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

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

2.5. Statistical analysis

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

3. Results

3.1. Seasonal changes in the gonadosomatic index (GSI)

Seasonal changes in GSI are shown in Fig. 1. In female specimens, GSI began to increase in December, peaked
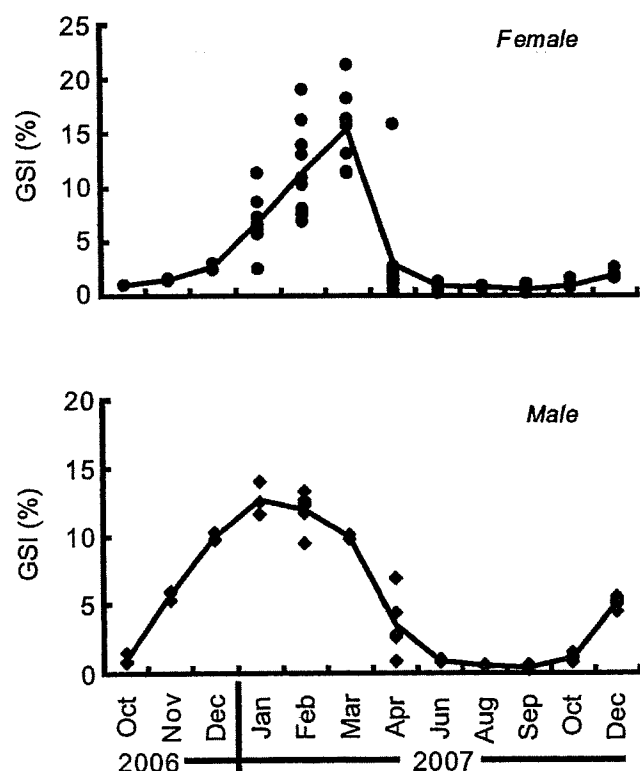

Fig. 1. Seasonal changes in the gonadosomatic index (GSI) in female (upper) and male (lower) specimens of T. poecilonotus. Data are shown by individual values (symbols) and mean of each month (bend of sequential line). White, light gray, and gray zones indicate 'ordinary period', 'maturation period',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

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

3.2. Seasonal changes in toxicity per gram of each tissue

3.2.1. Females

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

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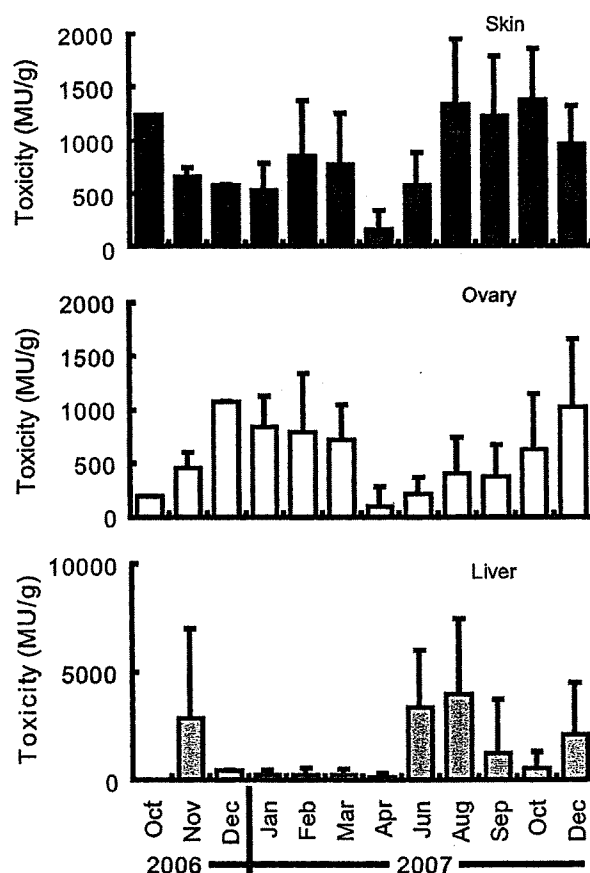

Fig. 2. Seasonal changes in the toxicity (MU/g) of skin (upper), ovary (middle), and liver (lower) in the female specimens of T. poecilonotus. Data are shown by mean (column) and standard deviation (SD, error bar) of each month.

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

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

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

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

3.2.2. Males

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

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

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

3.3.1. Female

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

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

The plasma TTX content (p-TTX = b-TTX + f-TTX) ranged between 1.75 and 15.1 MU/ml, the levels being much lower than that in the other three tissues. Although p-TTX was significantly higher (Student's t-test, $p < 0.05$) in the ordinary period than in the maturation period, it generally showed large fluctuations throughout the year, which did not clearly correspond to changes in tissue toxicity; even the decline just after spawning in April was not observed in
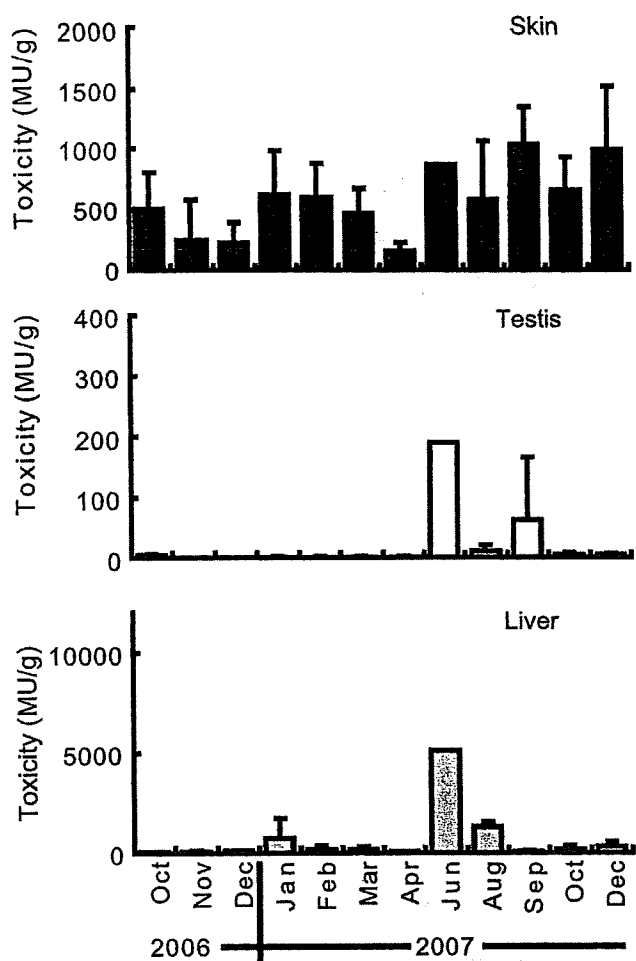
Fig. 3. Seasonal changes in the toxicity (MU/g) of skin (upper), testis (middle), and liver (lower) in the male specimens of *T. poecilonotus*. Data are shown by mean (column) and SD (error bar) of each month.

