Table 1. Toxicity and tetrodotoxin (TTX) content of gastropods from coastal waters of Okinawa Prefecture, Japan

Spemen No.	Species "Japanese name"	Month of collection	Muscle					Viscera					Whole body (combined muscle and viscera)				
			Weight (g)	Toxicity[*1] (MU/g)	TTX amount[*2] (MU/g)	Total toxicity[*1] (MU/indivi.)	Total TTX amount[*2] (MU/indivi.)	Weight (g)	Toxicity[*1] (MU/g)	TTX amount[*2] (MU/g)	Total toxicity[*1] (MU/indivi.)	Total TTX amount[*2] (MU/indivi.)	Weight (g)	Toxicity[*1] (MU/g)	TTX amount[*2] (MU/g)	Total toxicity[*1] (MU/indivi.)	Total TTX amount[*2] (MU/indivi.)
1	*Nassarius glans*	Mar. 2009	2.83	461	243	1,300	688	1.27	189	33.8	240	42.9	—	—	—	—	—
2	"kinshibai"	May 2009	2.04	375	249	765	508	0.80	98.6	43.9	78.9	35.1	—	—	—	—	—
3		Jun. 2009	0.38	47.6	9.59	18.1	3.64	0.12	<20	7.01	—	0.841	—	—	—	—	—
4			0.76	206	26.9	157	20.4	0.11	<20	13.3	—	1.46	—	—	—	—	—
5			2.47	45.9	11.8	113	29.1	0.83	<20	9.91	—	8.23	—	—	—	—	—
6			1.89	39.6	8.16	74.8	15.4	0.68	<20	4.17	—	2.84	—	—	—	—	—
7	*Nassarius coronatus*	Jan. 2009	—	—	—	—	—	—	—	—	—	—	0.83	11.1	5.51	9.23	4.57
8	"iboyofubai"		—	—	—	—	—	—	—	—	—	—	1.17	<5.64	1.90	6.60	2.22
9			—	—	—	—	—	—	—	—	—	—	1.05	<5	<0.1	—	—
10			—	—	—	—	—	—	—	—	—	—	1.29	<5	<0.1	—	—
11			—	—	—	—	—	—	—	—	—	—	1.33	<5	<0.1	—	—
12	*Zeuxis* sp.[*3] "kagerouyofubai"	Feb. 2009	—	—	—	—	—	—	—	—	—	—	0.13±0.16 (n=6)	12.7	2.69	0.16±2.06	0.34±0.44
13	*Niotha albescens*[*3] "awamushiro"	Mar. 2009	—	—	—	—	—	—	—	—	—	—	0.30±0.30 (n=6)	<10	5.08	—	1.52±1.50
14	*Oliva annulata*	Mar. 2009	1.14	<10	<0.1	—	—	0.67	10.8	8.90	7.24	5.96	—	—	—	—	—
15	"satsumabina"		1.00	<10	<0.1	—	—	0.85	<10	<0.1	—	—	—	—	—	—	—
16			0.94	<10	<0.1	—	—	0.63	<10	<0.1	—	—	—	—	—	—	—
17			0.97	<10	<0.1	—	—	0.58	<10	<0.1	—	—	—	—	—	—	—
18			1.35	<10	<0.1	—	—	0.98	<10	<0.1	—	—	—	—	—	—	—
19		May 2009	1.08	<10	<0.1	—	—	0.66	<10	<0.1	—	—	—	—	—	—	—
20			0.56	<10	<0.1	—	—	0.68	<10	<0.1	—	—	—	—	—	—	—
21			1.00	<10	<0.1	—	—	0.88	<10	<0.1	—	—	—	—	—	—	—
22	*Oliva concavospira*	Jan. 2009	1.35	6.65	3.23	8.98	4.36	0.39	—	<0.1	—	—	—	—	—	—	—
23	"hekomimakura"		1.05	<5	<0.1	—	—	0.49	—	<0.1	—	—	—	—	—	—	—

[*1]: Toxicity score was determined by mouse bioassay.
[*2]: TTX amount was determined by LC-MS.
[*3]: Combined specimens were examined.
—: Not examined or not calculated.

