

Maturation-associated changes in toxicity of the pufferfish *Takifugu poecilonotus*

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Abstract

From October 2006 to December 2007, wild specimens of the pufferfish *Takifugu poecilonotus* (93 females, 45 males) were collected from the Ariake Sea. Tissue toxicity was examined by mouse bioassay, and tetrodotoxin (TTX) content in the blood plasma by enzyme-linked immunosorbent assay. The relationship between toxicity and maturation was investigated based on changes in the gonadosomatic index: December-March in females and November-March in males, the 'maturation period'; April, 'just after spawning'; and the other months, the 'ordinary period'. Toxicity of both sexes was high throughout the year, but sharply declined in April. In all tissues examined (skin, liver, and ovary) other than testis, toxicity exceeded 1000 MU/g or 10,000 MU/individual in many individuals. Seasonal profiles of tissue toxicity differed markedly between sexes. In females, liver toxicity was high during the ordinary period, and ovary toxicity was high during the maturation period. In males, little maturation-associated change in the toxin distribution was observed. Plasma TTX levels were similar between the sexes (1.59-15.1 MU/ml), and fluctuated largely throughout the year without corresponding changes in tissue toxicity. The percentage of TTX binding to high molecular-weight substances in the plasma varied in association with maturation; the binding ratio fluctuated at relatively low levels during the ordinary period, and stabilized at a high level during the maturation period.

Keywords: Pufferfish; *Takifugu poecilonotus*; tetrodotoxin; enzyme-linked immunosorbent assay (ELISA); gonadosomatic index (GSI); maturation

1. Introduction

Many marine pufferfish of the family Tetraodontidae possess a potent neurotoxin, tetrodotoxin (TTX). In toxic species inhabiting Japanese coastal waters, the liver and ovary usually have strong toxicity, whereas the muscle and testis are weakly toxic or non-toxic (Noguchi and Arakawa, 2008), indicating sexual differences in pufferfish toxicity, and that maturation may affect toxin kinetics in the pufferfish body. TTX is originally produced by marine bacteria and distributes over a wide variety of animals, including pufferfish, gobies, blue-ringed octopuses, carnivorous gastropods, starfish, toxic crabs, horseshoe crabs, flat worms, and ribbon worms (Miyazawa and Noguchi, 2001). TTX is exogenous in pufferfish and is derived from the food chain that consists of these TTX-bearers (Noguchi and Arakawa, 2008). The transfer, accumulation, and elimination mechanisms of TTX taken up into the pufferfish body via food organisms remain unclear. Various types of toxin administration experiments performed with pufferfish have revealed important information on uptake and inter-tissue transfer of TTX in the pufferfish body (Matsui et al., 1981, Watabe et al., 1987, Yamamori et al., 2004, Honda et al., 2005, Kono et al., 2008, Ikeda et al., 2009). In these experiments, however, non-matured, non-toxic cultured fish were used, and the influence of aging or maturation was not considered. Although TTX-binding proteins have been found in the blood plasma of toxic pufferfish (Matsui et al., 2000; Yotsu-Yamashita et al., 2001), and may be involved in the transportation mechanism, little information is available on their distribution, seasonal variation, or functions other than TTX binding. In our studies to clarify the roles of TTX-binding high molecular-weight substances in the accumulation mechanisms of TTX in pufferfish and the effect of maturation, we collected the pufferfish *Takifugu poecilonotus* periodically from the Ariake Sea and investigated maturation-associated changes in tissue toxicity, as well as the amount and forms of TTX in the blood plasma.

2. Materials and methods

2.1. Pufferfish specimens

From October 2006 to December 2007, wild specimens of the pufferfish *T. poecilonotus* (93 females and 45 males) (Table 1) were collected from the Ariake Sea (off Minamishimabara, Nagasaki Prefecture, Japan), and transported live to the laboratory of Nagasaki University. After blood was withdrawn from the portal vein using a syringe

precoated with sodium heparin, each fish was dissected to obtain the skin, liver, and gonads (ovary/testis), which were then extracted with 0.1% acetic acid according to the official guidelines of the Japan Food Hygiene Association (2005), and analyzed with a toxicity assay using mice.