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

3.3.2. Male

Seasonal changes in toxicity (MU/individual) of each tissue and in plasma TTX in males are shown in Fig. 5. Skin toxicity largely exceeded that of the other tissues, except for June, in which the liver toxicity was extremely high. As

a whole, the tissue toxicities of males were significantly lower (Student's t-test, $p < 0.05$) than those of females; the level of liver, gonad, and skin toxicity was about 1/4, 1/400, and 1/1.5 that of the female specimens, respectively (Table 3).

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

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

4. Discussion

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

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

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

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

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

Data are shown as mean ± SD.
Asterisks indicate significant differences between the two measured values in each column (Student's t-test, $p < 0.05$).
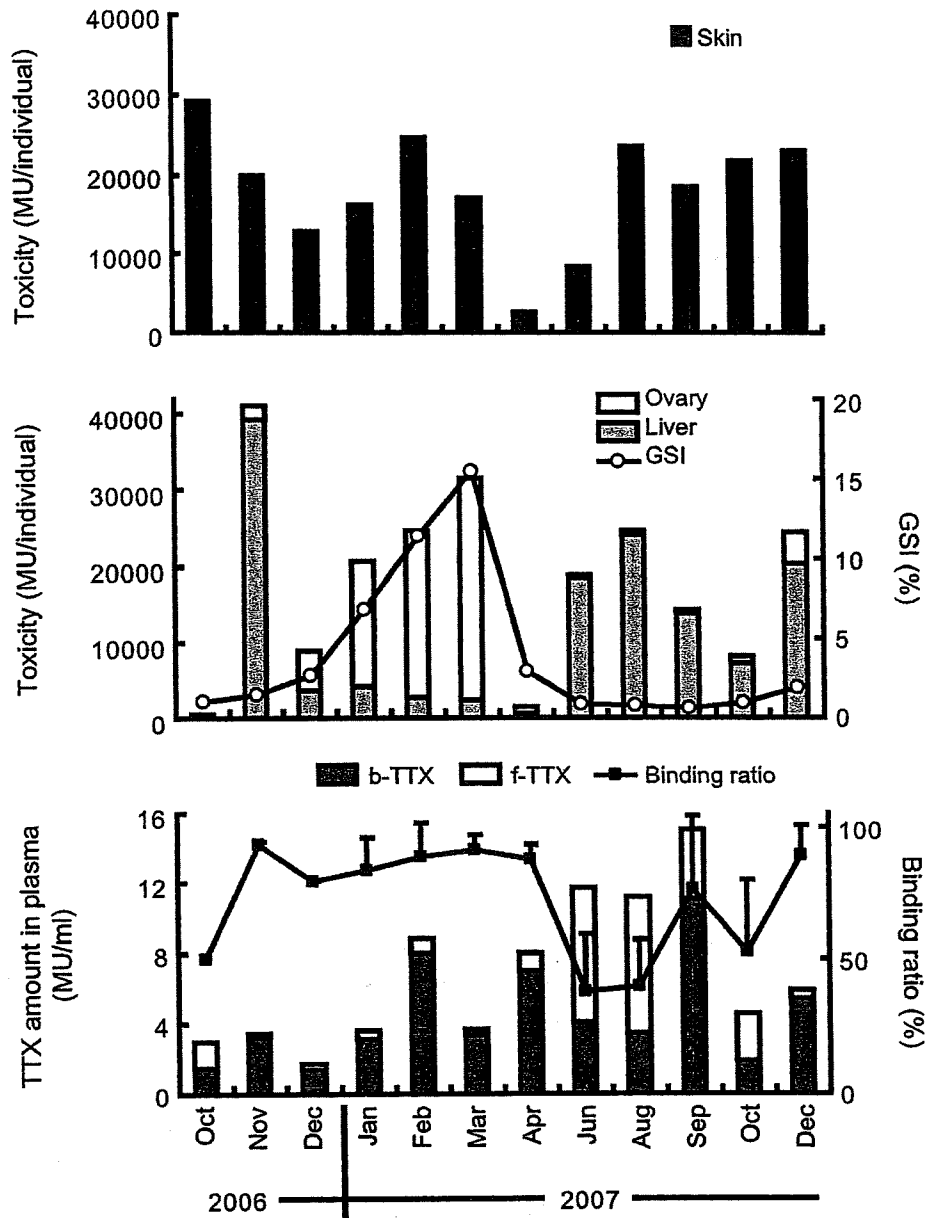
Fig. 4. Seasonal changes in the toxicity (MU/individual) of the skin (upper) and ovary/liver with GSI (middle), and in the TTX amount of blood plasma (lower) in the female specimens of *T. poecilonotus*. The sum of free TTX (f-TTX) and TTX binding to high molecular-weight substances (b-TTX) was considered as a total TTX amount in plasma (p-TTX), and the percentage of b-TTX in p-TTX was calculated as the binding ratio. Data are shown by mean of each month (column or symbol on sequential line). Error bars (SD) for data other than the binding ratio are omitted to avoid confusion.

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

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

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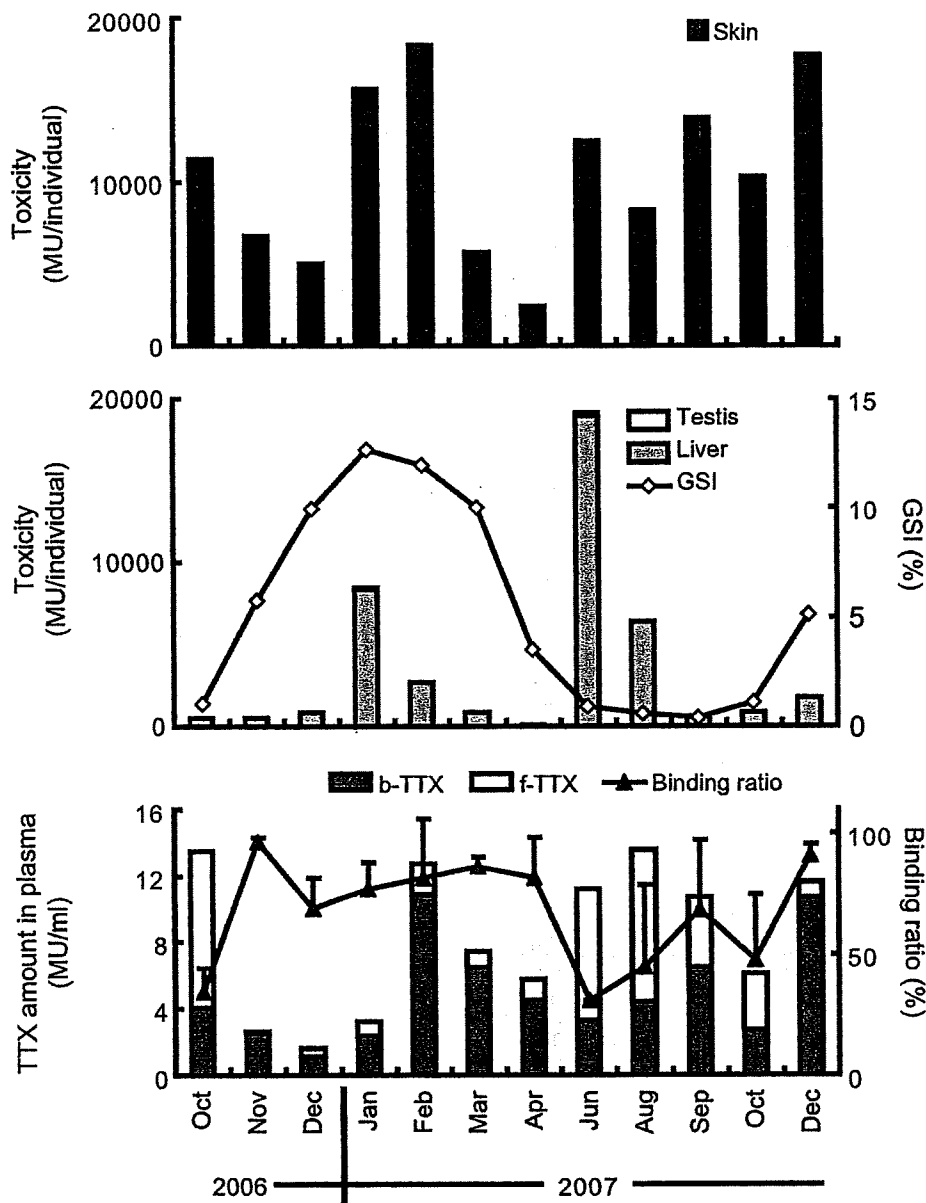Data are shown as mean ± SD.
†O: ordinary period; M: maturation period.