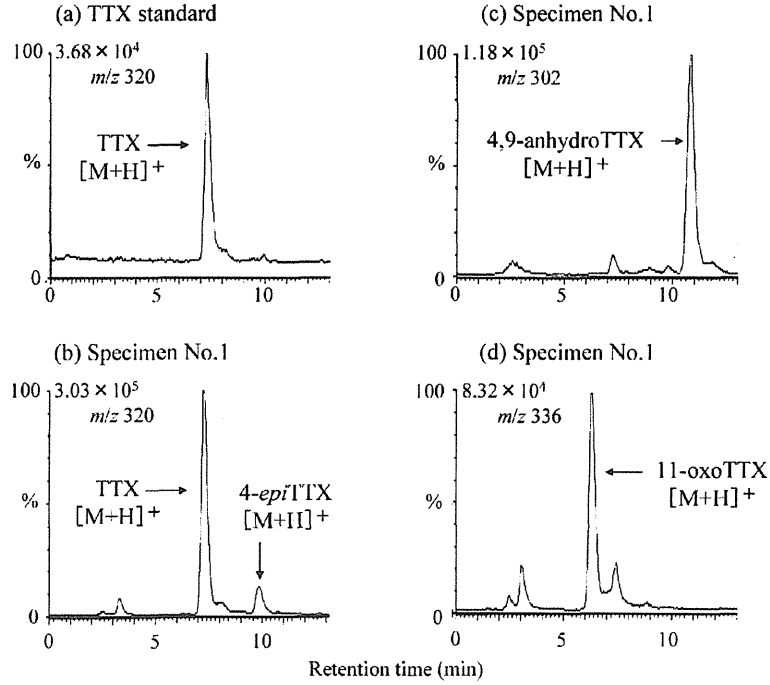

Fig. 2. LC-MS chromatograms at *m/z* 320 (a, b), 302 (c) and 336 (d) obtained from TTX standard (0.002 MU) (a) and muscle (equivalent to 0.08 mg) of specimen No. 1 (b, c, d) (see Table 1 for specimen No.)
LC-MS[20] was carried out on an Alliance LC-MS system (Waters) equipped with a Zspray™ MS 2000 detector using a Mightysil RP-18 GP column (ϕ2.0×250 mm) with 30 mmol/L heptafluorobutyric acid in 1 mmol/L ammonium acetate buffer (pH 5.0) as the mobile phase. The flow rate was 0.2 mL/min. The eluate was introduced into the ion source of MS, ionized by electrospray ionization (ESI) in the positive mode, and monitored through the MassLynx™ NT operating system.

45%，サツマビナの内臓では82%，イボヨフバイの可食部では50および34%，カゲロウヨフバイの合一可食部では21%程度をTTXが占めていると判断された．長崎県産キンシバイの場合，平均的には筋肉で総毒力の約65%，内臓では約60%をTTX，残余毒力の相当部分を11-oxoTTXが占めている[4]．今回のキンシバイでも，一部の筋肉からTTXに加えて4,9-anhydroTTX，4-*epi*TTX，11-oxoTTX[35),36)]が検出されたことから（Fig. 2），これらのTTX誘導体，特に比毒性がTTXと同等，あるいはそれより高い11-oxoTTX[35)]が残余毒力に大きく寄与しているものと推察される．この点については，11-oxoTTXの標準品がないため，本成分の精製や毒性評価，他の有毒種の場合も含めて現在検討中である．

一方，キンシバイ各個体のTTX量（MU/g）は，筋肉が内臓より1.2～7.2倍高い値を示した．すなわち，これらの個体では総TTX量（MU/個体）の74～94%が筋肉に偏在していたことになる（Table 1）．長崎および熊本県産キンシバイでは毒の63～99%が筋肉に偏在している[4),5)]．また台湾産キンシバイでは85%の個体で筋肉の毒力が中腸腺より1.7～8.3倍高いという[16)]．沖縄県産キンシバイの毒蓄積パターンは，これまで報告のある同種巻貝とよく類似していた．

