and Science, Tsukuba (NSMT), and the paratype at the Kagoshima University Museum (KAUM).

Torquigener albomaculosus sp. nov

(New English name: White-spotted Pufferfish; New Japanese name: Amami-hoshizora-fugu) (Figs. 2, 3, 4, 5, 6, 7)

Torquigener sp.: Kawase et al. 2013: 1, fig. 1 (Amami-oshima Island, same as the type locality).

Holotype. NSMT-P 118118, male, 87.8 mm SL (109 mm TL), Katetsu Cove, south coast of Amami-oshima Island, Ryukyu Islands, 18 m depth, 21 May 2014.

Paratype. KAUM-I 61100, female, 90.5 mm SL (110 mm TL), same as the holotype but collected at 15 m depth.

Diagnosis. A species of *Torquigener* with the following unique combination of characters: dorsal-fin rays 9 (10); anal-fin rays 6; pectoral-fin rays 16 (dorsalmost ray nubbin-like and rudimentary); vertebrae 8 + 11 = 19; no solid, dark, longitudinal stripe nor longitudinal rows of dark spots on the mid-side of body from behind pectoral fin to caudal-fin base; no vertical markings on cheek; dorsal half of head and body covered with fine brown reticulations and many white spots; ventral half of head and body silvery white covered by many white spots from chin to above anal-fin origin; dorsal rim of eye light yellow; and many two-rooted spinules on head and body.

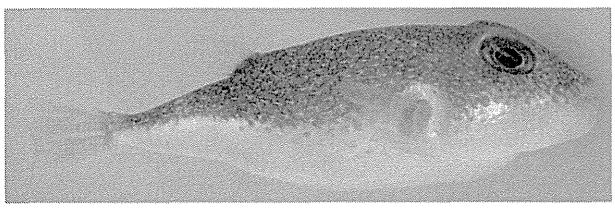

Fig. 3 The holotype of *Torquigener albomaculosus* sp. nov. immediately after collection from Katetsu Cove along the south coast of Amami-oshima Island in the Ryukyu Islands. Lateral view. Photograph by Tomohiro Yoshida

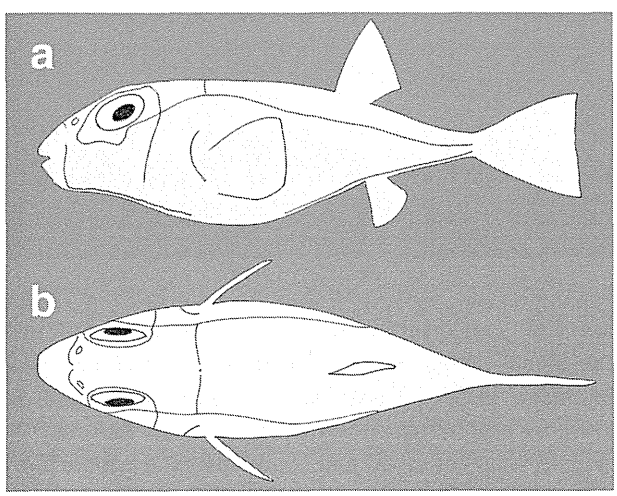

Fig. 4 Schematic illustration of lateral line system of *Torquigener albomaculosus* sp. nov. **a** Lateral view; **b** dorsal view

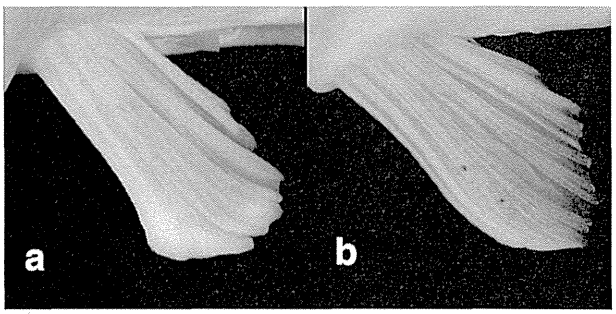

Fig. 5 Anal fin of preserved specimens of *Torquigener albomaculosus* sp. nov. **a** Holotype, NSMT-P 118118, male; **b** paratype, KAUM-I. 61100, female. Two small holes on anterior part of anal fin of the paratype were artificially made when the fin was extended for taking the photograph of Fig 1. Photographs by Eri Katayama

Description. Body proportions expressed as percentage of SL. Head length 38.4 (35.4), snout length 17.4 (18.2), snout to dorsal-fin origin 67.4 (68.3), snout to anal-fin origin 73.4 (72.4), body width at pectoral-fin base 30.4 (29.5), body depth at end of dorsal fin 15.5 (17.9), body depth at anal-fin origin 16.9 (18.3), depth of caudal peduncle 6.4 (6.9), length of caudal peduncle 23.3 (25.6), gill-opening length 11.7 (9.5), eye diameter 12.6 (12.2), bony interorbital width 7.0 (9.1), snout to anterior edge of nasal organ 11.2 (11.0), posterior edge of nasal organ to anterior edge of eye 5.1 (4.8), length of dorsal-fin base 7.3 (9.6), length of anal-fin base 4.6 (5.0), longest dorsal-fin ray 18.6 (17.9), longest anal-fin ray 12.0 (12.7), longest pectoral-fin ray 17.8 (18.1), caudal-fin length 24.7 (23.2).

