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Comparison of the localization of tetrodotoxin between wild pufferfish *Takifugu rubripes* juveniles and hatchery-reared juveniles with tetrodotoxin administration

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**Highlights**

► Localization of tetrodotoxin (TTX) in various tissues among wild pufferfish juveniles, hatchery-reared juveniles with or without TTX administration was investigated.

► Localization of TTX in hatchery-reared juveniles with TTX administration (skin, liver, olfactory, optic nerve, brain) coincides those in wild juveniles. ► TTX accumulation in the central nervous system is observed.
Abstract

To reveal the accumulation profile of tetrodotoxin (TTX) in pufferfish *Takifugu rubripes* juveniles, we compared the localization of TTX in various tissues among wild juveniles and hatchery-reared juveniles with or without TTX administration using immunohistochemical technique with anti-TTX monoclonal antibody. Immuno-positive reaction was observed in hepatic tissue, basal cell of skin and olfactory, olfactory epithelium, optic nerve and brain (optic tectum, cerebellum, medulla oblongata) of wild juveniles (body length: BL, 4.7-9.4 cm). TTX was detected in the same tissues as wild juveniles and epithelial cell layer of intestine of hatchery-reared juveniles (BL, 5.0-5.3 cm) to which TTX was orally administrated. No positive reaction was observed from the tissues of hatchery-reared juveniles without TTX administration. These results suggest that orally administrated TTX to the non-toxic cultured juveniles is accumulated in the same manner of wild juveniles. In addition, our study revealed that pufferfish accumulates TTX in the central nervous system.

Keywords: central nervous system, immunohistochemistry, pufferfish, *Takifugu rubripes*, tetrodotoxin (TTX).
1. Introduction

Marine pufferfish of the genus *Takifugu* contain a potent neurotoxin, tetrodotoxin (TTX, Noguchi et al. 2006a). TTX is thought to be originally produced by marine bacteria, and distributed over many taxa of animals including pufferfish, gobies, blue-ringed octopuses, carnivorous gastropods, starfish, toxic crab, horseshoe crabs, flat worms, and ribbon worms (Miyazawa and Noguchi 2001). Artificially raised grass puffer *Takifugu niphobles* and tiger puffer *Takifugu rubripes* becomes non-toxic when fed with non-toxic diets in an environment where the invasion of TTX-bearing organisms was eliminated (Matsui et al. 1982, Saito et al. 1984, Noguchi et al. 2006b), and such non-toxic pufferfish become toxic when fed with TTX-containing diets (Matsui et al. 1981, Honda et al. 2005, Kono et al. 2008). These evidences indicate that TTX in pufferfish is exogenous and is derived via the food chain that starts from TTX-producing bacteria (Noguchi and Arakawa 2008). However, it remains unclear that the transfer, accumulation, and elimination mechanisms of TTX accumulated in the pufferfish body from food organisms.

The distribution of TTX in the body of *Takifugu* spp. is species-specific except for liver and ovary (Noguchi et al. 2006a, Noguchi and Arakawa 2008). In *T. niphobles* at the spawning season, the amount of TTX in the ovary was high but non-toxic in the testes, whereas toxicity in skin and liver of male was higher than female (Itoi et al. 2012). Ikeda et al. (2010) reported that liver toxicity in the females of fine-patterned puffer *Takifugu poecilonotus* was high during the ordinary period, and ovarian toxicity was high during the maturation period. These evidences suggest that the TTX serves an antipredator function both for adults and for spawned eggs. Generally in wild
condition, the liver and ovary of T. rubripes adults are strongly toxic, whereas the muscle, skin and testes are non-toxic (Noguchi and Arakawa 2008). However, when TTX was administered intramuscularly to hatchery-reared T. rubripes juveniles, some TTX remain in the liver but most of the toxins are transferred to the skin (Ikeda et al. 2009). Predation is a major cause of mortality in T. rubripes juveniles (Shimizu et al. 2007, 2008; Nakajima et al. 2008). Shimizu et al. (2007, 2008) conducted release experiments in a salt pond mesocosm and clarified survival of non-toxic hatchery-reared T. rubripes juveniles was significantly lower than that of toxic wild juveniles. Thus, bearing of TTX in the skin of T. rubripes juveniles may be functional as predator defense. In addition, Shimizu et al. (2007, 2008) reported that fear response in the new environment of non-toxic hatchery-reared juveniles is different from that of toxic wild juveniles. These results indicate that TTX may have effects on behavior of the T. rubripes juveniles.