沖縄県産巻貝のうち，リュウテンサザエ科のヤコウガイ *Turbo marmorata* とチョウセンサザエ *Turbo argyrostoma*，ニシキウズカイ科のサラサバテイ *Tectus nilotica maxima* とギンタカハマ *Tectus pyramis* の中腸腺に2.0～20 MU/gのPSPを保有することがある[20)]．また，台湾産のアラレガイやオキナワハナムシロ，サメムシロはTTX主体の毒を持つものの，副成分としてPSPを含む[17)]．今回調査した全個体につき，PSPを対象としてHPLC蛍光分析を行ったが，同成分は全く検出されなかった（STX換算[24),37)]で0.06 μg/g（0.5 MU/g）未満）．

ま と め

沖縄県沿岸に分布する腐肉食性および肉食性小型巻貝8科15種64個体につき，マウス毒性を調べたところ，キンシバイ，サツマビナ，ヘコミマクラ，イボヨフバイ，カゲロウヨフバイの5種が有毒であった．このうち，キンシバイの毒力は総じて高く，すべての個体で筋肉にTTXが偏在し，同部位の最高毒力は460 MU/gに達した．過去のTTX中毒事例では，発症量を900～1,000 MUと推定した報告がある[3)]．すなわち，キンシバイでは内臓を除去しても，数グラム程度の喫食で中毒し，数十グラムでヒトの最小致死量（10,000 MU）[3)]に達する可能性がある．一方，イボヨフバイ，カゲロウヨフバイ，サツマビナ，ヘコミマクラはおおむね10 MU/gの毒力で，いずれもLC-MS分析にてTTXを保有することが明らかとなった．また，アワムシロもTTXを保有することが示された．以上の結果から，沖縄県産キンシバイは食品衛生上危険な種で，イボヨフバイ，カゲロウヨフバイ，サツマビナ，ヘコミマクラ，

アワムシロについては今後警戒を続ける必要があると結論した.

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Change in the transfer profile of orally administered tetrodotoxin to non-toxic cultured pufferfish *Takifugu rubripes* depending of its development stage

Ryohei Tatsuno [a], Miwako Shikina [a], Yoshiyuki Shirai [b], Junjie Wang [b], Kiyoshi Soyano [c], Gregory N. Nishihara [c], Tomohiro Takatani [b], Osamu Arakawa [b,*]

[a] Graduate School of Science and Technology, Nagasaki University, 1-14, Bunkyo-machi, Nagasaki 852-8521, Japan
[b] Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, 1-14, Bunkyo-machi, Nagasaki 852-8521, Japan
[c] Institute for East China Sea Research, Nagasaki University, 1551-7, Taira-machi, Nagasaki 851-2213, Japan

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ABSTRACT

To investigate the effects of growth (organ development) on tetrodotoxin (TTX) dynamics in the pufferfish body, TTX-containing feed homogenate was administered to 6- and 15-month old non-toxic cultured specimens of the pufferfish *Takifugu rubripes* at a dose of 40 mouse units (MU) (8.8 μg)/20 g body weight by oral gavage. After 24 h, the specimens were killed and the skin tissues (dorsal and ventral), muscle, liver, digestive tract, and gonads were separated. TTX content (μg/g) in each tissue, determined by liquid chromatography/mass spectrometry, revealed that the TTX distribution profile, particularly the TTX content of the liver, greatly differed between the two ages; the TTX score of 15-month old fish (3.3 μg/g) was nearly 5-fold that of 6-month old fish (0.68 μg/g). The total remaining TTX amount per individual (relative amount to the given dose) was 31% in 6-month old fish, of which 71% was in the skin, and 84% in 15-month old fish, of which 83% was in the liver. The gonadosomatic index (GSI) and hepatosomatic index (HSI) scores, and histologic observations of the gonads and liver suggest that although there is little difference in maturation stage between these two ages, there are clear distinctions in the developmental stage of the liver. The results suggest that the TTX dynamics in *T. rubripes* are linked to the development of the liver, *i.e.*, the TTX taken up into the pufferfish body via food organisms is eliminated or transferred mainly to the skin in young fish with an undeveloped liver, but as the fish grow and the liver continues to develop, most of the TTX is transferred to and accumulated in the liver.