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

(Fig. 2 and Table 2). Therefore, it is presumed that the TTX absorbed from toxic food organisms into the pufferfish body is transferred mainly to the liver and skin during the ordinary period, but is actively transported and accumulated into the ovary during the maturation period. Matsumoto/Nagashima et al. demonstrated that the liver tissue of *T. rubripes* is equipped with a specific TTX-uptake mechanism (Nagashima et al., 2003, Matsumoto et al., 2005, 2007), and using a pharmacokinetic model showed that TTX introduced into the pufferfish body is rapidly taken up into the liver via the blood (Matsumoto et al., 2008a, 2008b). We also found that TTX administered intramuscularly to non-toxic cultured specimens of *T. rubripes* was transferred first into the liver and then the skin via the blood (Ikeda et al., 2009). A similar result was obtained in

oral administration experiments (Kono et al., 2008), suggesting that, under natural conditions as well, pufferfish take up most of the ingested TTX into the liver first. During the ordinary period, some of the TTX taken up into the liver is gradually transferred to the skin, where it accumulates in the basal cells and/or TTX-bearing secretory glands or cells (succiform cells) of the epithelia (Kodama et al., 1986, Tanu et al., 2002, Mahmud et al., 2003a, 2003b), and is excreted by external stimuli under certain circumstances (Kodama et al., 1985, Saito et al., 1985). During the maturation period, the toxin transfer to the skin decreases somewhat, and most of the TTX taken up into the liver would be transported to the ovary, presumably with the precursors of yolk proteins that are synthesized in the liver (Wallace, 1985, Specker and Sullivan, 1994). The majority of the toxin

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

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

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

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

Acknowledgements

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Endo, R., 1984. Toxicological studies on puffer fishes: comparison of toxicities on the various species. J. Toxicol. Sci. 9 (Suppl. I), 1–11.

Fuchi, Y., Hoashi, K., Akaeda, H., Makino, Y., Noguchi, T., 1999. Toxicity of two species of puffer fish, *Takifugu pardalis* ("higanfugu") and *Takifugu poecilonotus* ('komonfugu") inhabiting the Kunisaki Coast, Oita Prefecture. J. Food Hyg. Soc. Jpn. 40, 80–89.

Honda, S., Arakawa, O., Takatani, T., Tachibana, K., Yagi, M., Tanigawa, A., Noguchi, T., 2005. Toxification of cultured puffer fish *Takifugu rubripes* by feeding on tetrodotoxin-containing diet. Nippon Suisan Gakkaishi 71, 815–820.

Ikeda, K., Murakami, Y., Emoto, Y., Ngy, L., Taniyama, S., Yagi, M., Takatani, T., Arakawa, O., 2009. Transfer profile of intramuscularly administered tetrodotoxin to non-toxic cultured specimens of the pufferfish *Takifugu rubripes*. Toxicon 53, 99–103.

Jang, J., Yotsu-Yamashita, M., 2006. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of puffer fish *Fugu pardalis*. Toxicon 48, 980–987.

Japan Food Hygiene Association, 2005. Puffer toxin. In: Environmental Health Bureau, Ministry of Health and Welfare. Shokuhin Eisei Kensa Shishin, Tokyo, pp. 661–673 (Manual for Methods for Food Sanitation Testing).

Kawatsu, K., Hamano, Y., Yoda, T., Terano, Y., Shibata, T., 1997. Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. Jpn. J. Med. Sci. Biol. 50, 133–150.

Kodama, M., Ogata, T., Kawamukai, K., Oshima, Y., Yasumoto, T., 1984. Toxicity of muscle and other organs of five species of puffer collected from the Pacific coast of Tohoku area of Japan. Bull. Jpn. Soc. Sci. Fish. 50, 703–706.

Kodama, M., Ogata, T., Sato, S., 1985. External secretion of tetrodotoxin from puffer fishes stimulated by electric shock. Mar. Biol. 87, 199–203.

Kodama, M., Sato, S., Ogata, T., Suzuki, Y., Kaneko, T., Aida, K., 1986. Tetrodotoxin secreting glands in the skin of puffer fishes. Toxicon 24, 819–829.

Kono, M., Matsui, T., Furukawa, K., Yotsu-Yamashita, M., Yamamori, K., 2008. Accumulation of tetrodotoxin and 4,9-anhydrotetrodotoxin in cultured juvenile kusafugu *Fugu niphobles* by dietary administration of natural toxic komonfugu *Fugu poecilonotus* liver. Toxicon 51, 1269–1273.

Mahmud, Y., Okada, K., Takatani, T., Kawatsu, K., Hamano, Y., Arakawa, O., Noguchi, T., 2003a. Intra-tissue distribution of tetrodotoxin in two marine puffers *Takifugu vermicularis* and *Chelonodon patoca*. Toxicon 41, 13–18.

Mahmud, Y., Arakawa, O., Ichinose, A., Tanu, M.B., Takatani, T., Tsuruda, K., Kawatsu, K., Hamano, Y., Noguchi, T., 2003b. Intracellular visualization of tetrodotoxin (TTX) in the skin of a puffer *Tetraodon nigroviridis* by immunoenzymatic technique. Toxicon 41, 605–611.

Matsui, T., Hamada, S., Konosu, S., 1981. Difference in accumulation of puffer fish toxin and crystalline tetrodotoxin in the puffer fish, *Fugu rubripes rubripes*. Bull. Jpn. Soc. Sci. Fish. 47, 535–537.

Matsui, T., Yamamori, K., Furukawa, K., Kono, M., 2000. Purification and some properties of a tetrodotoxin binding protein from the blood plasma of kusafugu, *Takifugu niphobles*. Toxicon 38, 463–468.