Recently the micro-distribution of TTX in the tissues of several puffer species was investigated by immunohistochemical techniques using anti-TTX monoclonal antibody (Tanu et al. 2002; Mahmud et al. 2003a,b; Ikeda et al. 2009; Itoi et al. 2012). Therefore, to reveal the accumulation profile of TTX in T. rubripes juveniles, we compared the localization of TTX not only in the skin and liver but also in brain and sensitive organ (olfactory and eye) which is responsible for behavior among wild juveniles, hatchery-reared juveniles with or without TTX administration using immunohistochemical technique with anti-TTX monoclonal antibody.

2. Materials and methods
2.1. Pufferfish

Wild juveniles of *T. rubripes* (body weight, 4.1-24.1 g; body length, 4.7-9.4 cm; n=5) were collected in the seashore sites in Kasaoka city, Okayama, Japan, in August 2008 and were transported to Research Center for Marine Invertebrates, National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Momoshima, Hiroshima, Japan. The wild juveniles were fed with the freezed krill *Euphausia* sp. once a day in an aerated 0.5 kl tank before immunohistochemical experiment. Non-toxic cultured *T. rubripes* (about two months old; body weight, 3.2±0.6 g; body length, 4.5±0.2 cm; n=500) were purchased from Yamaguchi Pref. Sea Farming Public Corporation, Japan and were transported to the same institute as wild fish. The non-toxic cultured juveniles were fed with the commercial diets (Otohime S2 and EP1, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) in an aerated 5 kl tank before TTX administration.

2.2. Preparation of TTX-containing diets

TTX was purified from the ovary of a wild-caught adult *T. rubripes* (body weight, 1.0 kg) according to the method of Ikeda et al. (2009) with a slight modification. In addition, the extract was partially purified with Bio-Gel P-2 column (Bio-Rad Laboratories Inc., Herucles, CA, USA) and the absorbed TTX by the gel was eluted with 0.05 M AcOH. TTX fraction was analyzed by LC/MS analysis on an alliance LC/MS system equipped with a ZSpray MS 2000 detector (Waters, Milford, MA, USA).
according to Nakashima et al. (2004). TTX was dissolved in distilled water at the toxicity of 7,600 MU/ml. The diet for the control group was commercial diet (Otohime EP1). For the TTX-feeding group, TTX solution was added to the control diet following the method of Honda et al. (2005), adjusting the concentration of TTX with 25 MU/g feed.

2.3. Toxin administration

The toxin administration was carried out for 5 days in July 2008. A total of 500 non-toxic cultured juveniles were randomly divided into two groups where one group was fed with commercial diets and the other was fed with TTX-containing diets. Fish were kept in 2 kl tank for each group with flow through system (2 kl/hour).

Fish were fed 6 times a day with 3-7% body weight on each diet group. Subsequently, 5 fish per group were randomly collected at 5 days after starting toxin administration, and immunohistochemical observation was performed.

2.4. Immunohistochemical observation

Wild juveniles and hatchery-reared juveniles with or without TTX administration were subjected to perfusion fixation (Oka and Ichikawa 1990, Amano et al. 1991). Fish were anesthetized with 300 ppm MS222 (3-aminobenzoate methanesulfonate, Sigma-Aldrich Cop., St., Louis, MO, USA). After the laparotomy of fish body, saline (1.35% NaCl) was injected into hepatic vein via intravenous drip. Blood and saline were discharged from snicked liver. Then, neutrally buffered formalin (4%) was
injected into ventricle until slowing down of spasms. Liver, skin, brain, olfactory and eye of fixed specimens were embedded in paraffin, followed by sectioning (5 μm in thickness). Subsequently, immunohistochemical observation was employed to recognize TTX in the section according to Tanu et al. (2002). Briefly, sections were deparaffinized and incubated with 10% hydrogen peroxide to remove endogenous peroxidase activity. After rinsing in PBS (137.0 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.4 mM KH₂PO₄), sections were incubated with 25% goat serum in PBS for blocking and subsequently were treated with the primary antibody (anti-TTX monoclonal antibody, Osaka Prefectural Institute of Public Health, Osaka, Japan). Following a wash with PBS, sections were incubated with the second antibody (EnVision+System-HRP Labelled Polymaer (DAB), Dako North America Inc., Carpinteria, CA, USA). As negative control, sections were treated with mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA) instead of the primary antibody. Sections were counter-stained by hematoxylin-eosin (HE) staining to observe the histological structure of tissues. Observation of immunoreactivity was done with a light microscope (Axioskop, Carl Zeiss Co., Ltd., Jena, Germany). Positive stain of TTX was recognized as a brown color.

