Puffer smells tetrodotoxin

Kogen Okita · Hideki Yamazaki · Kazutaka Sakiyama · Hikaru Yamane · Shinya Niina · Tomohiro Takatani · Osamu Arakawa · Yoshitaka Sakakura

K. Okita · H. Yamane · S. Niina · T. Takatani · O. Arakawa · Y. Sakakura (✉)

Graduate School of Science and Environmental Studies, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, Japan

e-mail: sakakura@nagasaki-u.ac.jp

H. Yamazaki · K. Sakiyama

Research Center for Marine Invertebrate, National Research Institute of Fisheries and Environment of Inland Sea, Incorporated Administrative Agency, Fisheries Research Agency, Momoshima, Hiroshima 722-0061, Japan

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Abstract Behavioral observation was conducted to test whether olfaction is functional to detect tetrodotoxin (TTX) in tiger puffer *Takifugu rubripes* using Y-maze. We placed either agarose carrier or one agarose and one agarose containing TTX (200 MU) at each head of the channel of Y-maze. Then 3 non-toxic hatchery-reared juveniles (body length, 5.6 ± 0.4 cm, *n* = 18) were released into the Y-maze and pecking behavior to carrier was observed for 3 h. The same procedure was tested for olfactory ablated juveniles and for juveniles received sham operation. Juveniles showed significant selectivity to TTX, except for olfactory ablated juveniles. These results indicate that pufferfish detects TTX by olfactory organ.

Keywords *Takifugu rubripes* · Tetrodotoxin · Sensing mechanism · Olfaction

Introduction

Marine pufferfish of the genus *Takifugu* contain a potent neurotoxin, tetrodotoxin (TTX, Noguchi et al. 2006a). Tiger puffer *Takifugu rubripes* and grass puffer *Fugu niphobles* possess TTX from fertilized eggs to adults (Matsui et al. 1982; Matsumura 1998; Noguchi and Arakawa 2008; Ikeda et al. 2009; Nagashima et al. 2010). Matsumura (1998) found that the toxin levels in embryos of *F. niphobles* increase from fertilization to hatching and concluded that TTX is produced by pufferfish. Other studies claimed that pufferfish accumulates TTX through the food chain (Yasumoto and Yotsu-Yamashita 1996; Noguchi et al. 2006a), which is originally produced by marine bacteria belonging to the genera *Vibrio* and *Shewanella* (Noguchi et al. 1986; Yasumoto et al. 1986; Narita et al. 1987; Matsui et al. 1990). It is still unclear whether pufferfish synthesize TTX or accumulate from food organisms, however, there are some reports that pufferfish are attracted to the exogenous TTX. Artificially raised *T. rubripes* becomes non-toxic when fed with non-toxic diets in an environment where the invasion of TTX-bearing organisms was eliminated (Saito et al. 1984, Noguchi et al. 2006b), and such non-toxic *T. rubripes* juveniles are attracted to TTX (Saito et al. 2000). Mature male of *F. niphobles* are also attracted to TTX (Matsumura 1995). These findings suggest that pufferfish is attracted to TTX throughout its life history, not only in spawning season but also in immature juveniles, however, the sensing mechanism of TTX in pufferfish is unknown.

Fishes detect chemical stimuli through at least two different channels of chemoreception: olfaction (smell) and gustation (Hara 1993). The distinction between these two senses in fishes is not always as clear as in air-breathing vertebrates, mainly because both olfaction and gustation of fishes are mediated by molecules dissolved in water (Hara 1993). However, fish possess differentiated olfactory organ and gustatory organ same as the terrestrial animals

(Hara 1993). In addition, these sensory organs have different sensitivity to chemicals and different central connections, respectively (Hara 1993). Matsumura (1995) reported TTX as a male-attracting pheromone at the time of spawning in *F. niphobles*. Since the olfactory system of fishes is highly sensitive to sex pheromones (Sorensen et al. 1988; Moore and Scott 1992; Lastein et al. 2006), mature male of *F. niphobles* was presumably attracted to TTX by olfactory. Here, we focused on the TTX sensing of immature *T. rubripes* juveniles and hypothesized that juvenile pufferfish senses TTX by olfactory organ as expected in the mature pufferfish. In this study, we investigated whether recognition of TTX is accompanied by olfaction using non-toxic *T. rubripes* juveniles with/without olfactory ablation in a Y-maze.