Matsumoto, T., Nagashima, Y., Takayama, K., Shimakura, K., Shiomi, K., 2005. Difference between tetrodotoxin and saxitoxin in accumulation in puffer fish *Takifugu rubripes* liver tissue slices. Fish Physiol. Biochem 31, 95–100.

Matsumoto, T., Nagashima, Y., Kusuhara, H., Sugiyama, Y., Ishizaki, S., Shimakura, K., Shiomi, K., 2007. Involvement of carrier-mediated transport system in uptake of tetrodotoxin into liver tissue slices of puffer fish *Takifugu rubripes*. Toxicon 50, 173–179.

Matsumoto, T., Nagashima, Y., Kusuhara, H., Ishizaki, S., Shimakura, K., Shiomi, K., 2008a. Pharmacokinetics of tetrodotoxin in puffer fish *Takifugu rubripes* by a single administration technique. Toxicon 51, 1051–1059.

Matsumoto, T., Nagashima, Y., Kusuhara, H., Ishizaki, S., Shimakura, K., Shiomi, K., 2008b. Evaluation of hepatic uptake clearance of tetrodotoxin in the puffer fish *Takifugu rubripes*. Toxicon 52, 369–374.

Miyazawa, K., Noguchi, T., 2001. Distribution and origin of tetrodotoxin. J. Toxicol. Toxin. Rev. 20, 11–33.

Nagashima, Y., Toyoda, M., Hasobe, M., Shimakura, K., Shiomi, K., 2003. In vitro accumulation of tetrodotoxin in pufferfish liver tissue slices. Toxicon 41, 569–574.

Ngy, L., Tada, K., Yu, C.-F., Takatani, T., Arakawa, O., 2008. Occurrence of paralytic shellfish toxins in Cambodian Mekong pufferfish *Tetraodon turgidus*: selective toxin accumulation in the skin. Toxicon 51, 280–288.

Noguchi, T., Arakawa, O., 2008. Tetrodotoxin – distribution and accumulation in aquatic organisms, and cases of human intoxication. Mar. Drugs 6, 220–242.

Saito, T., Noguchi, T., Harada, T., Murata, O., Hashimoto, K., 1985. Tetrodotoxin as a biological defense agent for puffers. Bull. Jpn. Soc. Sci. Fish. 51, 1175–1180.

Specker, J.L., Sullivan, C.V., 1994. Vitellogenesis in fishes: status and perspectives. In: Perspectives in Comparative Endocrinology. National Research Council of Canada, pp. 304–315.

Takita, T., Intong, S., 1991. Ecological studies on young puffers *Takifugu rubripes* and *T. xanthopterus* in Ariake Sound. Nippon Suisan Gakkaishi 57, 1883–1889.

Tanu, M.B., Mahmud, Y., Takatani, T., Kawatsu, K., Hamano, Y., Arakawa, O., Noguchi, T., 2002. Localization of tetrodotoxin in the skin of a brackishwater puffer *Tetraodon steindachneri* on the basis of immunohistological study. Toxicon 40, 103–106.

Wallace, R.A., 1985. Vitellogenesis and oocyte growth in nonmammalian vertebrates. In: Developmental Biology, vol. 1. Plenum Press, New York, pp. 127–177.

Watabe, S., Sato, Y., Nakaya, M., Nogawa, N., Oohashi, K., Noguchi, T., Morikawa, N., Hashimoto, K., 1987. Distribution of tritiated tetrodotoxin administrated intraperitoneally to pufferfish. Toxicon 25, 1283–1289.

Yamamori, K., 2002. Natural forms of tetrodotoxin in puffer fish. Nippon Suisan Gakkaishi 68, 922–923.

Yamamori, K., Kono, M., Furukawa, K., Matsui, T., 2004. The toxification of juvenile cultured kusafugu *Takifugu niphobles* by oral administration of crystalline tetrodotoxin. J. Food Hyg. Soc. Jpn. 45, 73–75.

Yotsu-Yamashita, M., Sugimoto, A., Terakawa, T., Shoji, Y., Miyazawa, T., Yasumoto, T., 2001. Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish. *Fugu pardalis*. Eur. J. Biochem 268, 5937–5946.

Toxins of Pufferfish That Cause Human Intoxications

Osamu ARAKAWA[1], Deng-Fwu HWANG[2], Shigeto TANIYAMA[1]
and Tomohiro TAKATANI[1]

[1]Faculty of Fisheries, Nagasaki University,
1-14 Bunkyo-mach, Nagasaki 852-8521, Japan
[2]Department of Food Science, National Taiwan Ocean University,
Keelung, Taiwan, Republic of China

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

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

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

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

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1. INTRODUCTION

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

2. PROPERTY OF TETRODOTOXIN (TTX)

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

The main symptoms of human intoxication include numbness of lips, tongue and the limbs, paresthesia, dysarthria, respiratory distress; death can occur due to respiratory failure in most critical cases (Noguchi and Ebesu, 2001). When a poisoning occurs, it is essential to transport the patient immediately to a well-equipped hospital. At present, there is no antidote or specific medication for TTX, and no fundamental treatment besides facilitating elimination of the toxin from the body,
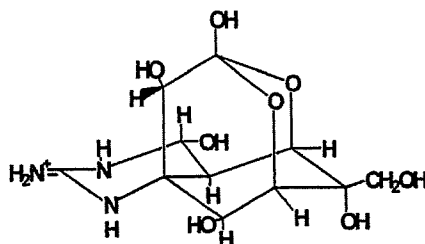
Fig. 1. Chemical structure of tetrodotoxin (TTX). Reprinted from *Marine Drugs* 6, Noguchi and Arakawa, Tetrodotoxin—distribution and accumulation in aquatic organisms, and cases of human intoxication, 220–242, 2008, Fig. 1.

