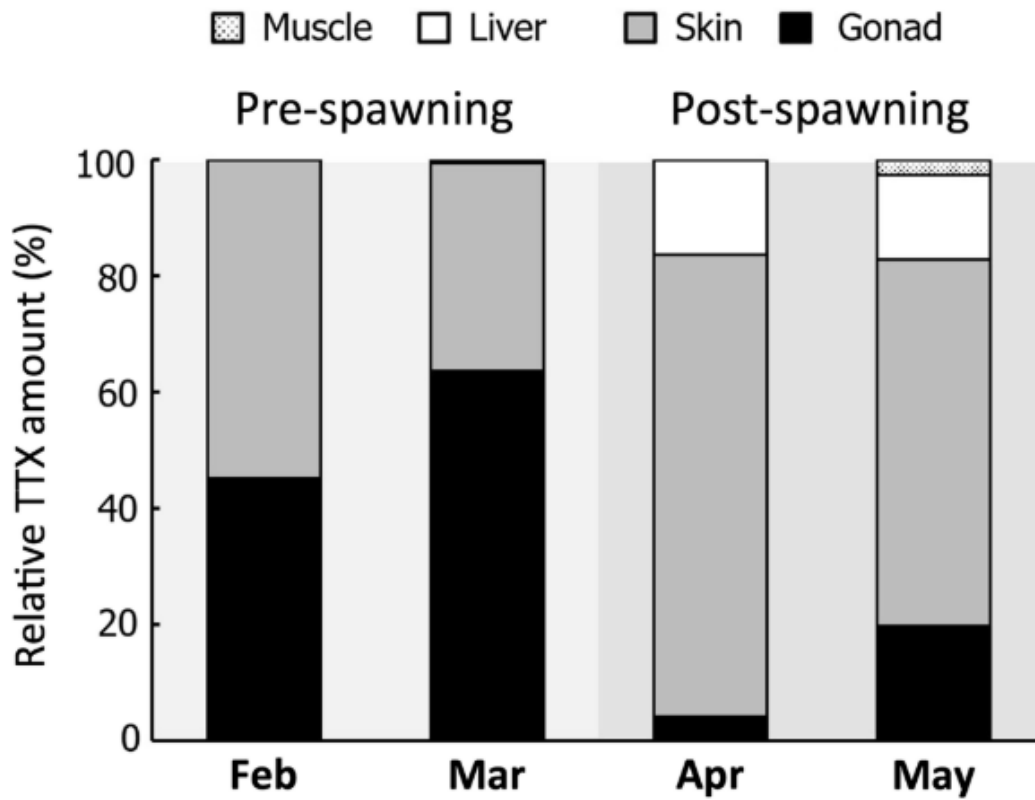
Fig. 23. Change in relative TTX amount (%) in each tissue before and after spawning (n=20).
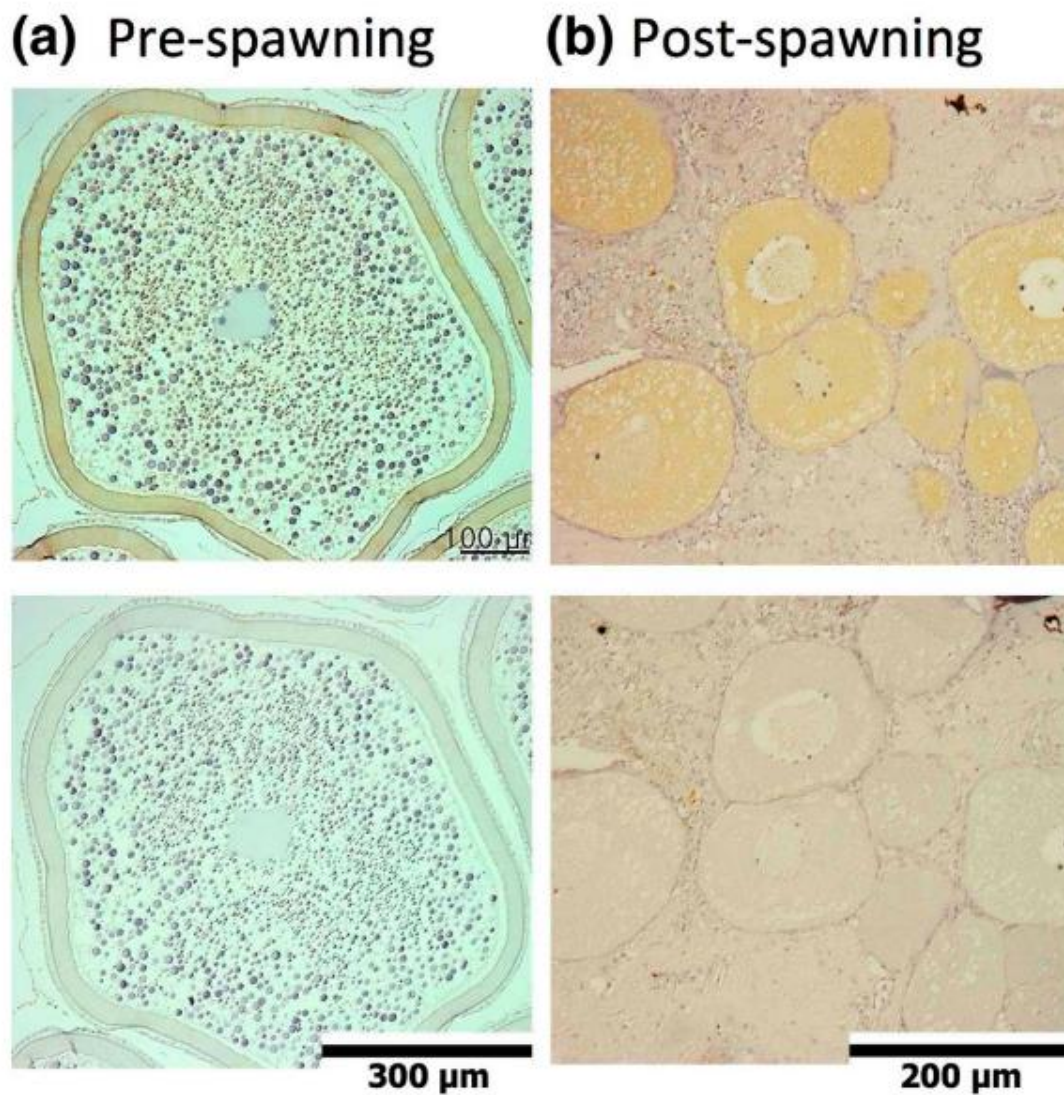
Fig. 24. Immunohistologic observation of a representative ovarian section before (a) and after (b) spawning, where TTX was visualized as a brown color (upper). No positive reaction of TTX was observed in negative control sections (lower).

