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 oblongate) 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 rublipes*, tetrodotoxin (TTX).

45 **1. Introduction**

46

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

61 The distribution of TTX in the body of *Takifugu* spp. is species-specific except for
62 liver and ovary (Noguchi et al. 2006a, Noguchi and Arakawa 2008). In *T. niphobles* at
63 the spawning season, the amount of TTX in the ovary was high but non-toxic in the
64 testes, whereas toxicity in skin and liver of male was higher than female (Itoi et al.
65 2012). Ikeda et al. (2010) reported that liver toxicity in the females of fine-patterned
66 puffer *Takifugu poecilonotus* was high during the ordinary period, and ovarian toxicity
67 was high during the maturation period. These evidences suggest that the TTX serves
68 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

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94 *2.1. Pufferfish*

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96 Wild juveniles of *T. rubripes* (body weight, 4.1-24.1 g; body length, 4.7-9.4 cm;
97 n=5) were collected in the seashore sites in Kasaoka city, Okayama, Japan, in August
98 2008 and were transported to Research Center for Marine Invertebrates, National
99 Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research
100 Agency, Momoshima, Hiroshima, Japan. The wild juveniles were fed with the freezed
101 krill *Euphausia* sp. once a day in an aerated 0.5 kl tank before immunohistochemical
102 experiment. Non-toxic cultured *T. rubripes* (about two months old; body weight,
103 3.2±0.6 g; body length, 4.5±0.2 cm; n=500) were purchased from Yamaguchi Pref. Sea
104 Farming Public Corporation, Japan and were transported to the same institute as wild
105 fish. The non-toxic cultured juveniles were fed with the commercial diets (Otohime
106 S2 and EP1, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) in an aerated 5 kl tank
107 before TTX administration.

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109 *2.2. Preparation of TTX-containing diets*

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111 TTX was purified from the ovary of a wild-caught adult *T. rubripes* (body weight,
112 1.0 kg) according to the method of Ikeda et al. (2009) with a slight modification. In
113 addition, the extract was partially purified with Bio-Gel P-2 column (Bio-Rad
114 Laboratories Inc., Herucles, CA, USA) and the absorbed TTX by the gel was eluted
115 with 0.05 M AcOH. TTX fraction was analyzed by LC/MS analysis on an alliance
116 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

141 injected into ventricle until slowing down of spasms. Liver, skin, brain, olfactory and
142 eye of fixed specimens were embedded in paraffin, followed by sectioning (5 μm in
143 thickness). Subsequently, immunohistochemical observation was employed to
144 recognize TTX in the section according to Tanu et al. (2002). Briefly, sections were
145 deparaffinized and incubated with 10% hydrogen peroxide to remove endogenous
146 peroxidase activity. After rinsing in PBS (137.0 mM NaCl, 2.7 mM KCl, 8.1 mM
147 Na_2HPO_4, 1.4 mM KH_2PO_4), sections were incubated with 25% goat serum in PBS for
148 blocking and subsequently were treated with the primary antibody (anti-TTX
149 monoclonal antibody, Osaka Prefectural Institute of Public Health, Osaka, Japan).
150 Following a wash with PBS, sections were incubated with the second antibody
151 (EnVision+System-HRP Labelled Polymaer (DAB), Dako North America Inc.,
152 Carpinteria, CA, USA). As negative control, sections were treated with mouse IgG
153 (Vector Laboratories Inc., Burlingame, CA, USA) instead of the primary antibody.
154 Sections were counter-stained by hematoxylin-eosin (HE) staining to observe the
155 histological structure of tissues. Observation of immunoreactivity was done with a light
156 microscope (Axioskop, Carl Zeiss Co., Ltd., Jena, Germany). Positive stain of TTX
157 was recognized as a brown color.
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159
160 **3. Results**
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162 Immunoreaction for TTX in each tissue of wild *T. rubripes* juveniles and
163 hatchery-reared juveniles with or without TTX administration is shown in Fig. 1. In
164 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