1. Introduction

Marine pufferfish generally possess a potent neurotoxin, tetrodotoxin (TTX). TTX is exogenous in pufferfish and is derived from the food chain that starts with marine bacteria (Noguchi and Arakawa, 2008), but the transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. TTX administration experiments conducted to elucidate these mechanisms have revealed that when non-toxic cultured specimens of *Takifugu rubripes* or *Takifugu niphobles* are reared with a TTX-containing diet, the toxins are efficiently accumulated into the liver and ovary (Matsui et al., 1981; Yamamori et al., 2004; Honda et al., 2005; Kono et al., 2008a). Intramuscular administration of TTX to similar pufferfish specimens, however, is first transferred to the liver and then to the skin via the blood, but toxin

* Corresponding author. Tel./fax: +81 95 819 2844.
E-mail address: arakawa@nagasaki-u.ac.jp (O. Arakawa).

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transfer to the gonads differs greatly between male and female fish (Kono et al., 2008b; Ikeda et al., 2009; Wang et al., 2011, 2012). Nagashima et al. (2003) and Matsumoto et al. (2005, 2007, 2008a,b) demonstrated that, unlike general non-toxic fish, the liver tissue of *T. rubripes* is equipped with a specific TTX-uptake mechanism, and using a pharmacokinetic model showed that TTX introduced into the pufferfish body is rapidly taken up into the liver via the blood.

Wild adult specimens of *T. rubripes* generally have TTX in the liver and ovary, but the skin, muscle, and testis are non-toxic (Tani, 1945). In juveniles, however, the toxin is occasionally detected in the skin. In addition, TTX administration experiments using non-toxic cultured juveniles revealed that a lot of toxin is transferred to the skin (Honda et al., 2005; Ikeda et al., 2009). Some studies suggest that the liver toxicity of wild *T. rubripes* increases as the fish age (Kanoh et al., 1984; Fuchi et al., 1986). Therefore, the toxin transfer/accumulation profiles inside the pufferfish body may differ depending on the stage of growth. To date, there have been no such studies of the toxin dynamics in pufferfish. Here, to elucidate the transfer, accumulation, and elimination mechanisms of TTX, we administered TTX by oral gavage according to the method of Wang et al. (2012) to non-toxic *T. rubripes* of two different ages, and compared the toxin distribution inside the body after 24 h.

2. Materials and methods

2.1. Pufferfish specimens

Non-toxic cultured 6-month old (body length; 12.3 ± 0.4 cm, body weight; 53.5 ± 6.9 g, $n = 8$ [5 males and 3 females]) and 15-month old (21.9 ± 0.7 cm, 328 ± 36 g, $n = 5$ [3 males and 2 females]) *T. rubripes* specimens were used for the TTX administration experiment as described below.

2.2. Preparation of TTX-containing feed homogenate

The toxicity of TTX purchased from Wako (purity >90%, Japan) was calibrated using a mouse bioassay according to the official guidelines of the Japan Food Hygiene Association (2005). Lethal potency was expressed in mouse units (MU), where 1 MU (equivalent to ~220 ng TTX) was defined as the amount of toxin required to kill a 20-g male ddY strain mouse within 30 min after intraperitoneal administration.

The TTX standard was dissolved in water at a concentration of 280 MU/mL for 6-month old fish and 960 MU/mL for 15-month old fish, and mixed with an artificial feed for marine juvenile fish (Otohime C2, Marubeni Nisshin Feed Co., Ltd., Japan) at a ratio of 2:1 (vol:wt), homogenized, and then the homogenate (~187 MU/mL for 6-month old fish and ~640 MU/mL for 15-month old fish) was used for the oral gavage administration (Wang et al., 2012), as described below.

2.3. TTX administration experiment

A Teflon tube (outer diameter × length = 3 × 70 mm) connected to a 5-mL syringe was inserted through the mouth into the digestive tract of each fish as deeply as possible, and 0.6–1 mL (112–640 MU; equivalent to 40 MU (8.8 μg)/20 g body weight) of TTX-containing feed homogenate was squeezed into the digestive tract. Immediately after toxin administration, the fish were returned to a 200-L aerated tank and then collected 24 h later.

2.4. TTX quantification

Skin tissues (dorsal and ventral), muscle, liver, digestive tract, and gonads (testis or ovary) of each specimen were extracted with 0.1% acetic acid (Japan Food Hygiene Association, 2005). Each tissue extract was filtered through an HLC-DISK membrane filter (0.45 μm; Kanto Chemical Co., Inc., Japan) and then liquid chromatography/mass spectrometry analysis for TTX was performed according to the method of Nakashima et al. (2004).