Body moderately elongate, rounded dorsally and flattened ventrally in cross-section, tapering posteriorly to laterally compressed caudal peduncle. A longitudinal skin fold extending on ventrolateral corner of body from chin to ventral part of caudal-fin base. Mouth small, terminal; lips thin, covered with many short papillae; chin prominent. Nasal organ a short, erect papilla well before eye, with two well-separated openings, posterior opening much larger than anterior opening, inner surface with several well-developed flaps around circumference. Eye large, 3.0 (2.9) in HL, elliptical, and dorsally adnate, ventral rim at level of dorsal end of gill opening; interorbital region slightly concave. Gill opening a slightly curved slit extending ventrally from level of ventral rim of eye to level of two-thirds down pectoral-fin base.

Dorsal fin slightly rounded, third dorsal-fin ray longest, dorsal-fin origin just behind a vertical through vent; anal fin slightly rounded, much smaller than dorsal fin, second anal-fin ray longest, first and second anal-fin rays of male

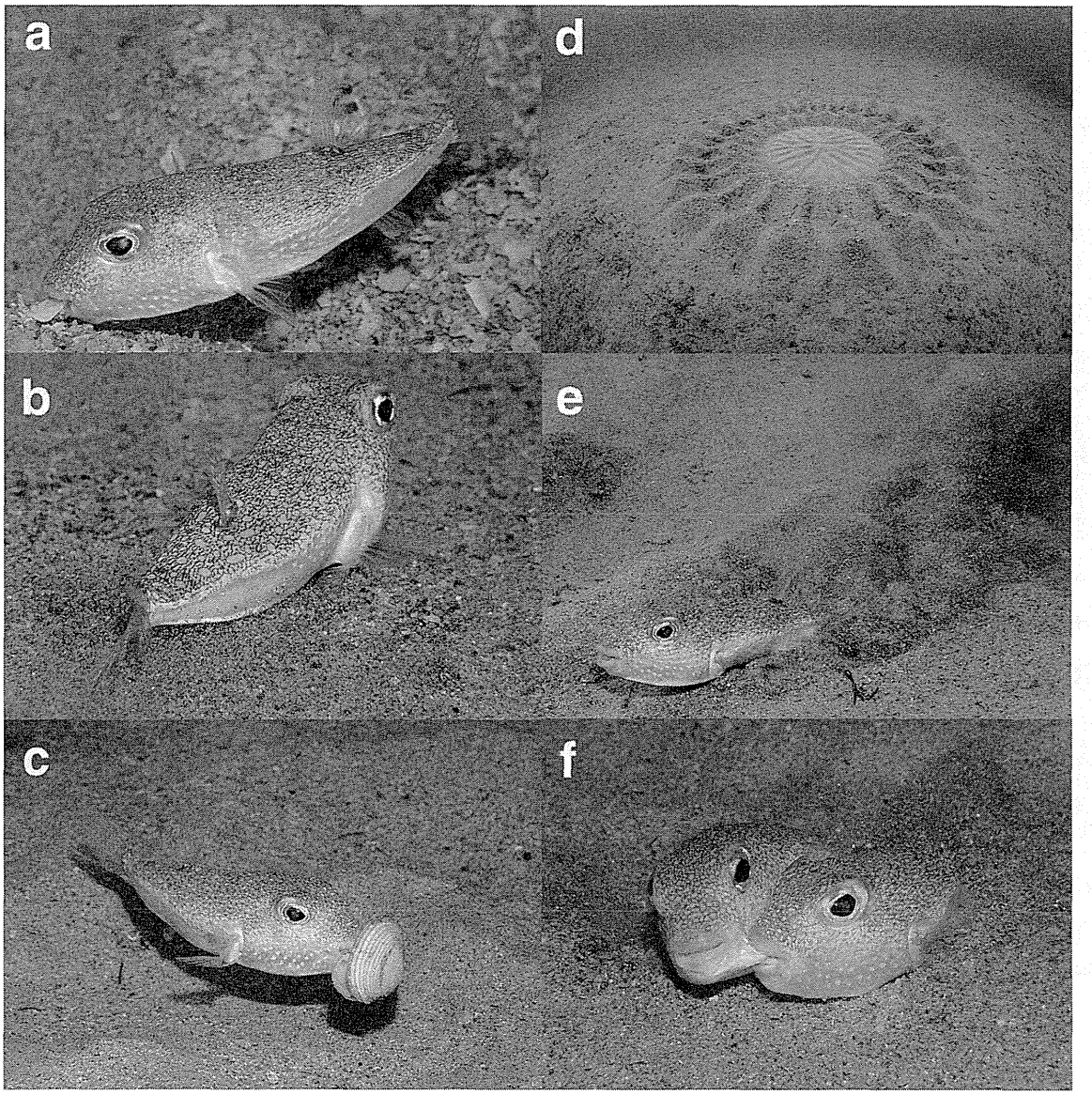

Fig. 6 Underwater photographs of *Torquigener albomaculosus* sp. nov. and its spawning nest. **a** *Torquigener albomaculosus* placing a piece of shell on a ridge of a spawning nest, 11 July 2012; **b** posterior view of a male of *T. albomaculosus* making a trough on a spawning nest, 28 May 2011; **c** a male of *T. albomaculosus* bringing a dead bivalve in a spawning nest; **d** a spawning nest (mystery circle) of *T. albomaculosus* found at 26 m depth on a sandy bottom along the south coast of Amami-oshima Island in the Ryukyu Islands, 28 May 2011; **e** a male digging a trough by vibrating anal fin and posterior half of body, 28 May 2011; **f** a male (right) biting on the left cheek of a female (left) while they were spawning, 13 July 2012. Photographs by Yoji Okata

thickened and united distally but these rays normal in female (Fig. 5), anal-fin origin just below posterior end of dorsal fin; pectoral fin rounded, first pectoral-fin ray rudimentary and nubbin-like, second pectoral-fin ray longest, dorsal end of pectoral-fin base at level between ventral rim of eye and mouth; caudal fin almost truncate, slightly concave.