Chapter V

General discussion

In this chapter, all results from the last 4 chapters are summarized, and comprehensively discussed.

First of all, I focused on the inter-species differences of toxin accumulation. In wild, marine pufferfish contain TTX as the major toxic component, while freshwater pufferfish contain PSP instead. It could be due to the insufficiency of the TTX or STX-containing food in their habitats. To clarify the differences of the toxin selectivity between marine and freshwater pufferfish, *in vivo* TTX/PSP administration experiments were conducted using artificially reared nontoxic specimens of *T. pardalis* and *P. suvattii*. In *T. pardalis*, little dcSTX, and in *P. suvattii*, little TTX was retained in any organ other than the intestine. It is clear that toxin selectivity is present in pufferfish according to their habitat. There are three possible reasons for this: (1) the intestine serves as a barrier allowing little toxin to be absorbed into the body; (2) the toxin is absorbed into the body from the intestine but is quickly decomposed and/or excreted without being taken up into the liver and skin; and (3) the toxin is absorbed into the body from the intestine and taken up into certain organs but is converted to other analogues that were not detectable in the present study. Nagashima et al. (2003) and Matsumoto et al. (2005), demonstrated that the liver tissues of

marine pufferfish of the genera *Takifugu* and *Lagocephalus* take up considerable amounts of TTX, unlike general marine fish, but do not take up PSP like general marine fish. These findings support the second possibility but still reamins the first possibility. TTX and its analogues 4-*epi*TTX and 4,9-anhydroTTX are converted to each other and exist in equilibrium in an approximate ratio of 8:1:1 in an aqueous acidic solution (Nakamura et al, 1985). Under the analytical conditions of the present study, 4-*epi*TTX was detectable with TTX but was below the LOQ in any of the organs examined. Yasumoto et al. and Yotsu-Yamashita et al. (Yotsu-Yamashita, 2001; Jang et al, 2007; Yotsu-Yamashita et al, 2013; Kudo et al, 2014; Ueyama et al, 2018) separated many TTX analogues from various pufferfish, and they estimated the biosynthetic and metabolic pathways of TTX through a series of oxidations as 5,6,11-trideoxyTTX→5,11-dideoxyTTX or 6,11-dideoxyTTX→5-deoxyTTX, 11-deoxyTTX or 6-deoxyTTX→TTX→11-*nor*TTX-6-ol→11-oxoTTX. It is possible that TTX administered to the pufferfish is oxidized to 11-*nor*TTX-6-ol or 11-oxoTTX. These analogues, as well as 4,9-anhydroTTX, are all minor components coexisting with TTX in pufferfish (Jang et al, 2006; Yotsu-Yamashita et al, 2013; Kudo et al, 2014; Jang et al, 2010; Puilingi et al, 2015), and it is unlikely that all TTX taken up into the body is converted to these analogues. On the other hand, conversions of PSP components (hydrolysis of *N*-sulfocarbamoyl toxins and reductive elimination of the