2.5. Gonadosomatic index (GSI) and hepatosomatic index (HSI)

The GSI of each specimen was calculated from its gonad weight (GW) and body weight (BW) according to the following equation: GSI = 100 × GW/BW, and the HSI of each specimen was calculated from its liver weight (LW) and BW according to the following equation: HSI = 100 × LW/BW.

2.6. Histologic observation

Tiny tissue blocks from the liver and gonads of representative specimens fixed in 10% neutral buffered formalin for 7 days were dehydrated through an ascending series of ethanol (70%–100%), cleared in xylene, and embedded in paraffin. The embedded tissues were sectioned with a microtome at a thickness of 3–5 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.

2.7. Statistical analyses

Student's *t*-test was applied to the toxin content data of each tissue in both age groups. A generalized linear model with a binomial distribution and a logit link-function was applied to examine if age was a significant factor in determining relative TTX amount and HSI using a χ^2-test.

3. Results

3.1. Transfer profile of TTX

In both the 6- and 15-month old fish, TTX administered inside the digestive tract was transferred to the skin, liver, and ovary, but not the muscle and testis. As no sex difference was recognized in the TTX content (μg/g) of each tissue other than the gonads (data not shown), the content was compared between age groups without distinguishing between females and males. The toxin transfer/remaining profiles of the skin, liver, and digestive tract differed remarkably between age groups (Fig. 1). In the 6-month old

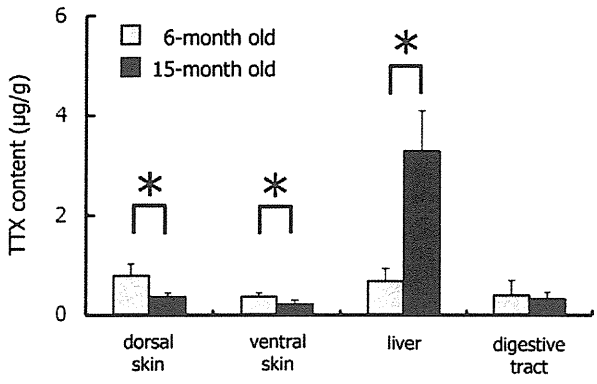

Fig. 1. Comparison of TTX content (μg/g) in each tissue between 6- and 15-month old fish. Data are shown as the mean (column) and SD (error bar) of each tissue in each age. Asterisks indicate significant differences (*t*-test, $p < 0.05$).

fish, TTX in the dorsal and ventral skin, and liver (0.37–0.79 μg/g) was almost the same level as that in the digestive tract (0.39 μg/g), whereas in the 15-month old fish, TTX content was remarkably higher in the liver (3.3 μg/g) than in the other tissues, although the TTX content of the skin (0.22–0.35 μg/g) was the same as that of the digestive tract (0.33 μg/g). The TTX content was significantly higher ($p < 0.05$) in the liver of the 15-month old fish than in the 6-month old fish, and significantly higher in the dorsal and ventral skin of the 6-month old fish than in the 15-month old fish ($p < 0.05$).

The TTX content of the ovary was 2.4–3.2 μg/g ($n = 3$) in the 6-month old fish, and 0.05 and 2.2 μg/g ($n = 2$) in the 15-month old fish, and there was no significant difference between the two age groups.

The amount of remaining toxin in the body per individual (TTX amount of each tissue is shown as stacked bars, and expressed as a value relative to the given dose) is shown in Fig. 2. For comparison without distinguishing between females and males, the TTX amount in the ovary (all less than 5%) was excluded from the graph. The total remaining toxin amount was significantly higher in the 15-month old fish (84%) than in the 6-month old fish (31%, $p < 0.05$). The toxin distribution profiles were also greatly different between age groups; 71% of the total remaining toxin was distributed in the skin and 21% in the liver in the 6-month old fish, while 83% was retained in the liver and 14% in the skin in the 15-month old fish.