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

3. DISTRIBUTION OF TTX IN AQUATIC ORGANISMS

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

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

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

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

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

4. ACCUMULATION OF TTX IN PUFFERFISH

Many years of studies on TTX have revealed that (1) pufferfish toxicity shows remarkable individual and regional variations, (2) TTX is distributed over various organisms, including food animals of pufferfish, (3) the trumpet shell accumulates TTX by ingesting toxic starfish, (4) marine bacteria primarily produce TTX, (5) pufferfish become non-toxic when they are fed TTX-free diets in an environment in which the invasion of TTX-bearing organisms is completely prevented (Matsui et al., 1982; Saito et al., 1984; Noguchi et al., 2004, 2006b) (Fig. 2), and (6) such non-toxic pufferfish efficiently accumulate TTX when orally administered TTX (Matsui et al., 1981; Yamamori et al., 2004; Honda et al., 2005a; Noguchi et al., 2006a; Kono
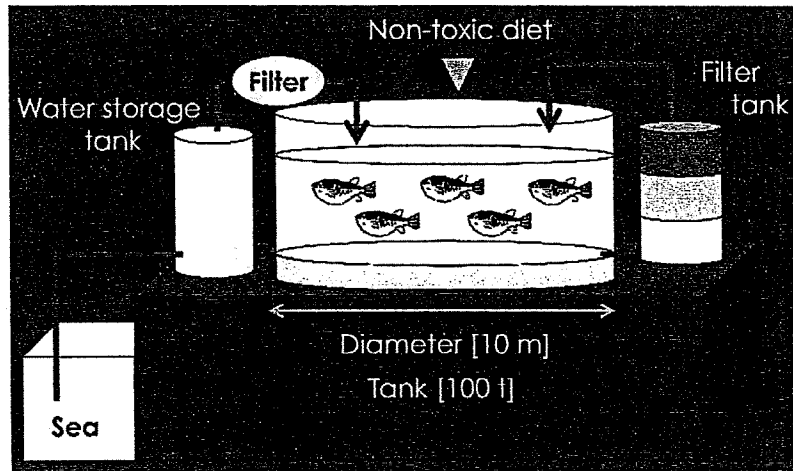
Fig. 2. Production of non-toxic pufferfish by land culture, in which seawater is thoroughly filtered before pouring into a tank to prevent toxic organisms from entering into it. Redrawn after *Japan Food Science*, **44**, Arakawa et al., 42–47, 2005, Fig. 3. © JSFS.

et al., 2008), suggesting that pufferfish do not synthesize TTX, but accumulate it through the food chain, which starts from marine bacteria.

The transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. Recently, we found that TTX administered intramuscularly to non-toxic cultured specimens of *T. rubripes*, which possesses TTX mainly in the liver in nature, was transferred first into the liver and then the skin via the blood (Ikeda et al., 2009). A similar transfer was observed when PSP was administered to non-toxic cultured specimens of the freshwater pufferfish *T. turgidus* that has PSP in the skin (Ngy et al., 2008b). The amount of toxin transferred to the liver, however, was very little in *T. turgidus*, and more than 90% of the toxin remaining in the body was transferred/accumulated in the skin. Interestingly, when *T. turgidus* specimens were administered the same dosage of TTX, all died within 3 to 4 h, and more than half of the TTX administered remained in the muscle in the dead specimens. Matsumoto/Nagashima et al. demonstrated that unlike general non-toxic fish, the liver tissue of *T. rubripes* is equipped with a specific TTX-uptake mechanism (Nagashima et al., 2003; Matsumoto et al., 2005, 2007), and using a pharmacokinetic model showed that TTX introduced into the pufferfish body is rapidly taken up into the liver via the blood (Matsumoto et al., 2008a, 2008b). These facts indicate that marine pufferfish that ingest TTX are endowed with a mechanism by which they transport TTX specifically and actively, and freshwater pufferfish that ingest PSP are endowed with a mechanism that processes PSP. TTX/PSP-binding proteins have been isolated from the blood plasma of marine pufferfish (Matsui et al., 2000; Yotsu-Yamashita et al., 2001), and may be involved in the transportation mechanism. Very recently, we investigated seasonal changes in tissue toxicity, as
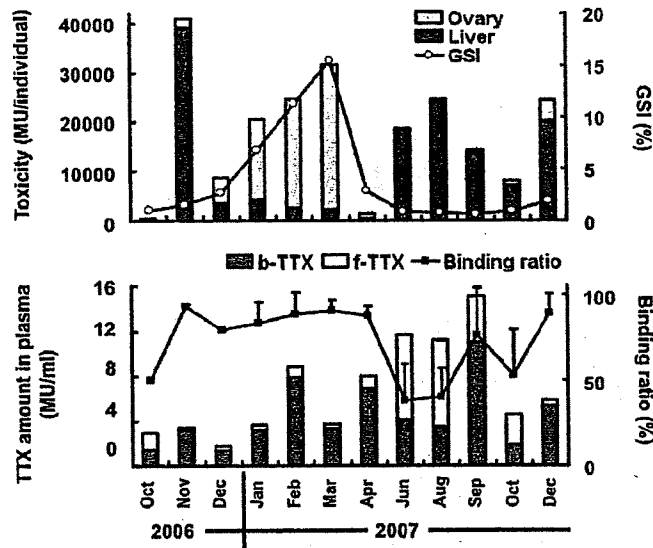
Fig. 3. Seasonal changes in the toxicity (MU/individual) of the skin (upper) and ovary/liver with GSI (middle), and in the TTX amount of blood plasma (lower) in the female specimens of *Takifugu poecilonotus.* The sum of free TTX (f-TTX) and TTX binding to high molecular-weight substances (b-TTX) was considered as a total TTX amount in plasma (p-TTX), and the percentage of b-TTX in p-TTX was calculated as the binding ratio. Data are shown by mean of each month (column or symbol on sequential line). Error bars (SD) for data other than the binding ratio are omitted to avoid confusion. Redrawn after *Toxicon*, 55, Ikeda et al., Maturation-associated change in toxicity of the pufferfish *Takifugu poecilonotus*, 289–297, 2010, Fig. 4, with permission from Elsevier.

well as the amount and forms of TTX in the blood plasma using wild specimens of the pufferfish *T. poecilonotus* from the Ariake Sea, and demonstrated that the maturation greatly affects the inter-tissue transfer and/or accumulation of TTX via the blood stream in nature (Ikeda et al., 2010) (Fig. 3).

5. PHYSIOLOGIC FUNCTION OF TTX IN TTX-BEARING ORGANISMS

As described above, a wide variety of organisms including pufferfish possess TTX. Physiological functions of TTX in these organisms can be estimated by elucidating the distribution of TTX in their body. In marine pufferfish and flatworms, the amount of toxins in the eggs are generally very high (Miyazawa and Noguchi, 2001). In addition, pufferfish and newts have TTX-bearing glands or secretory cells (succiform cells) in their skin (Tanu et al., 2002; Tsuruda et al., 2002; Mahmud et al., 2003a,b) (Fig. 4), and secrete TTX by external stimuli (Kodama et al., 1985; Saito et al., 1985a; Tsuruda et al., 2002), suggesting that they possess TTX as a biologic defense agent to protect themselves or their eggs from predators. On the other hand, the blue-ringed octopus and ribbon worms possess TTX in the posterior salivary

Fig. 4. TTX-bearing gland observed in epidermis of the skin section in *Takifugu vermicularis* under light microscope (×100). The positive stain to TTX-antibody results in brown color. Stronger TTX antigen-antibody reaction was recognized at cytoplasm of the gland (arrow head). Modified from *Toxicon* 41, Mahmud et al., Intra-tissue distribution of tetrodotoxin in two marine puffers *Takifugu vermicularis* and *Chelonodon patoca*, 13–18, 2003, Fig. 1, with permission from Elsevier.

gland and proboscis, respectively, and are believed to utilize the toxin to capture prey (Sheumack and Howden, 1978; Tanu et al., 2004). Recently, we observed that when non-toxic cultured pufferfish were fed with TTX-containing diets, their immune function was activated (Honda et al., 2005b), though the mechanism remains unclear.