189 orally administrated, while no positive reaction was observed from the tissues of
190 hatchery-reared juveniles without TTX administration.
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192 *4.1. TTX in liver and skin*
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194 We confirmed TTX in the liver and skin of *T. rubripes* juveniles which is generally
195 strongly toxic and non-toxic in wild adults, respectively (Noguchi and Arakawa 2008).
196 TTX was also detected at hepatic tissue and basal cells along the basement membrane of
197 epidermis in wild juveniles and hatchery-reared juveniles with TTX administration.
198 These results imply that *T. rubripes* juveniles accumulate TTX in the liver same as
199 adults but accumulation of TTX in basal cells is restricted in the juvenile stage. In *T.*
200 *niphobles* at the spawning season, the amount of TTX in the ovary was high but
201 non-toxic in the testes, whereas toxicity in skin and liver of male was higher than
202 female (Itoi et al. 2012). Ikeda et al. (2010) reported that liver toxicity in the females
203 of *T. poecilonotus* was high during the ordinary period, and ovarian toxicity became
204 high during the maturation period. These evidences suggest that the TTX serves an
205 antipredator function both for adults and for spawned eggs. Since predation is a major
206 cause of mortality in *T. rubripes* juveniles (Shimizu et al. 2007, 2008; Nakajima et al.
207 2008), bearing of TTX in the skin of *T. rubripes* juveniles may be functional as predator
208 defense. *T. niphobles*, *T. poecilonotus*, panther puffer *T. pardalis* and vermiculated
209 puffer *T. snyderi* secrete large amount of TTX immediately after being stimulated by
210 electric shock (Kodama et al. 1985). *T. niphobles*, *T. pardalis* and *T. snyderi* secrete
211 TTX from the skin when they were stimulated by handling (Saito et al. 1985).
212 Cultured *T. rubripes*, which were artificially toxified by feeding with toxic puffer liver,

213 also release TTX in such case (Saito et al. 1985). However, exocrine glands or
214 gland-like structures are not found in the skin of *T. rubripes*, whereas *T. niphobles*, *T.*
215 *poecilonotus* and *T. pardalis* possess TTX secreting glands (Kodama et al. 1986). In
216 addition, we observed that TTX in *T. rubripes* juveniles remained at basal cells and did
217 not reach succiform cells, which presumably excrete TTX in adult pufferfish. These
218 results indicate that *T. rubripes* juveniles possess TTX in the skin not only for predator
219 defense but also for any other reason. Adult *T. rubripes* accumulate large amount of
220 TTX (up to 1000 MU/g) in the liver (Noguchi and Arakawa 2008). However, when
221 TTX was administered intramuscularly to hatchery-reared *T. rubripes* juveniles, some
222 TTX remain in the liver but most of the toxins are transferred to the skin (Ikeda et al.
223 2009). These results suggest that the liver of *T. rubripes* juveniles has lower capacity
224 for TTX than that of adults, and that excess TTX for liver may be transferred to the skin
225 in juveniles.
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227 *4.2. TTX in brain and sensitive organ*
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229 We clarified intracellular distribution of TTX in the brain in both wild juveniles and
230 hatchery-reared juveniles with TTX administration. Watabe et al. (1987) had reported
231 that TTX exists at the brain in hatchery-reared juveniles after tritium-labeled TTX
232 administration. However, they detected TTX by radiation measurement at tissue level,
233 thus distribution of TTX in the brain was unclear, and they paid little attention to TTX
234 in the brain rather than distribution of TTX in liver and skin. It is believed that large
235 molecules like TTX cannot cross the blood-brain barrier (BBB, Soong and Venkatesh
236 2006). Teleost fishes, like other vertebrates, have BBB (Soengas and Aldegunde 2002).

237 Therefore, the central nervous system of *T. rubripes* is unlikely to be exposed to TTX.
238 However the present study suggests TTX presumably passed through the BBB and was
239 transferred to the central nervous system (CNS) of *T. rubripes* juveniles. The tight
240 junctions among brain capillary endothelial cells in the CNS of higher vertebrates are
241 thought to be responsible for the BBB that impedes the passive diffusion of solutes from
242 the blood into the extracellular space of the CNS (Ohtsuki 2009). Therefore drugs in
243 circulating blood are transported to the CNS through endothelial cells by transcellular
244 transport (Ohtsuki 2009). TTX binding protein in the blood of pufferfish takes part in
245 TTX transfer and transport (Matsui et al. 2000, Yamamori 2002). The liver of *T.*
246 *rubripes* has ability to aggressively take up TTX from blood (Nagashima et al. 2003,
247 Matsumoto et al. 2007) and membrane transport protein may play a role in transport of
248 TTX (Nagashima et al. 1999, Matsumoto et al. 2007). These evidences indicate that
249 TTX is transported to the brain through membrane transport protein. However it is
250 unclear TTX passes the BBB is whether by active transport or by facilitated diffusion
251 (passive transport).