3. Results

Immunoreaction for TTX in each tissue of wild *T. rubripes* juveniles and hatchery-reared juveniles with or without TTX administration is shown in Fig. 1. In the wild juveniles (Fig. 1A), positive immunoreactions were observed in the liver, skin,
olfactory, optic nerve and brain. In the liver, TTX was localized at hepatic tissue. The epidermal layer of the skin was comprised of two distinct cell types, basal cells and succiform cells, and no exocrine gland or gland-like structure were observed. Positive reactions for TTX were localized at basal cells along the basement membrane of epidermis. No positive reaction was observed in succiform cells of epidermis. In the olfactory, TTX was detected not only in basal cells but also in olfactory epithelium which is directly responsible for detecting odors. All brain sections were stained weakly. In particular, higher signals were obtained in the optic tectum, cerebellum (purukinje cells and molecular layer) and medulla oblongate. On the other hand, no positive reaction was observed from the all tissues of negative control that is hatchery-reared juveniles without toxin administration (Fig. 1B). TTX was detected in the same tissues as wild juveniles and epithelial cell layer of intestine of hatchery-reared juveniles to which TTX was administered (Fig. 1C).

4. Discussion

In this study, we compared the localization of TTX in various tissues among wild _T. rubripes_ juveniles and hatchery-reared juveniles with or without TTX administration using immunohistochemical technique with anti-TTX monoclonal antibody. Immuno-positive reaction was observed in hepatic tissue, basal cell of skin and olfactory, olfactory epithelium, optic nerve and brain (optic tectum, cerebellum, medulla oblongate) of wild juveniles. TTX was detected in the same tissues as wild juveniles and epithelial cell layer of intestine of hatchery-reared juveniles to which TTX was
orally administrated, while no positive reaction was observed from the tissues of hatchery-reared juveniles without TTX administration.

4.1. TTX in liver and skin

We confirmed TTX in the liver and skin of *T. rubripes* juveniles which is generally strongly toxic and non-toxic in wild adults, respectively (Noguchi and Arakawa 2008). TTX was also detected at hepatic tissue and basal cells along the basement membrane of epidermis in wild juveniles and hatchery-reared juveniles with TTX administration. These results imply that *T. rubripes* juveniles accumulate TTX in the liver same as adults but accumulation of TTX in basal cells is restricted in the juvenile stage. In *T. niphobles* at the spawning season, the amount of TTX in the ovary was high but non-toxic in the testes, whereas toxicity in skin and liver of male was higher than female (Itoi et al. 2012). Ikeda et al. (2010) reported that liver toxicity in the females of *T. poecilonotus* was high during the ordinary period, and ovarian toxicity became high during the maturation period. These evidences suggest that the TTX serves an antipredator function both for adults and for spawned eggs. Since predation is a major cause of mortality in *T. rubripes* juveniles (Shimizu et al. 2007, 2008; Nakajima et al. 2008), bearing of TTX in the skin of *T. rubripes* juveniles may be functional as predator defense. *T. niphobles*, *T. poecilonotus*, panther puffer *T. pardalis* and vermiculated puffer *T. snyderi* secrete large amount of TTX immediately after being stimulated by electric shock (Kodama et al. 1985). *T. niphobles*, *T. pardalis* and *T. snyderi* secrete TTX from the skin when they were stimulated by handling (Saito et al. 1985). Cultured *T. rubripes*, which were artificially toxified by feeding with toxic puffer liver,
also release TTX in such case (Saito et al. 1985). However, exocrine glands or gland-like structures are not found in the skin of *T. rubripes*, whereas *T. niphobles*, *T. poecilonotus* and *T. pardalis* possess TTX secreting glands (Kodama et al. 1986). In addition, we observed that TTX in *T. rubripes* juveniles remained at basal cells and did not reach succiform cells, which presumably excrete TTX in adult pufferfish. These results indicate that *T. rubripes* juveniles possess TTX in the skin not only for predator defense but also for any other reason. Adult *T. rubripes* accumulate large amount of TTX (up to 1000 MU/g) in the liver (Noguchi and Arakawa 2008). However, when TTX was administered intramuscularly to hatchery-reared *T. rubripes* juveniles, some TTX remain in the liver but most of the toxins are transferred to the skin (Ikeda et al. 2009). These results suggest that the liver of *T. rubripes* juveniles has lower capacity for TTX than that of adults, and that excess TTX for liver may be transferred to the skin in juveniles.