Materials and methods

Experimental fish. Cultured *Takifugu rubripes* (about 2 months old; body length, 3.5 ± 0.2 cm; body weight, 1.6 ± 0.3 g; $n = 30$) were transported from Notojima Station, National Center for Stock Enhancement, Fisheries Research Agency, Notojima, Nanao, Ishikawa, Japan in July 2011 to Research Center for Marine Invertebrates, National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Momoshima, Hiroshima, Japan. Mass analysis of TTX confirmed juveniles were non-toxic (< 0.2 MU/g liver, skin, muscle, intestine and brain, respectively). Juveniles were fed with the commercial diets (Otohime S2 and EP1, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) in an aerated 5,000 L tank until behavioral observation.

Maturity and operation of T. rubripes juveniles. Non-treated juveniles (about 2 months old; body length, 5.6 ± 0.4 cm; $n = 18$) were tested for behavioral response to TTX as control.

Since gonadal sex differentiation in pufferfish begins at 8–9 weeks after fertilization when juveniles attain a body length of approximately 25 mm (Matsuura et al. 1994), the tested juveniles may be sexually differentiated. We ablated the olfactory of juveniles, and these juveniles were tested for the same behavioral observation, to investigate whether recognition of TTX is accompanied by olfaction. Juveniles were anesthetized with 300 ppm MS222 (3-aminobenzoate methanesulfonate, Sigma-Aldrich Co., St., Louis, MO, USA). Subsequently, fish were cauterized olfactory by a heating wire and were immediately transferred to another tank. In addition, we cauterized parietal of non-treated juveniles by the same way as olfactory ablated juveniles to investigate influence of operation to juveniles (sham operation).

Preparation of TTX-containing agarose. TTX was purified from the ovary of a wild-caught adult tiger puffer *T. rubripes* according to the method of Ikeda et al. (2009) with a slight modification. The extract of the ovary was partially purified with Bio-Gel P-2 column (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the absorbed TTX by the gel was eluted with 0.05 M acetic acid. The toxicity of the crude TTX 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 100 MU/ml. TTX solution (200 MU) was mixed in 2% agarose, adjusted to a volume 5 ml, and solidified at room temperature.

Elution of TTX from agarose carrier. The TTX-containing agarose was poured in a 50 ml glass beaker which was filled with distilled water, and 450 μl of the distilled water was sampled 15 min intervals for 3 h. The amount of TTX which was eluted from agarose carrier was determined by LC/MS analysis.

Experimental apparatus. The behavioral observations were conducted using a Y-maze channel (Fig. 1, each arm 50 × 20 cm). The Y-maze channel was constructed with polyvinyl

chloride (PVC) plate. The partition made of PVC plate was placed to prevent the test fish from entering the upstream area of channels (Fig. 1). The channel was filled with seawater to a depth of 5 cm. Illuminance at water surface of the each head of channel was adjust to 140 lx using fluorescent lamp to eliminate the influence of natural light. Two agarose carriers, or one agarose and one TTX-containing agarose, were placed at each head of channel (Fig. 1). A preliminary test following Saito et al. (2000) using a dye solution (alizarin red S, Wako Pure Chemical Industries, Ltd., Osaka, Japan) indicated that eluted TTX from agarose carrier reached the downstream section of the channel after 30 min from the set of carrier.

Testing procedure. We placed either agarose carrier at the both head of channel, or one agarose and one agarose containing TTX (200 MU) at each head of the channel (Fig. 1). We acclimated 3 hatchery-reared juveniles at the downstream of Y-maze for 30 min, then, pecking behavior to the agarose was observed for 3 h (Fig. 1). We also tested the same procedure for olfactory ablated juveniles and for juveniles received sham operation.