Two lateral lines on head and side of body (Fig. 4). The dorsalmost lateral line encircles the eye, with a preopercular branch terminating at level of ventral end of pectoral-fin base and a posteriorly directed branch coursing along the mid-lateral side of body from just behind ventroposterior part of eye to caudal-fin base, with a dorsally directed branch just above pectoral-fin base almost meeting in midline with its counterpart from the other side of the body. A short transverse element of lateral line in front of nasal organ almost meeting in midline with its counterpart from the other side of the snout. The ventral lateral line originates posterior to the mouth and then courses along the ventrolateral edge of body to caudal-fin base with an interruption ventral to the pectoral fin. Two-rooted spinules present on head and

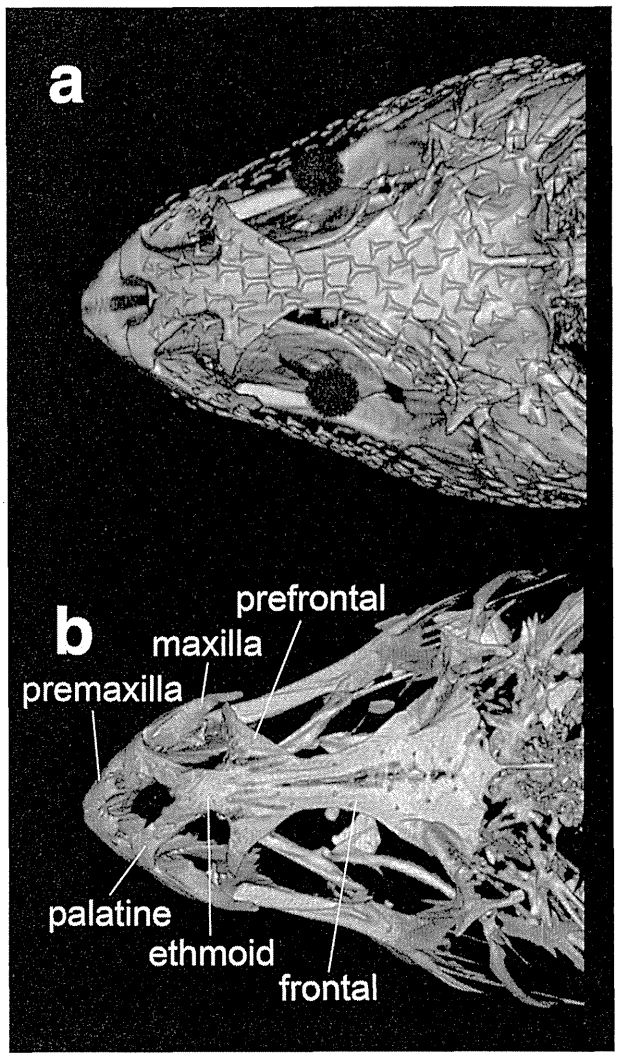

Fig. 7 CT scanning images of skull, associated bones and two-rooted spinules of *Torquigener maculosus* sp. nov. **a** Shallow layer image showing two-rooted spinules scattered over head and body. **b** deeper layer image showing skull and associated bones. Images made by Eri Katayama and Nozomi Kurihara

body (Figs. 2, 3, 7a), projecting from short, normally recessed papillae; extending on dorsal surface from just behind mouth to level of posterior end of dorsal fin and on lateral surface from cheek to level of posterior end of dorsal fin; 9 (10) spinules across dorsum immediately behind mid-dorsal branches of lateral lines; 19 (21) spinules across belly on a line between pectoral-fin bases; smaller spinules making two longitudinal rows along the dorsalmost lateral line from level of posterior end of dorsal fin to caudal peduncle, the dorsal row of spinules just above mid-body lateral line and the ventral row of spinules just below dorsalmost lateral line; two-rooted spinules also densely present on ventral surface from just behind chin to vent.

Color of fresh specimens before preservation (Fig. 2). Dorsal half of head and body covered by fine brown reticulations and many white spots; ventral half of head and body silvery white covered by many white spots from chin to above anal-fin origin; dorsal rim of eye light yellow; dorsal, anal and pectoral fins transparent with slight yellowish tinge; yellow smudge on anterior end of pectoral-fin base and axial part of pectoral fin base also light yellow; caudal fin transparent with yellowish brown dots on rays.

Color when alive (based on color photographs of holotype and paratype when alive, and underwater photographs of non-type individuals: Figs. 3, 6). Color of head, body, and fins almost same as the color of fresh specimens before preservation but network pattern on dorsal half of head and body bright yellowish brown; white spots on ventral half of body distinctive; light yellow spots forming a longitudinal row on mid-lateral body from behind pectoral fin to caudal-fin base.