sulfate group at C-11 or hydroxyl at N-1) occur in PSP-contaminated bivalves or toxic xanthid crabs, but in this case, dcSTX and STX are rather stable final products (Shimizu et al, 1981; Oshima et al, 1995; Samsur et al, 2006; Arakawa et al, 1998). In the present study, it could not exclude the possibility that TTX or dcSTX/STX are converted to nondetectable metabolites as the analysis target was limited to the administered toxins. It is unlikely, however, that TTX or dcSTX/STX, which are the main components of the toxins naturally harbored in pufferfish, are completely converted to other components in such a short period of time. Further studies are needed to evaluate the possibility that the TTX or STX analogues were not detectable. Yotsu-Yamashita et al (2013) investigated the PSTBP micro-distribution in the intestine of *T. pardalis,* and found that PSTBP presented in lamina propria mucosae, neighboring to epithelium cell. It was suggested that absorbed TTX from epithelium cell would be trapped by PSTBP for next step of transportation. Although PSTBP showed even a slightly higher binding ability to STX than TTX, in that case, STX should be absorbed into the body as equal as TTX in marine pufferfish. Therefore, probably STX is trapped and stay in the epithelium cell. But when large amount of STX appear, a few STX will be able to reach lamina propria layer and transported into the blood circulation. To confirm this hypothesis, immunohistochemical staining for TTX and STX of the intestine section of marine puffer species which is administered with STX

and TTX mixture, can be used to understand the micro-distribution of STX and TTX in the intestine. If STX was only found in the epithelium cells, then the hypothesis may be true. There is few research about PSTBP presence in the intestine of freshwater pufferfish, however, according unpublished researches on the presence of PSTBP genes in several freshwater pufferfish including (*Pao suvattii, Pao abe, Tetraodon cutucutia*), no PSTBP genes was found in their genome. This could be the key point for the block system for TTX in the freshwater pufferfish due to the lack of PSTBP. Consequently, it is highly possible that the intestine serves as a barrier selectively blocking the absorption of certain toxin in pufferfish. To confirm this hypothesis further, *in vitro* tissue incubation method which introduced in Chapter II could be used to evaluate the toxin uptake ability of the tissues in these two species. The presence of PSTBP in the intestine of freshwater pufferfish should also be investigated.

The TTX uptake profiles of the tissues based on the intra-species differences was also investigated. After TTX was absorbed into the pufferfish body through the intestine, the distribution of TTX varies depending on the growth stage. As mentioned before, on the basis of *in vivo* TTX administration experiment using *T. rubripes* of different ages, Tatsuno et al. (2013a) assumed that the growth-dependent changes in the toxin distribution between the skin and liver were due the undeveloped liver in young fish, making TTX less likely to accumulate in the liver than

in adult fish, and rather to transfer to the skin. Furthermore, Kiriake et al. (2016) performed an *in vitro* tissue slice incubation experiment and found no difference in the TTX uptake ability of the liver between young and adult fish. On the other hand, Matsumoto et al. (2015) administered TTX to nontoxic *T. rubripes* and revealed that some of the TTX that accumulates in the liver of *T. rubripes* is transferred to the gallbladder. These results presumed age-dependent differences in the ability to retain or metabolize TTX after uptake. These studies have focused on the TTX uptake ability or eliminating ability of the liver, and how the TTX uptake ability of other main toxin accumulation tissues changes at the same time were ignored. In Chapter II, *in vitro* tissue incubation experiments were used to evaluate the toxin uptake ability of the tissues in the young and adult *T. rubripes*. Firstly, given the skin and intestine showing similar uptake ability to the liver, this method is considered available for the evaluation of the TTX uptake ability of the skin and intestine. Furthermore, like in Kiriake et al. (2016), the TTX uptake ability in the liver did not differ significantly between young and adult fish. The TTX uptake ability of the intestine also differed little between young and adult fish. In contrast, the skin of the young fish took up about twice as much TTX as the skin of the adult fish. This finding strongly suggests that the TTX uptake ability of the skin is involved in the growth-dependent changes in the toxin distribution inside the body in *T. rubripes*, although the ability of the liver to retain and metabolize TTX