2.2. Assessment of gonadosomatic index (GSI)

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

2.3. Toxicity assay

Toxicity of each tissue extract from *T. poecilonotus* was determined by a mouse bioassay according to the official guidelines of the Japan Food Hygiene Association (2005). Lethal potency was expressed in mouse units (MU), where 1 MU was defined as the amount of toxin required to kill a 20-g male ddY strain mouse within 30 min after intraperitoneal administration.

2.4. Quantification of TTX in blood plasma

The blood collected from each fish was centrifuged at 6000 g for 7 min (4°C), and the blood plasma obtained (200 µl) was ultrafiltered through a Microcon YM-50 membrane (cut-off 50,000 Da, Amicon). Phosphate buffered saline (10mM, 200µl) was added to the residue, and the mixture was ultrafiltered again through the same membrane. The operation was repeated one more time. The combined supernatant (low molecular-weight fraction) and the residue (high molecular-weight fraction) contain free TTX molecules (designated f-TTX) and the TTX molecules binding to high molecular-weight substances (designated b-TTX), respectively (Matsui et al., 2000). The low molecular-weight fraction was directly submitted to an enzyme-linked immunosorbent assay (ELISA) to determine the amount of f-TTX. To cut the binding between TTX and high molecular-weight substances, 0.1% acetic acid (400 µl) was added to the high molecular-weight fraction (Yamamori, 2002), and then the mixture was submitted to ELISA to quantify the amount of b-TTX. Preliminary experiments demonstrated that 0.1% acetic acid or TTX-binding substances in the high molecular-weight fraction did not affect the ELISA results (data not shown).

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 (average \pm SD: $15.5 \pm 3.4\%$) 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 ($12.7 \pm 1.2\%$) 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 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 (~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 ($p\text{-TTX} = b\text{-TTX} + f\text{-TTX}$) ranged between 1.75 to 15.1 MU/ml, the levels being much lower than that in the other three tissues. Although $p\text{-TTX}$ 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 the plasma TTX (Fig. 4 and Table 4). $b\text{-TTX}$ 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 $f\text{-TTX}$ decreased during the maturation period (Figs. 4). The changes of both binding ratio and $f\text{-TTX}$ 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 (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.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure captions

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).

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.

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.

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.

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.