3.2. Growth of the liver and gonads

The GSI was 0.04–0.19 in the 6-month old fish, and 0.16–0.33 in the 15-month old fish. Histologic observation of gonad sections (data not shown) indicated that most oocytes of the ovaries were in the perinucleolus stage and immature in both age groups. The testes were in the spermatogonial proliferation stage or resting stage, suggesting that the fish could produce a little sperm, but were not functional in males at either age. On the other hand, the HSI was significantly higher ($p < 0.05$) in the 15-month old fish (~10) than in the 6-month old fish (<6; Fig. 3). The HSI of wild mature *T. rubripes* specimens (body weight around 3 kg) was 6.8–7.9 (unpublished data), indicating that the HSI of the 15-month old fish was approximately the same level as that of adult fish. Moreover, hepatocyte size in the 15-month old fish (diameter ~25 μm) was larger than that in the 6-month old fish (~15 μm; Fig. 4).

4. Discussion

Wang et al. (2012) conducted a TTX administration experiment using artificial hybrid specimens of *T. rubripes* and *Takifugu porphyreus* ('torama'; 8-month old), and reported that the amount of TTX administered by oral gavage and transferred to the liver reached a maximum at

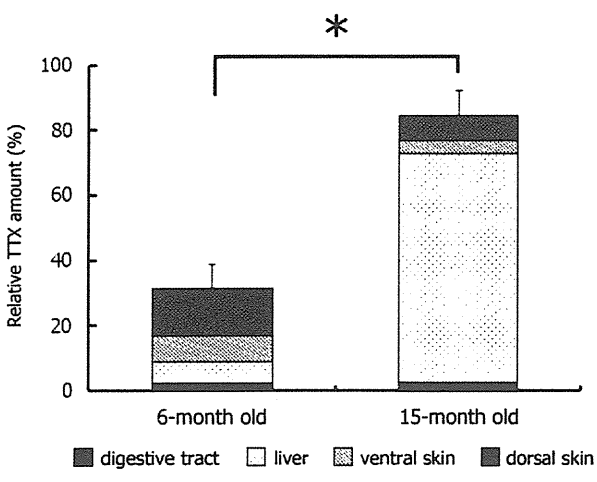

Fig. 2. Comparison of remaining TTX amount per individual (relative value to the given dose) between 6- and 15-month old fish. Data are shown as the mean (stacked column) and SD (error bar) of each age. Asterisk indicates a significant difference (χ^2-test, $p < 0.05$).

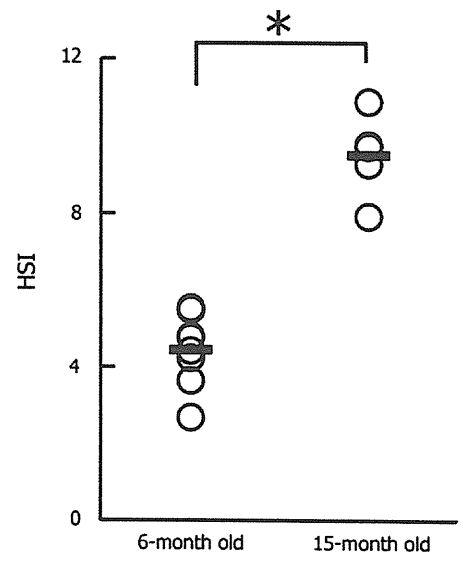

Fig. 3. Comparison of hepatosomatic index (HSI) between 6- and 15-month old fish. Data are shown as individual values (open circles) and the mean of each age (horizontal bars). Asterisk indicates a significant difference (χ^2-test, $p < 0.05$).

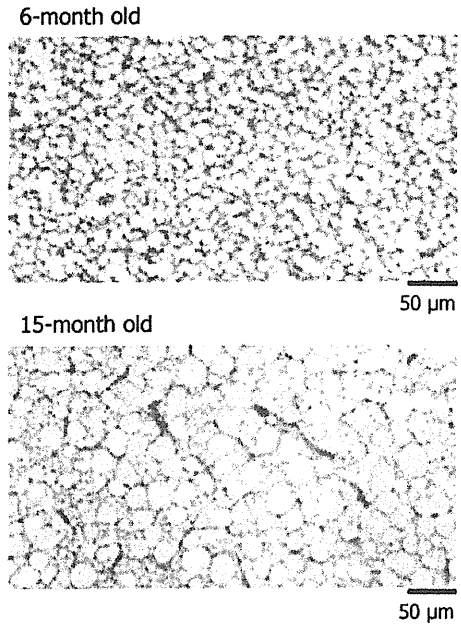

Fig. 4. Light micrographs of a representative hepatic section from 6-month old fish (upper) and 15-month old fish (lower).