requires further investigation. Based on the immunohistochemical staining, TTX was mostly distributed in the basal cells in both Chapters II and III, therefore, the basal cells could be the key point to the difference of the TTX uptake ability in the skin between the young and adult *T. rubripes*. To further clarify the TTX uptake profiles in the liver and skin, TTX was administered intramuscularly to immature specimens of *T. rubripes* at different dose of administration in Chapter III. The toxin transfer profile after 24 h differed greatly between the skin and liver. Ikeda et al. (2009) administered TTX intramuscularly to 4-month-old *T. rubripes* (7.1 ± 0.6 cm, 13.2 ± 3.4 g) at a dose of 11 mg/individual (0.84 µg/g body weight), and determined the TTX concentration of each tissue for up to 168 h after administration. At 24 h, the toxin concentration of the skin was ≥ 2.2 µg/g and that in liver was ≤ 0.33 µg/g, which is greatly different from the result of the MD group (0.98 ± 0.51 µg/g and 1.70 ± 0.98 µg/g) whose dose (0.69 µg/g body weight) was closest to that administered by Ikeda et al. (2009) among the three groups in the present study. It is possible that the TTX concentration of the liver was substantially lower in the Ikeda et al. study because they tested younger fish (4 months) than in the present study (8 months). Considering that the TTX transfer/accumulation profiles significantly differ between the liver and skin at different dose of administration, it is indicated that the molecular mechanisms involved in the transfer/accumulation of TTX differ between the skin and liver tissues.

In addition, we found that in an *in vivo* TTX administration experiment using *T. rubripes*, TTX-positive signals were obtained in the whole hepatic parenchymal cells and pancreatic exocrine cells in the liver only when administered at a high dose (300 μg/fish). It seems that TTX overflowing from the hepatic cytoplasm was transferred to the pancreatic exocrine cells. However, in Chapter II, when temporal changes are considered, the reverse would be true. In this case, given that pancreas is embeded in the inflowing vessels to the liver (Akiyoshi et al., 2001), pancreatic exocrine cells may be involved in the TTX uptake toward the hepatic cells.

Many pufferfish species showed specific TTX accumulation in the ovary during maturation period. Ikeda et al. (2010) investigated seasonal changes of tissue toxicity in wild *T. flavipterus* specimens based on the GSI of the group of each month. In Chapter IV, we collected wild *T. pardalis* during maturation period, and investigated the changes of the internal distribution and intra-ovarian micro-distribution of TTX based on the maturation stage of oocytes of each specimen. In Group I, from Yv to Yg-1, the TTX concentration of the ovary greatly increased, and from Yg-1 to Yg-3 the TTX amount in the ovary greatly increased. The toxic goby also exhibits this maturation-related increase in toxin amount in the ovary (Tatsuno et al. 2013b), as does *T. flavipterus* (Ikeda et al. 2010) and *T. alboplumbeus* (Itoi et al. 2016). Although the composition of the prey species of pufferfish changes with season and/or growth stage (Takita and

Intong 1991), it is unclear whether food intake and/or its composition changes with maturation. Further investigations could clarify whether, not only the toxin accumulation ability of the ovary itself but also the change in TTX supply via food organisms is involved in the conspicuous increase in the amount of toxin in the ovary during the maturation period. In Group II, when spawning is complete, the TTX previously provided to the ovary begins to transfer instead to the liver. Considering the continuity between Yg-3 of Group I and the pre-spawning stage of Group II, the TTX concentration in the ovary during the pre-spawning stage in Group II seems to be too low. Although the reason for this is unclear, in addition to very large individual differences, it is possible that the population at the sampling location was replaced by a population from another location. While the age of the fish specimens was not considered in the present study, it might have had some effect on the findings. In the immunohistochemical observation of the ovarian sections, clear positive TTX reactions were seen only in the nucleus in immature oocytes at the perinucleolus stage, and in the nucleus and yolk vesicles in the inadequately matured oocytes at the yolk vesicle stage, suggesting that the TTX that entered into the oocytes is first taken up into the nucleus. In the yolk globule stage, however, we observed both oocytes in which positive TTX reactions were observed and not observed in the nucleus. Although the reason for such phenomena remains to be elucidated, it is possible that TTX loses reactivity with the