TTX-bearing organisms such as toxic marine pufferfish, the goby *Y. criniger*, the xanthid crab *A. floridus*, and the newt *C. pyrrhogaster* show extremely high resistance to TTX, i.e., the MLD of TTX administered intraperitoneally to these animals is 300 to 1,000 times (more than 10,000 times in the newt) greater than that of mice (Noguchi and Hashimoto, 1973; Koyama et al., 1983; Saito et al., 1985b; Arakawa, 2001). In contrast, non-toxic marine pufferfish show medium resistance to TTX (MLD, 13 to 15 times greater than mice), and in general fish show resistance as low as mice (Saito et al., 1985b). The mechanism of TTX resistance in pufferfish and newts has been explained based mainly on the TTX-resistant sodium channels found in the animals, in which the aromatic amino acid commonly located in the p-loop region of domain I in TTX-sensitive sodium channels is replaced by a nonaromatic amino acid, resulting in their extremely low affinity to TTX (Kaneko et al., 1997; Yotsu-Yamashita et al., 2000; Venkatesh et al., 2005; Maruta et al., 2008). Garter snakes and clams can also acquire similar sodium channel mutation-based TTX/PSP resistance by interacting with their toxic food organisms, TTX-bearing newts and PSP-producing dinoflagellates, respectively (Geffeney et al., 2005; Bricejl et al., 2005).

Toxic small gastropods are also endowed with high resistance to TTX, and secrete TTX as defense or attack agent (Hwang et al., 1990a,b, 1992b). Interestingly, they were significantly attracted by TTX, while non-toxic species showed a negative response (Hwang et al., 2004). The more toxic species appeared to prefer TTX, indicating that TTX is an attractant for the toxic gastropods. Matsumura (1995) found that TTX was mostly distributed in the surface of pufferfish eggs, and might act as a

Table 1. Food poisonings due to animal natural toxins (Total score of 2002–2006, Ministry of Health, Labour and Welfare).

Causative food	Causative toxin	Number of incident	Number of patient	Number of death
Pufferfish of Tetraodontidae	Tetrodotoxin	166	223	13
Ciguateric fish	Ciguateric toxins	17	67	0
Gastropods of Buccinidae	Tetramine	16	38	0
Boxfish (Pufferfish of Ostraciidae)	Palytoxin-like toxin	3	6	0
Prickleback	Dinogunellin	1	4	0
Marine turtle	Unknown	1	1	0
Unknown		19	31	1
Total		223	370	14

pheromone to attract the male fish.

Very recently, Sakakura et al. (unpublished) found that when TTX was administered to artificial-reared non-toxic juveniles of *T. rubripes*, their ecological behavior became similar to that of wild juveniles, and it was more difficult for predators to prey on them. This suggests a possibility that TTX is involved in the control of information transmission in the central nervous system of pufferfish. Sodium channel mutations and/or presence/absence of TTX in the cranial nervous system may exert a great influence on the physiology and ecology of pufferfish.

6. TTX INTOXICATION DUE TO PUFFERFISH

According to the statistics of Japanese Ministry of Health, Labour and Welfare (Table 1), 116 incidents of pufferfish poisoning has occurred in Japan, involving 223 patients and 13 deaths during the 5 years from 2002 to 2006. One of the more recent poisoning cases is described below. In October 2008, a 69 year-old male died at a hospital in Isahaya, Nagasaki Prefecture. He stated that he cooked a "usubahagi" (a sort of thread-sail filefish "kawahagi"), caught by himself, and ate its raw meat (sashimi) in a dip of the liver and soy sauce mixture. About 30 minutes after ingestion, he felt numbness in the limbs, and after 30 minutes, vomited, and fell into a coma, before being transported by an ambulance to the hospital. The doctor confirmed his death about 4 hours after ingestion, and initially provided the following diagnosis: 'ciguatera due to the ingestion of "kawahagi" liver, a possibility of TTX is not denied'. Thereafter, it was determined that the patient cooked a "kinfugu" with the "usubahagi", but the liver was missing among the leftovers. We investigated the leftovers, and revealed that the "usubahagi" was non-toxic, but the "kinfugu" was actually a highly toxic species, "komonfugu" *T. poecilonotus*, and 600 MU/g of TTX was detected in the skin. Furthermore, 0.7 MU/mL, 2 MU/mL, and 45 MU/g of TTX was detected in the blood, urine, and vomit of the patient, respectively, allowing us

to conclude that the present poisoning was a TTX intoxication due to the mistaken ingestion of *T. poecilonotus* liver.

To clarify the cause of pufferfish poisoning, identification of causative species, as well as investigation of leftover fish toxicity are essential. Although the species identification is usually carried out based on the morphological characteristics such as pattern of the skin, shape of the fins, and distribution of the small spines, several methods using proteins or genes have also been established (Chen and Hwang, 2002; Chen et al., 2002a,b, 2003, 2004; Ishizaki et al., 2005), and species can be identified even from a small tissue fragment. Analytical techniques of TTX have also been advanced, and TTX can be detected not only in the leftovers but also in the blood and urine of the patient (Kawatsu et al., 1999; Kurono et al., 2001; O'Leary et al., 2004; Akaki and Hatano, 2006; Tsai et al., 2006).

In Taiwan and China, although people do not eat pufferfish as often as the Japanese, many food poisoning cases due to ingestion of wild pufferfish have occurred. According to the records of TTX poisoning in Taiwan, that some cases are caused by the mistaken ingestion of the muscle tissue of a pufferfish species with toxic muscle, and by ingesting puffer roe that had been sold as fake dried mullet roe called "karasumi", or by ingesting a dried dressed fish fillet produced from toxic pufferfish by a food processing company (Du et al., 1999; Hwang et al., 2002a; Hsieh et al., 2002, 2003; Hwang and Noguchi, 2007). In countries outside of East Asia, people generally do not have a custom of eating pufferfish, and poisonings do not occur as frequently.

7. TTX INTOXICATION DUE TO MARINE ANIMALS OTHER THAN PUFFERFISH

In July 2007, a food poisoning incident due to the scavenging gastropod "kinshibai" *Nassarius glans* (Fig. 5) suddenly occurred in Nagasaki, Nagasaki Prefecture. The patient was a 60 year-old female, who developed symptoms such as a feverish feeling in the limbs, abdominal pain, and hectic flush and edema in the face 15 minutes after ingestion, and was administered an intravenous drop at a clinic nearby her home. Thereafter, her condition worsened, developing dyspnea, paralysis in the whole body, and mydriasis; she was finally transported to an emergency hospital. The patient required an artificial respirator for the first 3 days, but recovered enough to take breakfast on the 4th day. However, she unexpectedly relapsed after lunch, fell into respiratory arrest, and was equipped with the respirator again. Afterwards, she gradually recovered, and was discharged from the hospital 3 weeks later.