Data analysis. In behavioral experiments, we calculated the average frequency of pecking behavior of *T. rubripes* to the agarose carrier per individual, following the comparison among 3 treatments was analyzed statistically with two-way ANOVA, followed by Tukey-Kramer test. The level of significance was set at $p < 0.05$. Statistical analysis was performed by Stat View J 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Elution of TTX from agarose carrier. 40 MU (20 %) of TTX was eluted from TTX-containing agarose at the start of observation (30 min after from acclimatization) and the amount of TTX

reached to 80 MU (40 %) until the end of the observation.

Behavioral observation. In control test, *T. rubripes* juveniles pecked agarose with very low frequency in 3 h (0.0–3.7 times). In contrast, the juveniles without surgery and the juveniles with sham operation showed significant selectivity to TTX (135.9 ± 85.1 times and 178.6 ± 103.0 times, respectively; two-way ANOVA; $F = 16.188$, $p < 0.0001$, treatments; $F = 55.261$, $p < 0.0001$, carriers; $F = 13.809$, $p < 0.0001$, treatments×carriers interaction; Tukey-Kramer *post hoc* test, $p < 0.05$), while the olfactory ablated juveniles were not attracted to TTX (3.3 ± 5.8 times).

Discussion

In this study, we compared the behavioral response to TTX among olfactory ablated *Takifugu rubripes* juveniles, juveniles with sham operation and juveniles without surgery, using Y-maze. Olfactory ablated puffer juveniles were not attracted to TTX, whereas juveniles without surgery were attracted to TTX. Juveniles with sham operation also showed positive selectivity to TTX, indicating that influence of operation on juveniles is negligible. During the behavioral observation in Y-maze, elution of TTX (200 MU) from agarose carrier was estimated as 40 MU (2.8×10^{-8} mol) at the start of observation (30 min after from acclimatization) and the elution of TTX reached to 80 MU (5.6×10^{-8} mol) until the end of the observation. A preliminary test using alizarin red S (342.26), of which molecular weight is similar to TTX (319.27), indicated that TTX reached the downstream section of the channel at the start of observation and spread all over the Y-maze channel during observation. Therefore, *T. rubripes* juveniles could recognize TTX ranging $3.7–5.6\times10^{-9}$ mol/L, if 40 MU and 80 MU

of TTX spread equally at the section of one channel in Y-maze where a TTX-containing agarose was placed (5 L = 50 × 20 × 5 cm) and all over the Y-maze channel (15 L), respectively. Saito et al. (2000) also reported that attraction of *T. rubripes* juveniles to TTX. In their experiment, one gelatin and one TTX-containing gelatin (100–400 MU) were placed at each end of an aquarium (90 × 30 × 20 cm) and one juvenile was placed in the center of the aquarium and was attracted to TTX. In the experimental design by Saito et al. (2000), *T. rubripes* juveniles could recognize TTX at a concentration of up to 2.6×10^{-8} mol/L, if 100 MU (7.0×10^{-8} mol) of TTX eluted from gelatin spread equally to the half of the aquarium (2.7 L = 45 × 30 × 20 cm), which is higher concentration than our study. Synthesizing these results and the evidence, we concluded that *T. rubripes* juveniles sense TTX by olfactory organ. Matsumura (1995) reported that mature male *F. niphobles* was attracted to 1.5×10^{-11} mol/L of TTX, and assumed that TTX is functional as pheromone. Since the olfactory system of fishes is highly sensitive to sex pheromones (Sorensen et al. 1988; Moore and Scott 1992; Lastein et al. 2006), mature male of *F. niphobles* was presumably attracted to TTX by olfactory. These evidences suggest that pufferfish senses TTX by olfactory organ from juveniles to adults.