Distribution. *Torquigener albomaculosus* has been observed by local SCUBA divers at depths from 10 m to 27 m on the sandy bottom along the south coast of Amami-oshima Island of the Ryukyu Islands. According to these SCUBA divers, another population of *T. albomaculosus* has recently been found around 30 m depth in the northern part of Amami-oshima Island. Judging from distributions of other tropical species of *Torquigener*, *T. albomaculosus* will probably be found in the tropical region of the West Pacific.

Etymology. The specific name, *albomaculosus*, refers to many white spots on the body.

Remarks. The new pufferfish is classified in the genus *Torquigener* by having the following combination of characters: eye dorsally adnate only; chin well developed (Fig. 2); two openings on nasal organ; ventrolateral skin fold extending behind pectoral fin to caudal-fin base; frontals elongate and narrow across the orbit, not more than twice the width of the mid-dorsal surface of ethmoid at the posterior margin of prefrontals (Fig. 7b); and the dorsal surface of ethmoid extending well forward of the anterior margin of prefrontals. The revision of 12 Australian species of *Torquigener* (see Hardy 1983a) was followed by descriptions of additional seven new species from various regions of the Indo-Pacific (Hardy, 1983b, 1984, 1989; Hardy and Randall 1983). *Torquigener albomaculosus* brings the total number of species of *Torquigener* to 20. The most helpful characters distinguishing *T. albomaculosus* from all other species of the genus are coloration of the head and body provided in the Diagnosis above.

To the best of our knowledge, *T. albomaculosus* is unique in building large geometric circles on sandy bottoms. Kawase et al. (2013) reported on the reproductive behavior of *T. albomaculosus* showing how this pufferfish builds its circles.

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Biliary excretion of tetrodotoxin in the cultured pufferfish *Takifugu rubripes* juvenile after intramuscular administration

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ABSTRACT

Marine pufferfish of the family Tetraodontidae accumulate a considerable amount of tetrodotoxin (TTX), mainly in the liver and ovary. The detailed distribution of TTX in pufferfish body tissues, however, remains poorly understood. Here we investigated the tissue distribution and biliary excretion of TTX in cultured pufferfish *Takifugu rubripes* juveniles (6-month-old, 81.5 ± 2.0 g body weight) for 24 h after intramuscular administration of 0.25 μg TTX/g body weight into the caudal muscle. The blood TTX concentration was 0.53 ± 0.15 μg/mL at 1 h, and gradually decreased to 0.05 ± 0.01 μg/mL at 24 h after administration ($p < 0.05$). The TTX concentration in the liver declined from 1.59 ± 0.10 μg/g at 1 h to 0.48 ± 0.21 μg/g at 24 h ($p < 0.05$). In contrast, the TTX concentration in the skin increased from 0.27 ± 0.04 μg/g at 1 h to 0.48 ± 0.08 μg/g at 24 h ($p < 0.05$). The concentration of TTX in the bile remarkably increased from 0.08 ± 0.03 μg/mL at 1 h to 0.39 ± 0.05 μg/mL at 8 h ($p < 0.05$) and remained at almost the same level at 24 h. These findings indicate that TTX was excreted from the liver into the gallbladder bile in the pufferfish *T. rubripes* juveniles.

1. Introduction

Marine pufferfish of the family Tetraodontidae generally accumulate a considerable amount of tetrodotoxin (TTX) that selectively blocks the voltage-gated sodium channels in muscle and nerve tissue, causing severe and sometimes fatal food poisoning (Kao, 1982; Halstead, 1988; Narahashi, 2001). In wild adult pufferfish, TTX mainly localized in high levels in the liver and ovary, although the distribution and accumulation of TTX is species-specific (Hashimoto, 1979; Noguchi et al., 2006a; Bane et al., 2014). Pufferfish mainly acquire the toxin through the food chain associated with biologic magnification (Yasumoto and Yotsu-Yamashita, 1996; Noguchi and Arakawa, 2008), based on findings that artificially hatched and grown pufferfish are non-toxic when reared with non-toxic diets (Matsui et al., 1981; Lin et al., 1998; Noguchi et al., 2006b), and that they become toxic when reared with TTX-containing diets (Matsui et al., 1981; Yamamori et al., 2004; Kono et al., 2008). The detailed biologic function involved in the tissue distribution of TTX in pufferfish body, however, remains poorly understood.

We used a new approach to evaluate the TTX uptake mechanism in the pufferfish liver. Liver tissue slices prepared from non-toxic cultured *Takifugu rubripes* adults accumulated TTX over time, when the slices were incubated in TTX-containing medium. The amount of TTX accumulated in the liver tissue slices did not decrease even when further incubated in medium without TTX (Nagashima et al., 2003). Other studies revealed a specific accumulation of TTX preferentially over saxitoxins in liver tissue slices (Matsumoto et al., 2005) and the involvement of the carrier-mediated transport system in the TTX uptake mechanism by the pufferfish *T. rubripes* liver (Matsumoto et al., 2007). Furthermore, we investigated the pharmacokinetics of TTX in *T. rubripes* adults, and observed that most of the TTX given by single administration was transferred into the liver within a relatively short time

Abbreviations: LC-MS/MS, liquid chromatography tandem mass spectrometry; TTX, tetrodotoxin; STX, saxitoxin; PSTBP, pufferfish saxitoxin and tetrodotoxin binding protein.