4.2. TTX in brain and sensitive organ

We clarified intracellular distribution of TTX in the brain in both wild juveniles and hatchery-reared juveniles with TTX administration. Watabe et al. (1987) had reported that TTX exists at the brain in hatchery-reared juveniles after tritium-labeled TTX administration. However, they detected TTX by radiation measurement at tissue level, thus distribution of TTX in the brain was unclear, and they paid little attention to TTX in the brain rather than distribution of TTX in liver and skin. It is believed that large molecules like TTX cannot cross the blood-brain barrier (BBB, Soong and Venkatesh 2006). Teleost fishes, like other vertebrates, have BBB (Soengas and Aldegunde 2002).
Therefore, the central nervous system of \textit{T. rubripes} is unlikely to be exposed to TTX. However, the present study suggests TTX presumably passed through the BBB and was transferred to the central nervous system (CNS) of \textit{T. rubripes} juveniles. The tight junctions among brain capillary endothelial cells in the CNS of higher vertebrates are thought to be responsible for the BBB that impedes the passive diffusion of solutes from the blood into the extracellular space of the CNS (Ohtsuki 2009). Therefore, drugs in circulating blood are transported to the CNS through endothelial cells by transcellular transport (Ohtsuki 2009). TTX binding protein in the blood of pufferfish takes part in TTX transfer and transport (Matsui et al. 2000, Yamamori 2002). The liver of \textit{T. rubripes} has the ability to aggressively take up TTX from blood (Nagashima et al. 2003, Matsumoto et al. 2007) and membrane transport protein may play a role in transport of TTX (Nagashima et al. 1999, Matsumoto et al. 2007). These evidences indicate that TTX is transported to the brain through membrane transport protein. However, it is unclear whether TTX passes the BBB is whether by active transport or by facilitated diffusion (passive transport).

Fear response of non-toxic hatchery-reared \textit{T. rubripes} juveniles is different from that of toxic wild juveniles, and release experiment into the pond with predators revealed that survival of hatchery-reared fish with no TTX was significantly lower than that of wild juveniles (Shimizu et al. 2007, 2008). Parasitic diseases such as white spot disease (Ishitani et al. 1996), myxosporean emaciation disease (Takami 2012) and cannibalism (Nagao et al. 1993) occur in the cultured \textit{T. rubripes} juveniles which are non-toxic. TTX administration to these non-toxic juveniles enhances immunopotentiating effect (Honda et al. 2005) and reduces agonistic interactions (Saito et al. 2002). We detected high concentration of TTX at the molecular layer and
purkinje cells in brain which serve as the sole output of the cerebellar cortex (Voogd and Glickstein 1998) of the cerebellar corpus in the cerebellum. The piscine cerebellar corpus is thought to be homologous with the vermal part of the cerebellum of higher vertebrates (Ito 1978). Thus, it is possible that the piscine cerebellar corpus plays a role in motor learning and motor control. *T. pardalis* has TTX-resistant and STX-resistant Na\(^+\) channels in the skeletal muscle, and Na\(^+\) channels in the brain and skeletal muscle of *T. pardalis* are lower affinity for TTX than that of rat (Yotsu-Yamashita et al. 2000). If this is the case in *T. rubripes* which is the same genus of *T. pardalis*, TTX may be functional in the brain without blocking Na\(^+\) channels. Synthesizing these results and evidences, we presume that TTX transferred to the central nervous system is physiologically functional to *T. rubripes* juveniles. We observed accumulation of TTX in the sensitive organs such as olfactory and eye in wild juveniles and hatchery-reared juveniles with TTX administration. TTX is reported to attract *T. rubripes* juveniles (Saito et al. 2000), while sensing mechanism of TTX has not been clarified.

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**Conflict of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**
The authors declare that this manuscript complies with the Elsevier Ethical Guidelines for Journal Publication.

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Figure legends

Fig. 1. Immunoreactivity for TTX in the liver, skin, intestine, olfactory, optic nerve and brain section of (A) wild Takifugu rubripes juveniles, (B) hatchery-reared T. rubripes and (C) hatchery-reared juvenile with TTX administration. The first line of photographs (TTX) represent anti-TTX antibody. The positive stain to TTX-antibody results in a brown color (arrow heads). The second line of photographs (NC) represents negative control with mouse IgG. Alphabetical letters in photographs indicate a, hepatic tissue; b, pancreatic tissue; c, hepatic portal vein; d, epidermis; e, succiform cells; f, basal cell; g, dermis h, epithelial cell layer; i, lamina propria; j, olfactory epithelium; k, molecular layer; l, purkinje cells; m, granular cell layer. Scale bars indicate 50 μm.
(A) Wild juveniles

Liver Skin Intestine Olfactory Optic nerve

TTX NC

Okita et al. Fig. 1(A)
(B) Hatchery-reared juveniles (TTX 0 MU/g feed)

Okita et al. Fig. 1 (B)
(C) Hatchery-reared juveniles (TTX 25 MU/g feed)

Okita et al. Fig. 1(C)