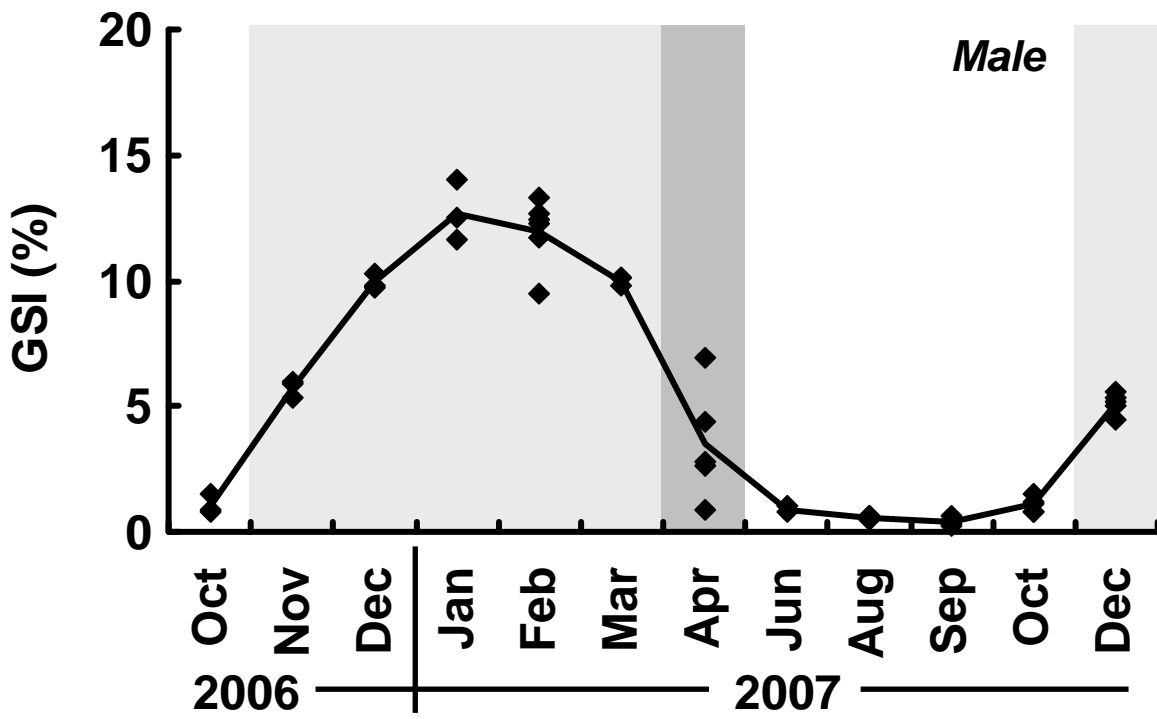
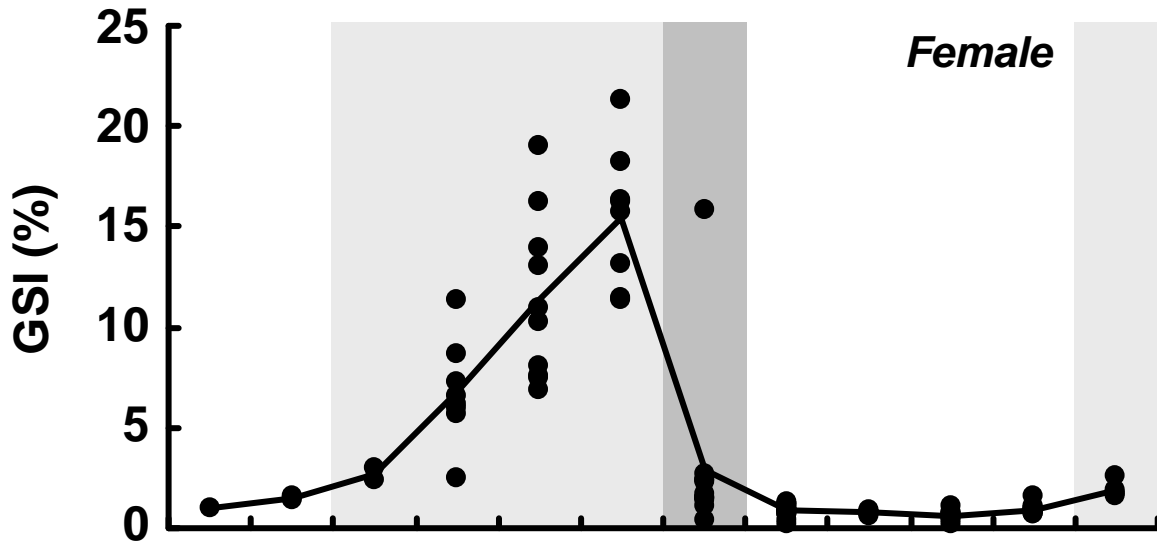