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0041-0101/

regardless of the administration route. At a dosage of 0.25 mg TTX/kg body weight into the hepatic vein, hepatic portal vein, or gastrointestinal tract, the TTX amount in the liver at 300 min after administration accounted for 84 ± 6%, 70 ± 9%, or 49 ± 17% of the total TTX amount applied, respectively. Comparison of the area under the blood concentration-time curves following the single administration of 0.25 mg TTX/kg body weight in the hepatic vein and gastrointestinal tract estimated the bioavailability of TTX to be 62%. These findings indicate that TTX is well absorbed in the circulation from the gastrointestinal tract and predominantly accumulated in the liver (Matsumoto et al., 2008a). An integration plot analysis revealed that the hepatic uptake clearance of TTX in *T. rubripes* adults is remarkably low compared with the hepatic portal vein blood flow rate, indicating the negligible hepatic first-pass effect of TTX in pufferfish. It should be noted that the liver-specific distribution is not achieved by the hepatic first-pass effect, but rather by the removal of the toxin from the circulation involving a hepatic transmembrane transport-limited process (Matsumoto et al., 2008b). Several studies have examined the pharmacokinetic properties of TTX in adult specimens.

Tatsuno et al. (2013) reported differences in the transfer profile of orally administered TTX to non-toxic cultured pufferfish *T. rubripes* in the development stage and adult stage. When TTX-containing feed homogenate (0.44 mg TTX/kg body weight) was administered by oral gavage to 6- and 15-month-old specimens, the TTX distribution profile greatly differed between the two ages after 24 h. The total remaining TTX amounts in 6-month-old fish and 15-month-old fish were 31% and 84% of the given dose, respectively. The young fish had TTX mainly in the skin, whereas the adults had TTX exclusively in the liver. In addition, when non-toxic cultured specimens of *T. rubripes* (4-month-old) were intramuscularly injected with crystalline TTX (10 μg/individual with body weight of 13.2 ± 3.4 g), the concentration of TTX in the liver gradually decreased, and most of the toxin remaining in the body accumulated in the skin after only 12 h (Ikeda et al., 2009). These findings suggest that in *T. rubripes* juveniles TTX is excreted from the liver and transported via the circulation to the skin. Little is known, however, about the excretion mechanisms of TTX in the liver of marine pufferfish.

In the present study, we investigated the tissue distribution and biliary excretion of TTX in cultured *T. rubripes* juveniles after intramuscular administration of the toxin into the caudal muscle. We demonstrated that TTX is distributed to the circulating blood from the administration site, initially accumulated in the liver, and transferred to the skin over time, and at the same time, TTX is excreted from the liver into the gallbladder bile.

2. Materials and methods

2.1. Materials

Cultured marine pufferfish *T. rubripes* juveniles (6-month-old, 81.5 ± 2.0 g body weight, 15 individuals) were purchased from a local fish farmer in Kumamoto Prefecture, Japan, and used as the test fish. Crystalline TTX (Nacalai Tesque, Kyoto, Japan) was used in the administration experiment and as a standard for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. All other chemicals were of reagent grade.

2.2. TTX administration and sample preparation

The administration experiment was performed at the laboratory of the Prefectural University of Hiroshima. TTX was dissolved in modified Hank's balanced salt solution buffer (160 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 10 mM HEPES adjusted to pH 7.4 with NaOH solution) as reported previously (Matsumoto et al., 2008a). The test fish received an intramuscular administration of 0.25 μg TTX/g body weight into the caudal muscle as described previously (Matsumoto et al., 2011), and were maintained in a 90-L circular plastic tank of aerated artificial seawater at 20 °C. At 1, 4, 8, 12, and 24 h after administration, each group of three fish was randomly collected, ice-cold anesthetized, and dissected. The gallbladder bile, liver, spleen, kidney, skin, and muscle were immediately weighed and stored at −20 °C until use. The blood was obtained from the hepatic vein using a heparinized 1-mL disposable syringe (Terumo, Tokyo, Japan), and mixed with an equal volume of methanol/acetic acid solution to extract TTX as reported previously (Matsumoto et al., 2008a).

2.3. TTX extraction and quantification

TTX extraction was performed on the basis of the standard assay procedures for TTX (Kodama and Sato, 2005). TTX in the tissue samples was extracted with 0.1% acetic acid by heating in a boiling water bath for 10 min after ultrasonication for 1 min as reported previously (Matsumoto et al., 2008b). The extracts from the tissues other than gallbladder bile were defatted with dichloromethane and centrifuged at 2600×*g* for 30 min. The resulting supernatant was ultrafiltered (MWCO 5000). The filtrate was lyophilized and redissolved in 16 mM ammonium formate (pH 5.5). TTX was determined by the LC-MS/MS analysis according to the method of Nakagawa et al. (2006) with some modification. Briefly, the analytical column was a TSKgel Amide-80 (2.0 × 250 mm, 3 μm particle size, Tosoh, Tokyo, Japan) maintained at 35 °C. Mobile phases A and B were 16 mM ammonium formate (pH 5.5) and acetonitrile, respectively, and the flow rate was set at 0.2 mL/min. The elution profile was 55% B at 0–10 min, 90% B at 10–13 min, and 55% B at 13–20 min. The eluate was induced into the ion source of LC-MS/MS system composed of an 1100 HPLC unit (Agilent Technologies, Santa Clara, CA, USA) and an API2000 triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA). TTX was ionized by the positive ion mode, and the fragment ion at *m/z* 162 that results from the dissociation of the parent ion of TTX at *m/z* 320 was detected by the multiple reaction-monitoring mode. For the quantitation, intact blood, gallbladder bile, and tissue extracts were spiked with TTX standard.