Immediately after the incident, we investigated the leftover gastropods, and detected a maximum of 4,290 MU/g of TTX in the cooked muscles and digestive glands of *N. glans*. Moreover, during the subsequent investigations, an extremely high concentration of TTX and a putative derivative of TTX, i.e., a maximum of 10,200 MU/g (15,100 MU/individual) in the viscera and 2,370 MU/g (9,860 MU/individual) in the muscle, was detected in *N. glans* specimens collected from the same sea area as the leftovers (Taniyama et al., 2009a) (Nagasaki specimens in Fig. 6). In the present intoxication, the symptoms once recovered recurred after taking meals.
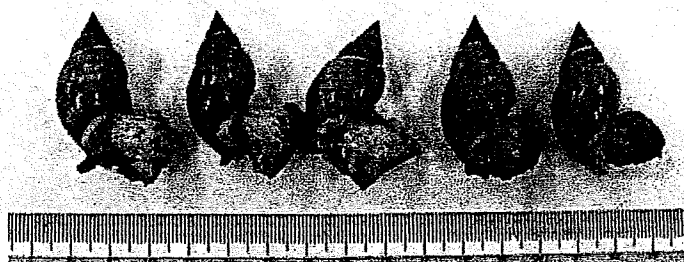
Fig. 5. Scavenging gastropod "kinshibai" *Nassarius glans*. Reprinted from Arakawa et al., *Koshu Eisei*, 73(5), 323–326, 2009. Fig. 2. © IGAKU-SHOIN Ltd.

Although the reason is not clear, it might be attributable to the situation where a highly toxic undigested tissue fragment of *N. glans* remaining in a digestive tract of the patient was digested and absorbed accompanying with the resumption of meals, and her respiratory center was again exposed to a high concentration of TTX. In July 2008, another poisoning incident due to *N. glans* occurred in Amakusa, Kumamoto Prefecture.

In China and Taiwan, people have a time-honored custom of eating small scavenging gastropods, and food poisoning due to these organisms has frequently occurred. At least 28 incidents were recorded during 1985–2004 in China, and 9 incidents during 1994–2006 in Taiwan, involving 233 patients and 24 deaths in total (Takatani et al., 2005; Hwang et al., 2007). In April 2004, a serious incident due to *N. glans* occurred in Taiwan, in which 2 of 6 poisoned patients died within 30 min after ingestion (Hwang et al., 2005). The causative species in China was identified as *Zeuxis samiplicutus* (Sui et al., 2002, 2003), while a total of 14 species of Nassariidae, Naticidae, and Olividae including *N. glans* were reported as the responsible gastropods for the poisonings in Taiwan (Hwang et al., 1995, 2002b, 2003, 2005, 2007; Shiu et al., 2003). Among them, *N. glans*, *N. papillosus*, *Z. scalaris*, and *Oliva miniacea* are also distributed in Japanese coastal waters.

From 1979 to 1987, 3 incidents of TTX intoxication due to the carnivorous gastropod *C. sauliae* occurred in Shizuoka, Wakayama and Miyazaki Prifectures, respectively (Narita et al., 1981; Maruyama et al., 1983). In this species, the toxin is localized at the digestive gland, and a total of 4 persons who had eaten not only the muscles but also gland were poisoned. In Taiwan, where at least 4 species of TTX-bearing gobies, *Yongeichthys nebulosus*, *Sillago japonica*, *Prachaeturichtys palynena*, and *Radigobius caninus*, are found, several fatal poisoning incidents and frequent deaths of duck, both due to ingesting the gobies have occurred (Lin et al., 1996, 2000). The highest toxicity scores of poisoning-related specimens of *Y. nebulosus* and *S. japonica* were 7,650 and 1,460 MU per individual, respectively (Lin et al., 1999). In some Southeast Asian countries, eggs of the horseshoe crab are used as a food, which can occasionally cause food poisonings. TTX and/or PSP were detected in the
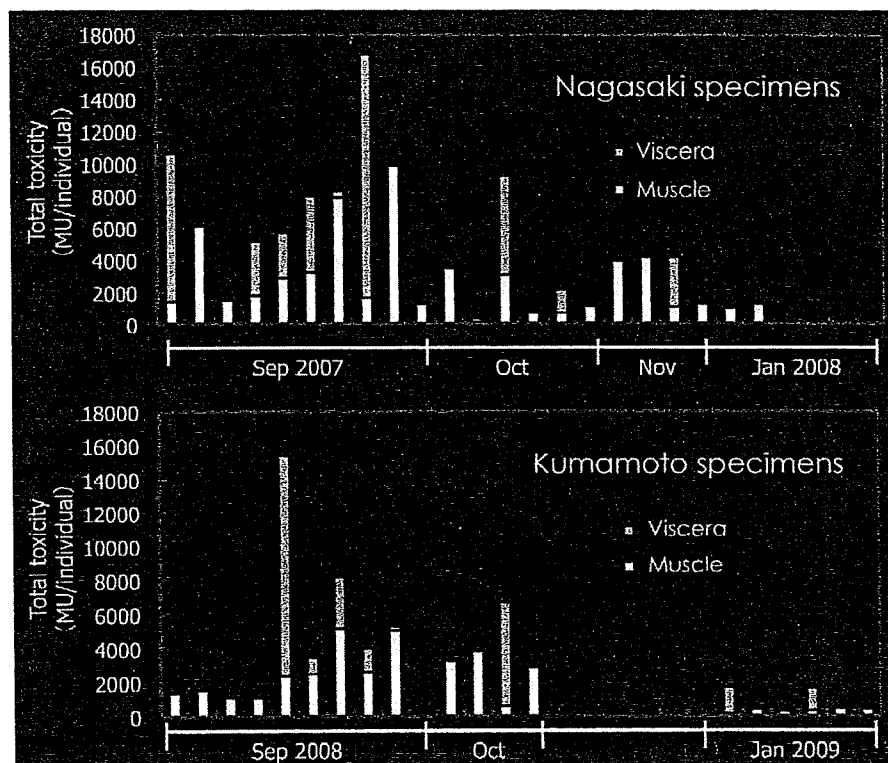
Fig. 6. Toxicity of *N. glans* specimens collected from Tachibana Bay, Nagasaki Prefecture (upper) and Miyanokawachi Bay, Kumamoto Prefecture (lower).

eggs and hepatic caecum of *Carcinoscorpius rotundicauda*, indicating that either of them or both are the causative agents (Fusetani et al., 1983; Kungsuwan et al., 1987; Tanu and Noguchi, 1999; Ngy et al., 2007).

8. PUFFERFISH POISONING DUE TO DIFFERENT TOXINS FROM TTX

In the Goto Islands, Nagasaki Prefecture, broiled boxfish with miso has long been eaten as a local delicacy. From October 1990 to October 2008, however, a total of 9 poisoning incidents due to ingestion of the dish occurred in Kagoshima, Nagasaki, Mie, and Miyazaki Prefectures, involving 13 patients and 1 death (Taniyama et al., 2009b). The causative species was identified as "hakofugu" *Ostracion immaculatus* (Fig. 7) from morphologic observations of the leftover fish in 2 of the incidents. *O. immaculatus* belongs to Tetraodontiformes, and is treated as pufferfish from a food hygienic point of view in Japan. A similar food poisoning due to boxfish has also occurred in Taiwan (Chen et al., 2001).