Fig. 1. GSI

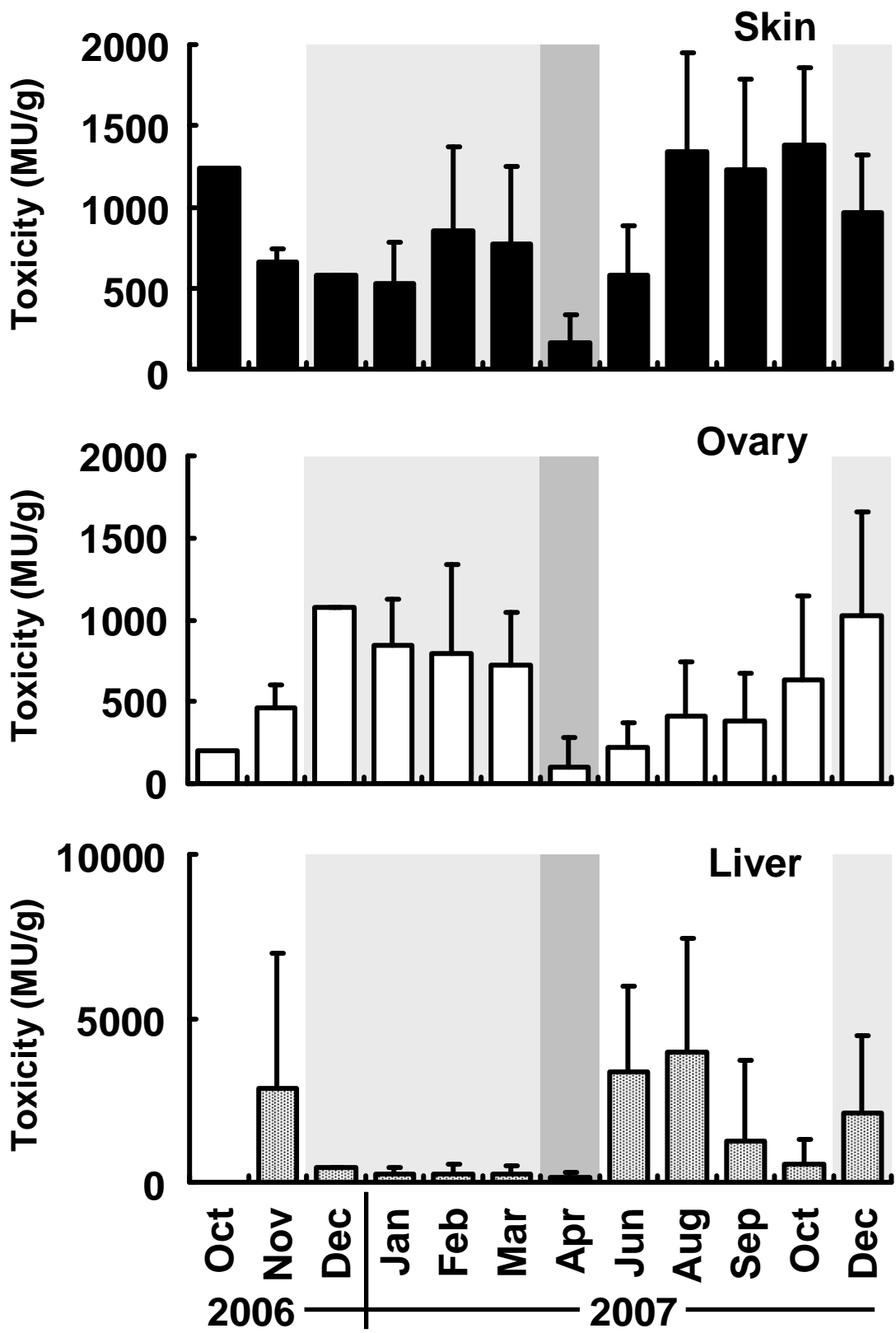


Fig. 2. Female

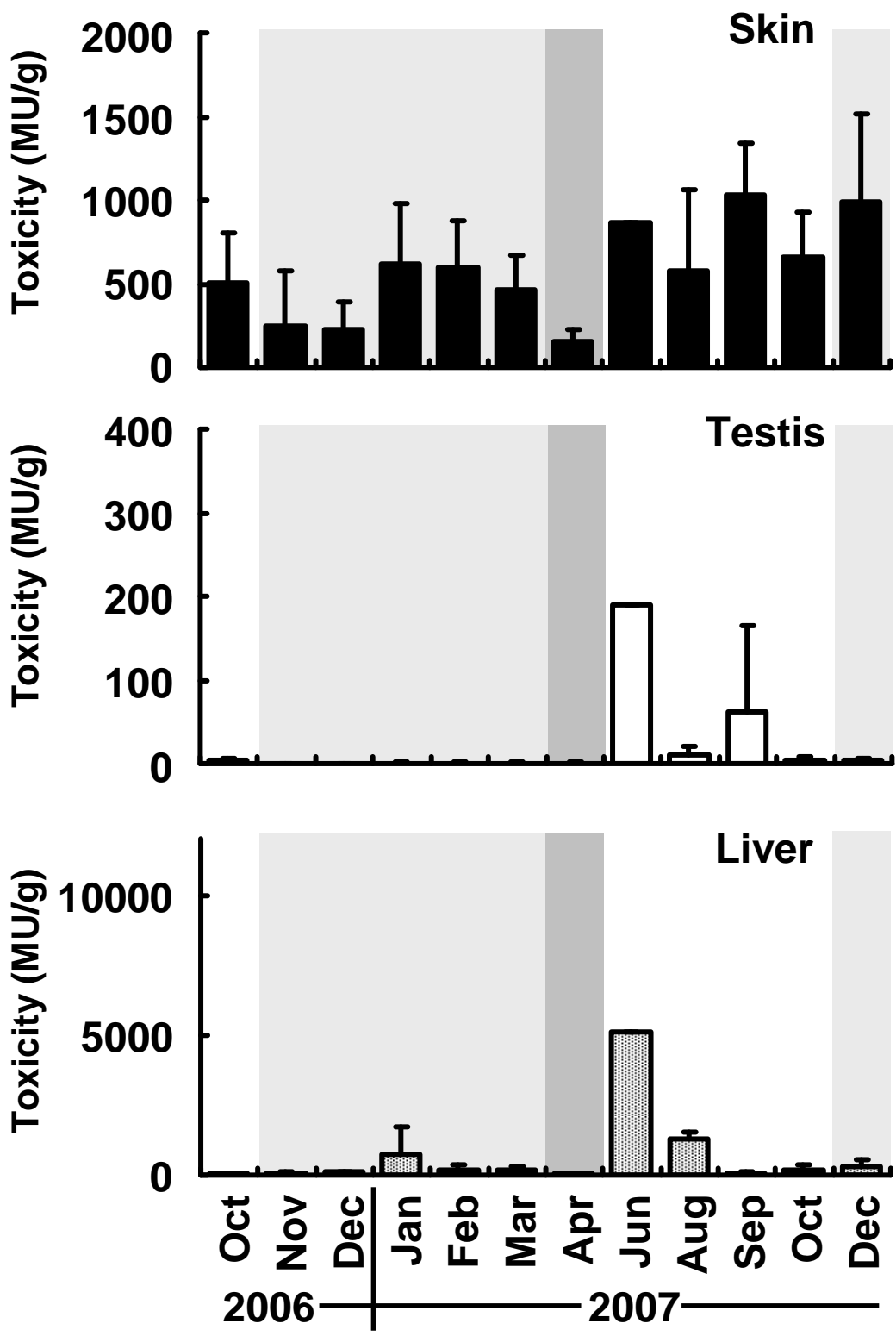


Fig. 3. Male

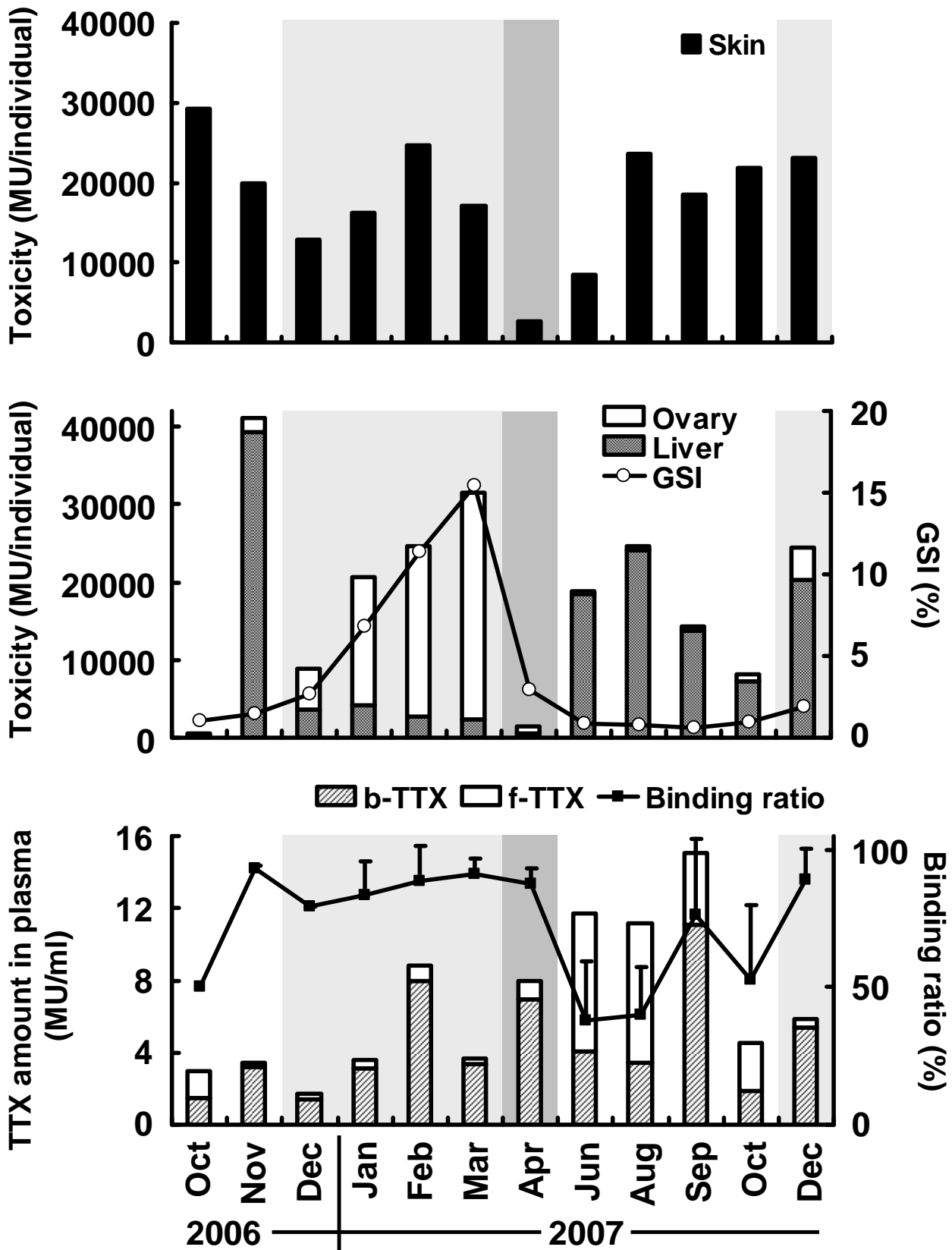


Fig. 4. Female

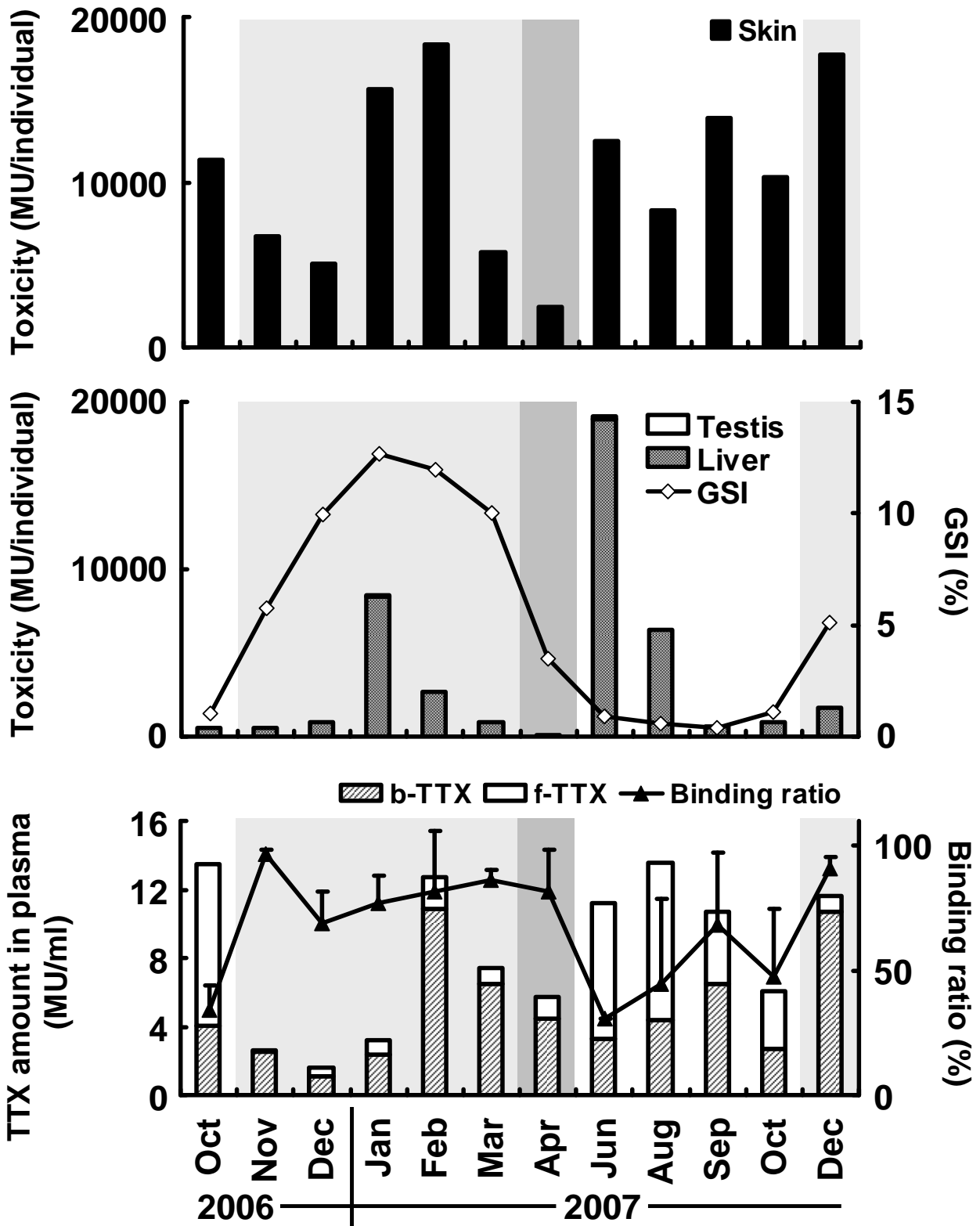


Fig. 5. Male

Table 1. Specification of *T. poecilonotus* specimens

Collection month	Sex	Number of specimens	Mean body weight (g)	Mean tissue weight		
				Skin (g)	Liver (g)	Gonad (g)
2006 Oct		1	198	24	12	2.0
		3	211	24	10	2.2
Nov		3	273	30	16	4.0
		3	227	26	14	13