2.4. Pharmacokinetic and statistical analyses

The intramuscular bioavailability (F_{im}), apparent distribution volume (V_d), absorption rate constant (k_a), elimination rate constant (k_{el}) were estimated from the following equation:

$$C = \frac{F_{im} D \cdot k_a}{V_d \cdot (k_a - k_{el})} \cdot \left(e^{-k_{el} \cdot t} - e^{-k_a \cdot t} \right) \quad (1)$$

where C is the blood TTX concentration, D is the amount of TTX administered into the caudal muscle, respectively. The blood TTX concentrations were fitted to the one-compartment model with first order absorption, Eq. (1) by an iterative nonlinear least-squares method using a MULTI program and the algorithm of the Damping Gauss Newton method (Yamaoka et al., 1981). Data are expressed as mean ± standard error (SE), and Tukey's test were used to analyze the significance of differences among the means at the 5% significance level.

3. Results

Blood and tissues from the cultured pufferfish *T. rubripes* juveniles used in the present study did not contain a detectable level of

TTX (<10 ng TTX/mL blood, <1 ng TTX/mL bile, and <10 ng TTX/g tissue).

The time-courses of TTX concentrations in the blood, spleen, kidney, liver, skin, and muscle after intramuscular administration into the caudal muscle at a dose of 0.25 μg TTX/g body weight are shown in Fig. 1. The blood concentration of TTX was 0.53 ± 0.15 μg/mL at 1 h after administration, gradually declining to 0.05 ± 0.01 μg/mL at 24 h, as observed previously (Matsumoto et al., 2011). There were significant differences between the value at 1 h and that at 8, 12, or 24 h ($p < 0.05$). The TTX concentration in the liver was 1.59 ± 0.10 μg/g at 1 h after administration, and changed thereafter to 0.80 ± 0.02 μg/g, 1.06 ± 0.12 μg/g, and 0.48 ± 0.21 μg/g at 8, 12, and 24 h after administration, respectively. There was a significant difference between the value at 1 or 4 h and that at 24 h, respectively ($p < 0.05$). The TTX concentration in the skin was 0.27 ± 0.04 μg/g, 0.50 ± 0.01 μg/g, 0.40 ± 0.03 μg/g, and 0.48 ± 0.08 μg/g at 1, 8, 12, and 24 h after administration, respectively ($p > 0.05$). The TTX concentration in the kidney was 0.14 ± 0.02 μg/g at 1 h and slowly decreased to 0.04 ± 0.01 μg/g at 24 h ($p < 0.05$). During the experiment, there was no significant change in the concentrations in the spleen (0.14 ± 0.08 to 0.04 ± 0.02 μg TTX/g, $p > 0.05$) or muscle (0.02 ± 0.00 to 0.04 ± 0.02 μg TTX/g, $p > 0.05$). In contrast, the TTX concentration in the bile markedly increased from 0.08 ± 0.03 μg/mL at 1 h to 0.39 ± 0.05 μg/mL at 8 h after administration ($p < 0.05$), and then the value remained steady at that level until the end of experiment at 24 h (Fig. 2), indicating that TTX was excreted from the liver into the bile.

TTX amounts accumulated in the tissues are summarized in Table 1. Although the amount of TTX in the liver was the highest among the tissues up to 4 h after administration, the toxin amount in the skin was predominant at 8 h after administration and later. The TTX amount in the skin increased over time as follows: 3.06 ± 0.54, 3.31 ± 0.65, 5.60 ± 0.23, 4.20 ± 0.25, and 6.07 ± 0.84 μg at 1, 4, 8, 12, and 24 h after administration, respectively. Comparison of the TTX amount at 24 h and that at 1 h revealed that the value decreased by 52% from 5.82 ± 1.05 μg to 2.81 ± 1.11 μg in the liver while it increased two-fold from 3.06 ± 0.54 μg to 6.07 ± 0.84 μg in the skin. The amount of TTX in the bile significantly increased from 0.01 ± 0.00 μg at 1 h to 0.09 ± 0.01 μg at 8 h after administration ($p < 0.05$), and thereafter the value was kept constant until the end of experiment at 24 h. On the other hand, the TTX amounts in the spleen and the muscle were unchanged, although that in the kidney was slightly decreased ($p < 0.05$). Throughout the experiment, the total amount of TTX was almost constant at around 50% of the administered dose.