252 Fear response of non-toxic hatchery-reared *T. rubripes* juveniles is different from
253 that of toxic wild juveniles, and release experiment into the pond with predators
254 revealed that survival of hatchery-reared fish with no TTX was significantly lower than
255 that of wild juveniles (Shimizu et al. 2007, 2008). Parasitic diseases such as white
256 spot disease (Ishitani et al. 1996), myxosporean emaciation disease (Takami 2012) and
257 cannibalism (Nagao et al. 1993) occur in the cultured *T. rubripes* juveniles which are
258 non-toxic. TTX administration to these non-toxic juveniles enhances
259 immunopotentiating effect (Honda et al. 2005) and reduces agonistic interactions (Saito
260 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

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

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295

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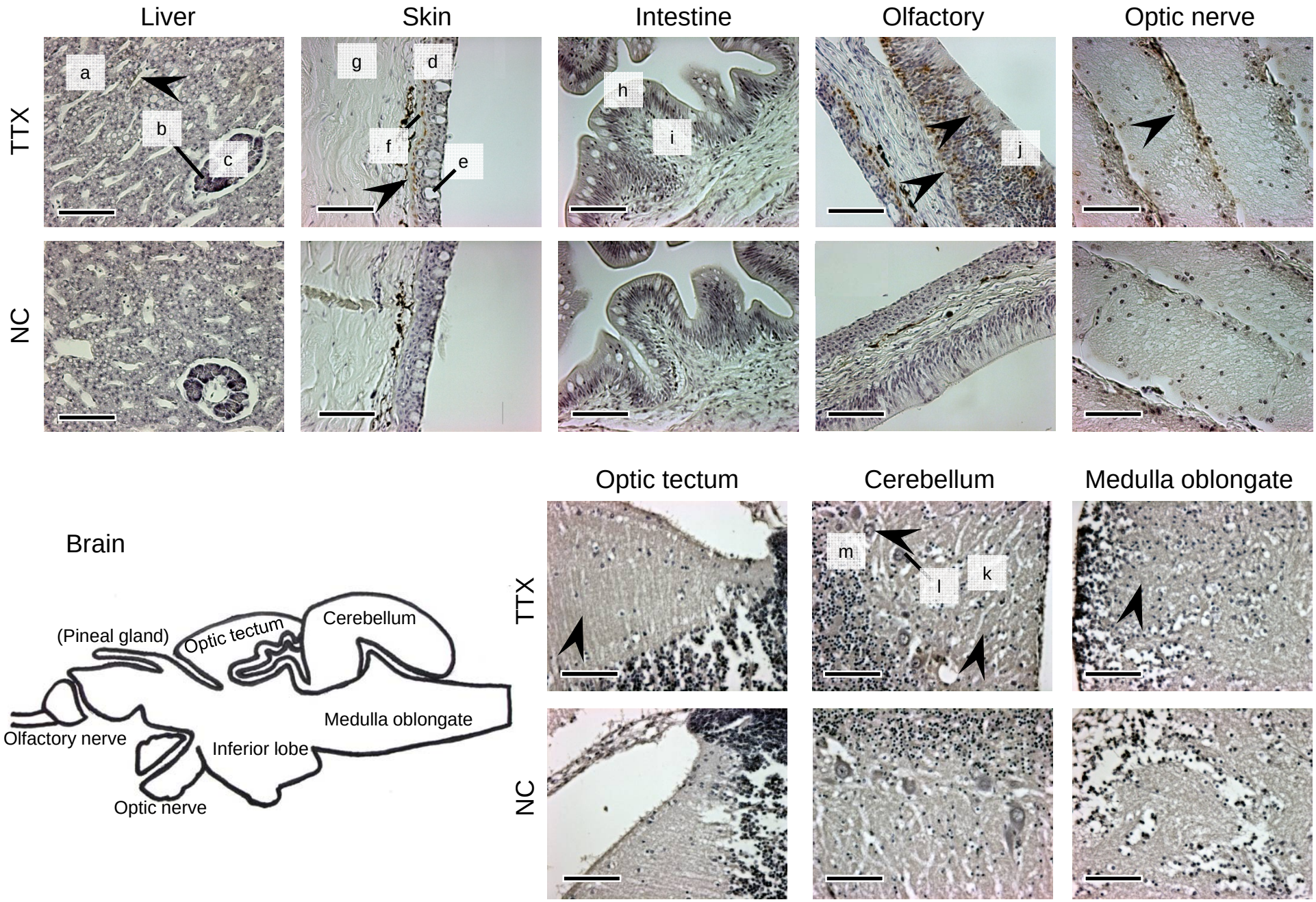
412 **Figure legends**

