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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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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.

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* become 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 testis, 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

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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 testis 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 7600 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/h).

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 Na_2HPO_4, 1.4 mM KH_2PO_4), 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 Labeled 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 counterstained by hematoxylin–eosin (HE) staining to observe the histological structure of tissues. Observation of immunoreactivity was done with a light microscope (Axioskop,