In this study, we focused on whether recognition of TTX is accompanied by olfaction, thus did not concern the sex of *T. rubripes* juveniles. Genetic sex of *T. rubripes* is determined by *Amhr2* which is sex-determining gene (Kamiya et al. 2012), and gonadal sex differentiation in *T. rubripes* begins at 8–9 weeks after fertilization when juveniles attain a body length of approximately 25 mm (Matsuura et al. 1994). Since the tested *T. rubripes* juveniles were about 60 mm in body length (2 months old), the juveniles may be sexually differentiated. Considering the evidences that TTX may be functional as a male-attracting pheromone at the time of spawning in *F. niphobles* (Matsumura 1995) and that toxicity of mature female

pufferfish is usually stronger than males because the ovary of mature female generally have strong toxicity (Noguchi and Arakawa 2008), TTX sensing of adult pufferfish can vary by sex. Therefore, it is possible that TTX sensing of *T. rubripes* juveniles is sexually different. In the future, the relationship of TTX sensing to sex of pufferfish is needed to investigate.

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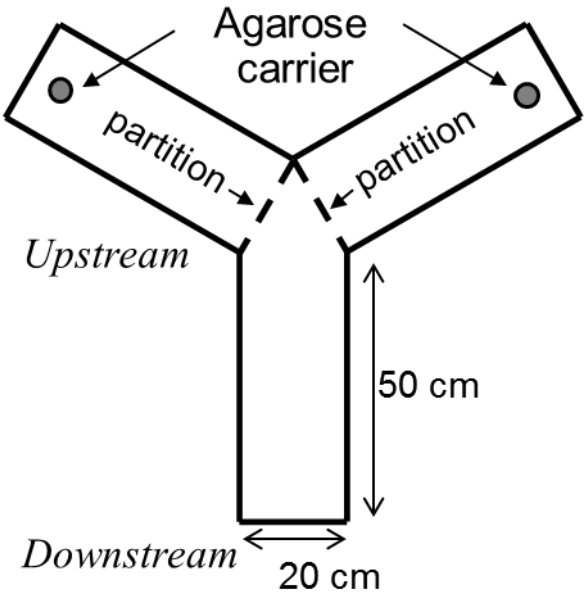

Fig. 1 Schematic drawing of the Y-maze used in this study. Three *Takifugu rubripes* juveniles were acclimatized in the downstream of Y-maze until the removal partitions

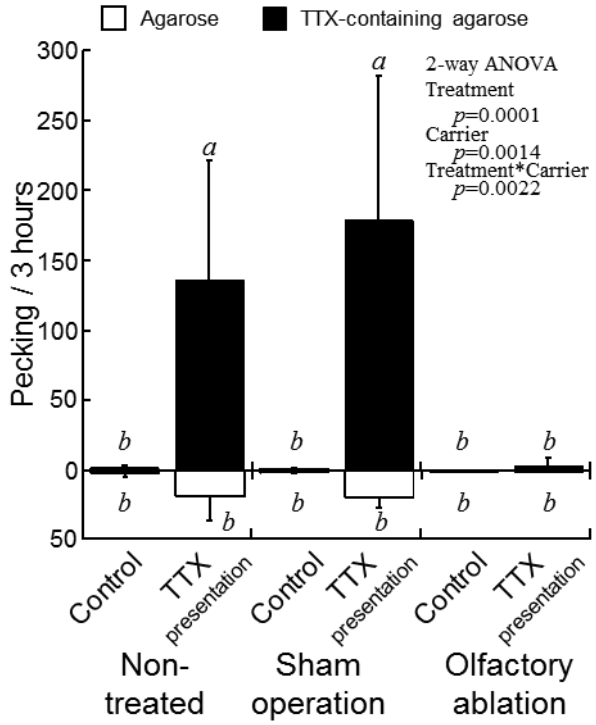

Fig. 2 Average frequency of pecking behavior of *Takifugu rubripes* juveniles to the agarose carrier. Columns are average number of pecking (n = 3). *Bars* indicate standard deviations. Different alphabetical letters on columns indicate significant differences (two-way ANOVA, $p < 0.05$; a > b > c, Tukey-Kramer test, $p < 0.05$)