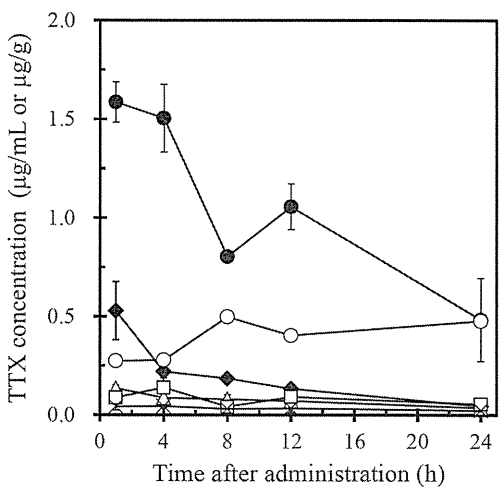

Fig. 1. Time-courses of TTX concentration in blood, spleen, kidney, liver, skin, and muscle of *Takifugu rubripes* juveniles after single intramuscular administration into caudal muscle at a dosage of 0.25 μg TTX/g body weight. TTX concentration in blood (◆) is shown as micrograms TTX per milliliter of blood. The concentrations in spleen (□), kidney (△), skin (○), muscle (×), and liver (●) are shown as micrograms TTX per gram of tissue, respectively. Each data and vertical bar represents the mean ± SE of three individuals.

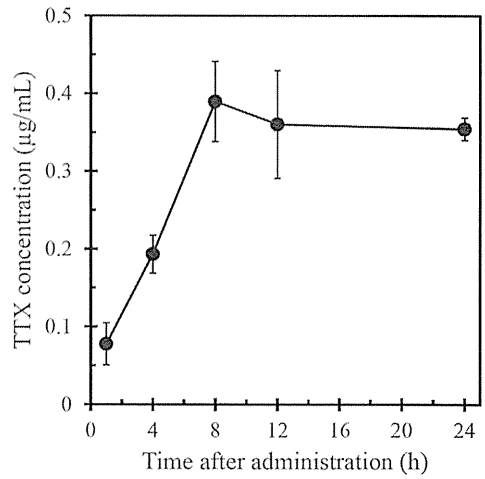

Fig. 2. Time-course of TTX concentration in gallbladder bile of *Takifugu rubripes* juveniles after single intramuscular administration into the caudal muscle at a dosage of 0.25 μg TTX/g body weight. TTX concentration in gallbladder bile (●) is shown as micrograms TTX per milliliter of bile. Each data and vertical bar represents the mean ± SE of three individuals.

The adsorption and elimination rate constants were evaluated by compartment model analysis. As illustrated in Fig. 3, the blood concentration of TTX was approximately described by the one-compartment model equation with first order absorption. Pharmacokinetic parameters are summarized in Table 2. The intramuscular bioavailability (F_{im}), apparent distribution volume (V_d), absorption rate constant (k_a), elimination rate constant (k_{el}) were estimated to be 82.0%, 360 mL/kg body weight, 4.19×10^{-1} min^{-1}, and 2.00×10^{-3} min^{-1}, respectively.

4. Discussion

In the present study, we investigated the tissue distribution and biliary excretion of TTX in cultured pufferfish *T. rubripes* juveniles after intramuscular administration, and demonstrated that TTX was distributed to the circulating blood from the administration site, initially concentrated into the liver, and then transferred to the skin over time. The amounts of TTX in liver accounted for 38.8% and 20.2% of the administered TTX at 4 h and 12 h, respectively. Our previous study using cultured *T. rubripes* adults (approximately 2-year-old), however, revealed that the hepatic accumulation reached 68 ± 4% that of the administered dose within 12 h after intramuscular administration (Matsumoto et al., 2011). These findings indicate that the tissue distribution of administered TTX clearly differs between *T. rubripes* juveniles and adults. Tatsuno et al. (2013) examined the TTX distribution profile of 6-month-old and 15-month-old cultured *T. rubripes* and suggested that TTX accumulation was related the development of the liver. These findings led us to hypothesize that a difference in liver function was involved in the accumulation of TTX between pufferfish juveniles and adults. That is, juveniles may have less ability to maintain TTX in the liver or greater ability to excrete the toxin from the liver, while adults have greater ability to accumulate more TTX in the liver or less ability to excrete TTX.

Table 1
Distribution of TTX in cultured pufferfish *Takifugu rubripes* juveniles after an intramuscular administration into the caudal muscle.

Time (h)	Body weight (g)	Dose (μg)	TTX amount (μg)							Total (% of dose)
			Spleen	Kidney	Skin	Muscle	Liver	Bile	Total	
1	77.7 ± 5.1[a]	19.4 ± 1.3[a]	0.02 ± 0.01[a]	0.15 ± 0.02[a]	3.06 ± 0.54[a]	0.97 ± 0.50[a]	5.82 ± 1.05[a]	0.01 ± 0.00[a]	10.03 ± 2.02[a]	51.0 ± 8.1[a]
4	83.3 ± 4.3[a]	20.8 ± 1.1[a]	0.02 ± 0.01[a]	0.11 ± 0.02[a,b]	3.31 ± 0.65[a]	1.08 ± 0.24[a]	8.07 ± 2.11[a]	0.04 ± 0.02[a,b]	12.63 ± 1.33[a]	60.8 ± 6.9[a]
8	81.0 ± 2.8[a]	20.3 ± 0.7[a]	0.01 ± 0.00[a]	0.09 ± 0.02[a,b]	5.60 ± 0.23[a,b]	0.77 ± 0.36[a]	3.22 ± 0.12[a]	0.09 ± 0.01[b]	9.78 ± 0.49[a]	48.2 ± 0.9[a]
12	76.5 ± 2.8[a]	19.1 ± 0.7[a]	0.01 ± 0.00[a]	0.08 ± 0.01[a,b]	4.20 ± 0.25[a,b]	0.75 ± 0.47[a]	3.85 ± 0.12[a]	0.09 ± 0.02[a,b]	8.97 ± 0.80[a]	46.9 ± 3.9[a]
24	89.2 ± 5.2[a]	22.3 ± 1.3[a]	0.01 ± 0.00[a]	0.05 ± 0.01[b]	6.07 ± 0.84[b]	0.59 ± 0.11[a]	2.81 ± 1.11[a]	0.10 ± 0.02[b]	9.64 ± 1.17[a]	43.5 ± 5.6[a]