413

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

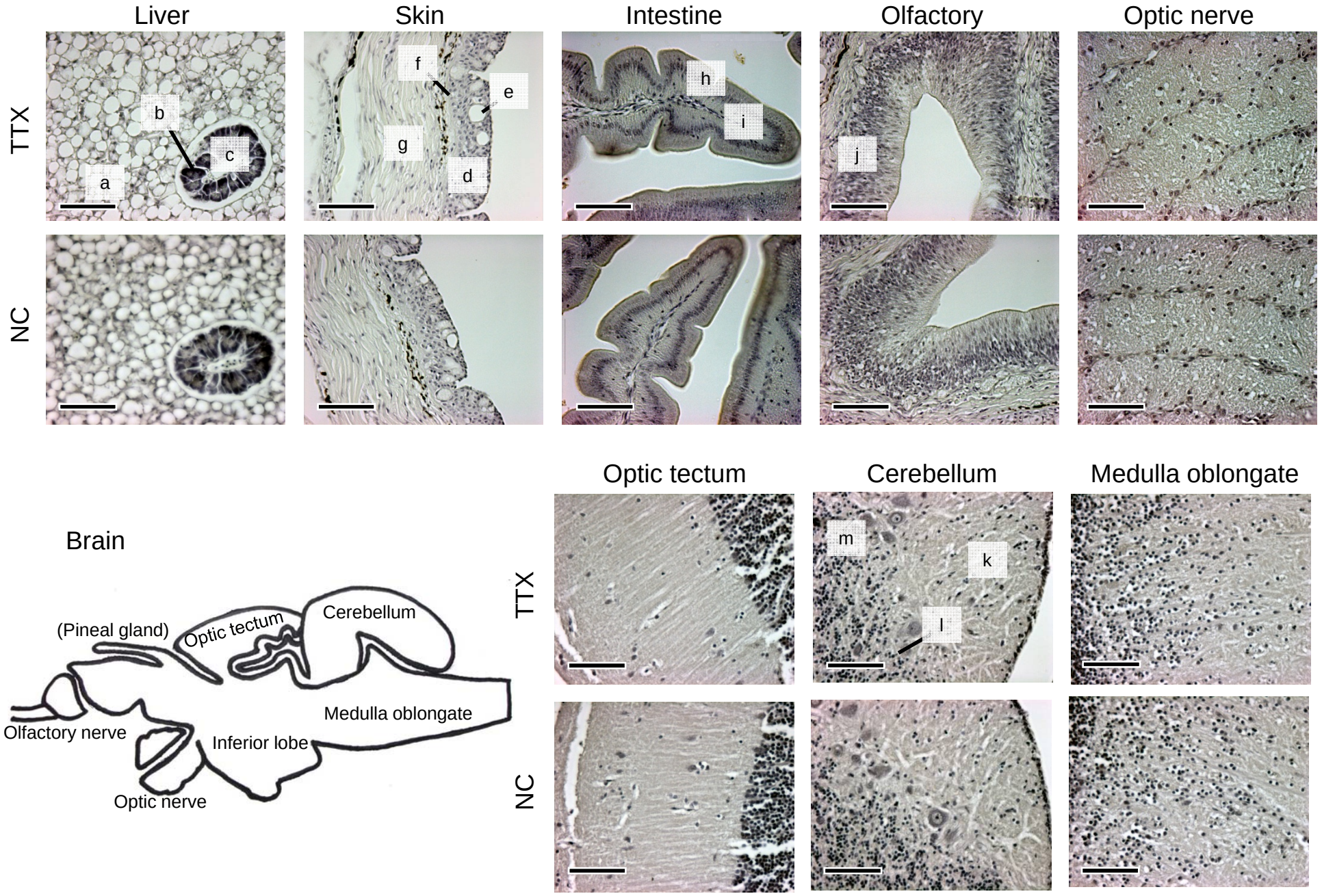
424

Okita et al. Fig. 1(A)



Okita et al. Fig. 1 (B)

(B) Hatchery-reared juveniles (TTX 0 MU/g feed)



Okita et al. Fig. 1(C)

(C) Hatchery-reared juveniles (TTX 25 MU/g feed)