A description of the fatal boxfish poisoning that occurred in Japan are as follows.
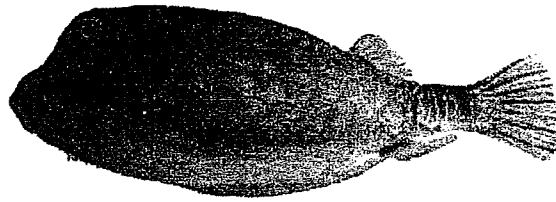
Fig. 7. Boxfish "hakofugu" *Ostracion immaculatus*. Reprinted from Arakawa et al. *Koshu Eisei*, 73(5), 323–326, 2009. Fig. 3. © IGAKU-SHOIN Ltd.

In August 2007, 4 individuals consumed broiled boxfish with miso in Goto, Nagasaki Prefecture, and 2 of them were poisoned. One patient exhibited lower-back pain and myoglobinuria, however the symptoms were relatively mild, and he recovered within 2–3 days. The other patient, however, developed severe muscle pain due to rhabdomyolysis, and fell into dysstasia. Moreover, he exhibited respiratory distress and myoglobinuria, and then died about 2 weeks later. Although the boxfish "umisuzume" *Lactoria diaphana* was suspected as a causative species, it could not be identified, because all of the leftovers had been disposed.

Boxfish poisoning is very similar to parrotfish poisoning (a unique variety of food poisoning that has sporadically occurred in Japan) (Noguchi et al., 1987; Arakawa et al., 1992; Taniyama et al., 2003a), and patients commonly show symptoms such as severe muscle pain accompanied with myoglobinuria and an abnormal rise of serum creatine phosphokinase (CPK), and the time course from the onset of the symptoms to recovery or death is relatively long. The causative substance is believed to be a palytoxin (PTX)-like toxin, as in the parrotfish poisoning (Noguchi et al., 1987; Taniyama et al., 2009b). A tropical/subtropical dinoflagellate *Ostreopsis* sp. (Fukuyo, 1981) is presumed to be one of the origins of the toxin, which has recently been found to inhabit the coastal waters of Kyushu and Honshu Islands in Japan (Taniyama et al., 2003b; Sagara, 2008). Bangladeshi freshwater pufferfish also possess PTX-like toxin, and have frequently caused food poisonings in the country (Mahmud et al., 2000; Taniyama et al., 2001).

Small pufferfish inhabiting rivers or inland waters in Southeast Asia such as *Tetraodon fangi*, *T. leiurus*, *T. suwatii*, and *T. turgidus* possess PSP mainly in their skin (Kungsuwan et al., 1997; Sato et al., 1997; Zaman et al., 1997; Ngy et al., 2008b). They are imported to Japan, and sold for ornamental purposes, but not used for food. In Thailand and Cambodia, however, food poisoning incidents due to the freshwater pufferfish have occasionally occurred with some fatalities. PSP is a group of neurotoxins produced by certain species of dinoflagellates, and the main component, STX, has almost equivalent molecular size and action mechanism to TTX (Deeds et al., 2008). Therefore, the symptoms of freshwater pufferfish poisoning is very similar to those of marine pufferfish poisoning, i.e., TTX poisoning. Floridian *Sphoeroides* pufferfish possess a large amount of PSP in the muscle, and caused 28 poisoning cases

during 2002–2004 (Landsberg et al., 2006). Several marine pufferfish from the Philippines (Sato et al., 2000) and "hoshifugu" *Arothron firmamentum* from Japanese coastal waters (Nakashima et al., 2004) are also known to possess PSP as a main toxin component in addition to TTX.

9. CONCLUSION

'Pufferfish toxin' generally indicates TTX, but as described above, gastropod poisoning due to TTX, or shellfish toxin poisoning and parrotfish poisoning-like poisoning due to pufferfish frequently occur, posing a great food hygienic issue in East and Southeast Asian countries. These toxins are all exogenous, and both pufferfish and gastropods are considered to obtain them from their toxic prey organisms, and accumulate the toxin in specific organs. Therefore, the toxicity of these toxic fish and shellfish may be greatly affected by a change in the marine environment, such as elevations in water temperature due to global warming. We need to enhance the information/collaboration network among East Asian countries to keep our eye on the diversity of TTX-bearing organisms, or of the toxins that pufferfish possess.

REFERENCES

Akaki, K. and K. Hatano. 2006. Determination of tetrodotoxin in puffer-fish tissues, and in serum and urine of intoxicated humans by liquid chromatography with tandem mass spectrometry. *Journal of the Food Hygienic Society of Japan* **47**: 46–50.

Arakawa, O., T. Noguchi and K. Hashimoto 1992. Parrotfish poisoning. *New Food Industry* **34**: 6–10.

Arakawa, K. 2001. Resistibility against TTX and PSP. In: *Studies on the Toxicity of a Japanese Newt Cynops pyrrhogaster*, Doctoral thesis, Nagasaki University, Nagasaki, pp. 50–53.

Bricelj, V.M., L. Connell, K. Konoki, S.P. MacQuarrie, T. Scheuer, W.A. Catterall and V.L. Trainer. 2005. Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP. *Nature* **434**: 763–767.

Brillantes, S., W. Samosorn, S. Faknoi and Y. Oshima. 2003. Toxicity of puffers landed and marketed in Thailand. *Fisheries Science* **69**: 1224–1230.

Chen, J. B., H.H. Pan and D.F. Hwang. 2001. Myoglobinuric acute renal failure following cardioversion in a boxfish poisoning patient. *Nephrol Dial Transpant* **16**: 1700–1701.

Chen, T.Y. and D.F. Hwang. 2002. Electrophoretic identification of muscle proteins in several puffer species. *Journal of Food Science* **67**: 936–942.

Chen, T.Y., C.Y. Shiau and D.F. Hwang. 2002a. Electrophoretic identification of muscle proteins in several puffer species with Coomassie blue/silver staining. *Fisheries Science* **69**: 1327–1330.

Chen, T.Y., Y.W. Hsieh, Y.H. Tsai, C.Y. Shiau and D.F. Hwang. 2002b. Identification of species and measurement of tetrodotoxin in dried dressed fillets of the puffer fish, *Lagocephalus lunaris*. *Journal of Food Protection* **65**: 1670–1673.

Chen, T.Y., C.Y. Shiau, T. Noguchi, C.I. Wei and D.F. Hwang. 2003. Identification of puffer fish species by native isoelectric focusing technique. *Food Chemistry* **83**: 475–479.

Chen, T.Y., C.Y. Shiau, C.I. Wei and D.F. Hwang. 2004. Preliminary study on puffer fish proteome: species identification of puffer fish by two-dimensional electrophoresis. *Journal of Agricultural and Food Chemistry* **52**: 2236–2241.

Deeds, J.R., J.H. Landsberg, S.M. Etheridge, G.C. Pitcher and S.W. Longan. 2008. Non-traditional vectors for paralytic shellfish poisoning. *Marine Drugs* **6**: 308–348.

Du, S.S., Y.M. Fu, Y.C. Shih, P.C. Chang, S.S. Chou, Y.H. Lue and D.F. Hwang. 1999. First report on suspected food poisoning with ingestion of dried seasoned fish fillet. *Journal of Food and Drug Analysis* **7**: 163–167.