Dec		2	176	21	9.2	4.8
		3	210	21	9.4	21
2007 Jan		8	278	31	16	19
		3	248	25	8.8	31
Feb		10	267	29	13	31
		6	280	32	15	34
Mar		8	243	22	9.2	39
		3	126	14	3.9	13
Apr		12	121	15	4.1	3.3
		5	113	16	3.4	4.1
Jun		17	124	15	6.2	1.1
		2	143	18	4.5	1.4
Aug		7	156	17	6.5	1.2
		3	155	19	4.7	0.9
Sep		8	121	13	4.4	0.9
		4	116	13	6.5	0.5
Oct		11	146	16	8.7	1.4
		5	123	15	6.6	1.3
Dec		6	194	22	8.9	3.7
		5	166	18	5.3	8.5

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

Sex classification	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	16300 ± 24600 ^a	385 ± 348 ^b	580 ± 691 ^{b*}	1010 ± 563 ^{a*}	16700 ± 12400
	M	34	584 ± 1190 ^b	6140 ± 11500 ^b	849 ± 440 ^a	18200 ± 15000 ^a	761 ± 429 ^{ab*}	19900 ± 12600
♂	O	17	909 ± 2320 ^a	3830 ± 8350 ^{ab}	41.9 ± 90.8 ^{c*}	29.2 ± 72.2 ^{b*}	730 ± 402 ^{ab}	11200 ± 5740
	M	23	267 ± 393 ^b	2450 ± 4520 ^b	1.04 ± 1.77 ^{c*}	13.3 ± 24.6 ^b	572 ± 410 ^b	13000 ± 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.

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/individual)	(MU/g)	(MU/individual)
♀	93	1410 ± 2290*	10600 ± 19700*	518 ± 454*	7080 ± 12400*	809 ± 550*	16100 ± 12800*
♂	45	485 ± 1460*	2710 ± 6070*	16.1 ± 58.2*	18.0 ± 47.8*	585 ± 416*	11100 ± 8300*

Data are shown as mean ± SD.

Asterisks indicate significant differences between the two measured values in each column (Student's *t*-test, $p < 0.05$).

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

Data are shown as mean ± 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.