24 h after toxin administration. Based on this finding, the collection time of the test fish in the present study was set at 24 h after toxin administration. A great difference in the TTX transfer profile, particularly the amount of toxin that transferred to the liver, was detected between 6- and 15-month old fish; *i.e.*, in the 15-month old fish, the TTX content (μg/g) of the liver was approximately 4- to 5-fold higher than that in the 6-month old fish (Fig. 1), and the relative TTX amount (%) was approximately 10-fold higher than that in the 6-month old fish (Fig. 2). The GSI/HSI scores (Fig. 3), and the histologic observations of the gonads/liver (Fig. 4) suggested little difference in the maturation stage between the two age groups, but there were clear distinctions in the developmental stage of the liver. Therefore, the different profiles of TTX transfer to the liver were considered to be due not to sexual maturation, but to the growth-mediated developmental stage of the liver. Ikeda et al. (2010) investigated the toxicity of the wild pufferfish *Takifugu poecilonotus*, and found that ovary toxicity rises remarkably with sexual maturation. In addition, Wang et al. (2011) reported that intramuscular administration of TTX to hybrid specimens produced by crossbreeding *T. rubripes* with *T. niphobles*, which matures earlier than *T. rubripes* ('kusatora'; 10-month old), is first taken up in the liver then transferred/accumulated to the skin in male specimens and to the ovary in female specimens. These findings suggest that TTX dynamics in the pufferfish body are strongly influenced by sexual maturation, and the present study further supports the effect of growth/development or aging.

Following TTX administration to 'torama' pufferfish (Wang et al., 2012), toxin transfer to the skin was first observed more than 24 h after toxin administration, but TTX was detected in the skin at 24 h in the present experiment. In contrast to the liver, TTX content and relative TTX amount in the skin tissues were significantly higher ($p < 0.05$) in the 6-month old fish than in the 15-month old fish (Figs. 1 and 2). In addition, they were significantly higher ($p < 0.05$) on the dorsal side than on the ventral side at both ages. In general, wild adult specimens of *T. rubripes* possess no toxin in the skin, but toxicity is occasionally detected in juveniles. Moreover, in a TTX administration experiment using *T. rubripes* juveniles (4-month old), most of the administered toxin was transferred to and eventually accumulated in the skin (Ikeda et al., 2009). Hence, it is presumed that during an early age, *T. rubripes* transport TTX mainly to their skin to accumulate or eliminate because the liver is undeveloped and has low TTX-accumulating ability, but the toxin amount in the skin decreases as they grow, as the liver develops, where TTX is then stored. As no histologic observation of the skin was conducted in the present study, it is not clear whether the developmental stage (difference in TTX-excreting ability) of the skin affects the TTX transfer profile, or why the toxin amount on the dorsal side was higher than that on the ventral side.

Here, we observed that toxin transfer to the ovary occurred despite the fact that the ovary was immature. In the toxin administration experiment using the artificial hybrid pufferfish 'kusatora' (Wang et al., 2011), the TTX content of the ovary reached about 8.8 μg/g at 24 h after toxin administration. In comparison, in the present study, the TTX content of the ovary (around 2.2 μg/g) was relatively low. Further studies are needed to examine the relationship between ovarian maturation and toxin accumulation.

Yotsu-Yamashita et al. (2001) isolated pufferfish saxitoxin- and TTX-binding protein (PSTBP) from the blood plasma of *Takifugu pardalis*, and suggested its involvement in the toxin transportation/accumulation mechanisms. They also indicated the presence of a PSTBP-like protein in *T. rubripes* blood plasma (Yotsu-Yamashita et al., 2010), although the mRNA was detected mainly in the liver tissue (Tatsuno et al., 2012), arousing our interest in the relationship between the developmental stage of the liver and the expression of the protein or its isoforms. Additional studies along this line are in progress.

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Conflict of interest

None declared.

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