Values at each time are the mean ± SE of three test fish, respectively. Means with a common superscript in the column do not differ significantly ($p > 0.05$).

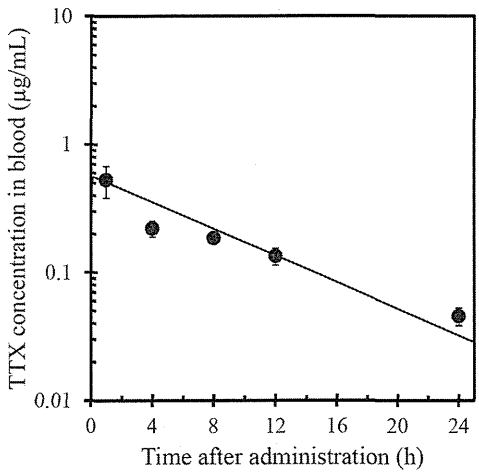

Fig. 3. Fitting plot of the predicted one-compartment model with first order absorption (line) of the observed blood TTX concentration-time profile (●). Each data and vertical bar represents the mean ± SE of three individuals.

Table 2
Pharmacokinetic parameters after the intramuscular administration of TTX into the caudal muscle in *Takifugu rubripes* juveniles.

Pharmacokinetic parameter	Value
Intramuscular bioavailability (F_{im})	82.0%
Apparent distribution volume (V_d)	360 mL/kg body weight
Absorption rate constant (k_a)	4.19×10^{-1} min^{-1}
Elimination rate constant (k_{el})	2.00×10^{-3} min^{-1}

The results of the present study provide evidence of the biliary excretion of TTX in the liver of *T. rubripes* juveniles. The biliary concentration of TTX increased over time up to 8 h after administration, and thereafter remained at almost the same level. The saturation of the TTX concentration in the bile also suggests a balanced state between the bile production rate and the biliary excretion of TTX. The apparent excretion rate of TTX into bile was estimated to be 8.17×10^{-1} ng/mL/min up to 8 h by the slope of the time course of the TTX concentration in the gallbladder bile in Fig. 2. It seems that the bile production rate of pufferfish juveniles in this study is rather low, because the bile production rate of fish was considerably lower than that of higher vertebrates (1.25 μL/min/kg body weight for rainbow trout *Oncorhynchus mykiss versus* 63, 69, and 83 μL/min/kg body weight for rat, mouse and rabbit, respectively) (Grosell et al., 2000; Clark and Smith, 1984). TTX accumulation was observed in the gallbladder of 1-year-old *T. rubripes* specimens 6 days after an intraperitoneal injection of [^{3}H] labeled-TTX at a dosage of 2.5 μg/g body weight (Watabe et al., 1987). Adult specimens of *T. rubripes* also appear to excrete TTX in the bile, as *T. rubripes* in the wild have toxic levels in the gallbladder bile (Endo, 1984). Together, these results reveal that TTX is concentrated in the bile juice via a transcellular transport pathway involving efflux transporters in the liver, re-absorbed into the circulating blood from the gastrointestinal tract, and returned to the liver in both *T. rubripes* juveniles and adults. However, it is likely that *T. rubripes* juveniles partly transfer the toxin to skin. We preliminarily performed pharmacokinetic analysis and showed the TTX disposition after intramuscular administration by the one-compartment model equation with first order absorption. Intramuscular bioavailability (F_{im}) and the ratio of rate constants (k_a/k_{el}) indicate the effective absorption of the toxin from the administration site into the body. The apparent distribution volume (V_d) was the same level in the case of bolus administration of TTX into the hepatic portal vein in *T. rubripes* adults reported previously (Matsumoto et al., 2008a). Further pharmacokinetic studies are necessary to evaluate the biliary excretion clearance and enterohepatic circulation of TTX in both juvenile and adult pufferfish *T. rubripes*.

TTX-binding proteins may also be related to the disposition of TTX in the pufferfish, since pufferfish saxitoxin and TTX-binding protein (PSTBP) in the plasma, as well as other plasma proteins (Matsumoto et al., 2010), are likely to function as TTX transfer mechanisms among the tissues in the marine pufferfish of the genus *Takifugu* (Yotsu-Yamashita et al., 2000, 2013). In addition, TTX-associated high molecular substances have been found and partially purified from toxic pufferfish liver (Kodama et al., 1983; Nagashima et al., 1993). Further studies are required to examine the involvement of high molecular substances in the hepatic accumulation of TTX and the expression stage of the proteins and/or their genes.

Conflict of interest

None declared.

Ethical statement

No ethical issues identified.

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