anti-TTX antibody by changing its existing form in some nuclei, or that TTX in the nucleus increases or decreases at the yolk globule stage. Kodama et al. (1983) found that TTX is released when a high molecular-weight fraction from the *T. flavipterus* liver is treated with RNase, suggesting a TTX-binding nucleic acid-related substance(s). Such a substance(s) might be expressed in some nuclei and bind with TTX, resulting in the disappearance of the positive reactions. Other than the nucleus, positive TTX reactions were observed mainly in the yolk vesicles in the yolk vesicle stage, and in the yolk globules at the yolk globule stage. Essentially similar results were obtained with *Takifugu vermicularis* (Mahmud et al. 2003) and *T. alboplumbeus* (Itoi et al. 2012). After pufferfish spawned, TTX that remain in the ovary is considered for alarming the predator as a defensive function. Therefore, TTX distributed around the egg membrane could be due to this function. Also TTX located in yolk globule may be passed to the next generation for defensive funtion, since yolk substances is the supply source of nutrition during the development of embryo. Although it is presumed that a large amount of TTX is transported to the ovary and accumulates in the oocytes as maturation progresses, many of the detailed mechanisms remain to be clarified.

However, all these phenomenon is likely related to the toxin-binding proteins. Yotsu-Yamashita et al. (2001, 2010) who separated a toxin-

binding protein, PSTBP from the blood plasma of *T. pardalis*, investigated the localization of PSTBP in the tissues of *T. pardalis* using an immunohistochemical technique, and concluded that PSTBP functions as a toxin transporter in the blood and is involved in toxin absorption in the intestine, as well as toxin accumulation in the liver, ovary, and skin (Yotsu-Yamashita et al., 2013). If this is the case, it is possible that toxin transportation to the skin reaches a plateau in Chapter III, because the protein binds to one TTX molecule as a dimer, so that binding with TTX could be saturated when a large amount of TTX is taken up into the blood circulation at once. The increase in the TTX concentration in the skin was slower than that of the liver, and this may be due to such saturation. Moreover, PSTBP localizes at the ovarian wall and yolk membrane of the oocytes (Yotsu-Yamashita et al. 2013), which is consistent with the TTX distribution in the ovary in wild *T. pardalis* in Chapter IV except cytoplasm near the egg membrane. The relation between the expression of PSTBP or PSTBP homologous proteins and maturation is unclear, and the extent of their involvement in maturation-dependent toxin transportation to the ovary remains to be elucidated. Furthermore, since PSTBP showed a binding ability to STX as well, it might be involved in the toxification mechanism in freshwater pufferfish, however, the presence or absence of these protein isoforms in freshwater pufferfish is unknown. In general, when maturation starts in fish, vitellogenin, a yolk protein precursor, is

synthesized in the liver by the induction of female hormones, and transported to the ovary via the bloodstream. Vitellogenin is then taken up by the oocytes by pinocytosis, decomposed to yolk proteins, and accumulated as yolk globules (Mommsen and Walsh 1988). Very recently, Yin et al. (2017) found a toxin-binding protein homologous to a vitellogenin subdomain in *T. pardalis* ovary, and suggested its involvement in the toxin accumulation in the ovary. Thus, the accumulation process of yolk proteins must be strongly related to the TTX transportation to the ovary and/or TTX accumulation in the oocytes.

In this study, it is indicated that (1) the TTX/PSP selectivity markedly differ beween marine pufferfish *T. pardalis* and freshwater pufferfish *P. suvattii,* suggesting that the intestine may serves as barrier; (2) the TTX uptake ability of the skin in *T. rubripes* varies with the develop of growth. (3) the toxin transfer profile to the skin and liver in *T. rubripes* is different when TTX is administered to *T. rubripes* at different concentrations, suggesting the difference of the transfer/accumulation mechanisms between the liver and skin; (4) a majority of TTX is transported and accumulated in the ovary in *T. pardalis* with the develop of the maturation stage. It is possible that the TTX uptake and accumulation in those tissues is influenced by different or multiple molecular mechanisms. As an example, TTX was transported by PSTBP in the blood circulation then taken up into the liver by the membrane protein on the hepatic cells, and

into the oocytes binding with the vitellogenin subdomain during maturation periods. Those toxin-binding proteins may be specifically responsible to the TTX uptake and accumulation in certain tissue depending on the species, growth/maturation stage of pufferfish. Clarifying the role of these toxin-binding proteins play in the TTX transfer/ accumulation mechanism would be required.

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