Title (Max 120 characters): Green spotted puffers can detect an almost nontoxic TTX analog odor using crypt olfactory sensory neurons (105 characters)

Running title (Max 40 characters): Green spotted puffers smell TTX analog (38 characters)

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Keywords (3~6)

Tetrodotoxin (TTX), TTX analog, crypt olfactory sensory neurons, chemoattractant, toxic puffer

Summary statement: 28 words (Max: 15~30 words))

Behavioral and immunohistochemical experiments suggest that an almost nontoxic TTX analog, 5,6,11-trideoxyTTX, acts as an olfactory chemoattractant for green spotted puffers, and crypt-type olfactory sensory neurons detect it.

Abstract (203 words; max 250 words)

Tetrodotoxin (TTX) is a well-known neurotoxin that functions as a defense substance for toxic puffers. Several behavioral studies reported that TTX attracts toxic puffers belonging to the genus *Takifugu*. Although our electrophysiological and behavioral studies showed that a TTX analog, 5,6,11-trideoxyTTX, acts as an olfactory chemoattractant for grass puffers (*T. alboplumbeus*), it is unclear whether toxic puffers are commonly attracted to 5,6,11-trideoxyTTX, and which types of olfactory sensory neurons (OSNs) detect 5,6,11-trideoxyTTX.

Here we investigated whether the green spotted puffer (*Dichotomyctere nigroviridis*), a phylogenetically different species from the grass puffer, is attracted to 5,6,11-trideoxyTTX. Administration of 5,6,11-trideoxyTTX attracted green spotted puffers, but TTX or vehicle did not. Furthermore, immunohistochemistry of the olfactory epithelium exposed to 5,6,11-trideoxyTTX with an antibody against phosphorylated ribosomal protein S6 (pS6), a neuronal activity marker, labeled oval cells with apical invagination. These oval cells were also labeled by the antibody against S100, a specific marker of crypt OSNs. Thus, our results suggest that 5,6,11-trideoxyTTX acts as an olfactory chemoattractant that is detected by crypt-type OSNs in the olfactory epithelium of green spotted puffers. Toxic puffers may use 5,6,11-trideoxyTTX as an olfactory chemoattractant involved in reproduction and parental care or as an olfactory cue of TTX-bearing organisms for effective toxification.

Introduction

Tetrodotoxin (TTX) is a well-known neurotoxin that functions as a defense substance for toxic puffers. TTX is believed to be accumulated in toxic puffer's bodies through the food chain, and is originally produced by bacteria such as *Pseudoalteromonas, Pseudomonas*, and *Vibrio* (Magarlamov et al., 2017; Noguchi et al., 2006). TTX has also been detected in other animals, such as flatworms, ribbon worms, crabs, and gobies (Noguchi and Arakwa, 2008). Recently, TTX-bearing animals and their DNA were detected in the intestinal contents of toxic puffers, which had been eating the egg plates of toxic flatworms containing highly concentrated TTX (Itoi et al., 2015, 2018; Okabe et al., 2019).

Several studies have reported that TTX attracts toxic puffers. For example, Matsumura (1995) reported that mature male grass puffers (*Takifugu alboplumbeus*) are attracted to TTX during the spawning season. Saito et al. (2000) also reported that juvenile tiger puffers (*T. rubripes*) were attracted to TTX, and Okita et al. (2013) reported that nose ablation eliminated the attraction of tiger puffers to TTX. Our electrophysiological and behavioral study has recently shown that an almost nontoxic TTX analog, 5,6,11-trideoxyTTX, acts as an olfactory chemoattractant for grass puffers (Noguchi et al., 2021). Research has only been conducted on toxic puffers of the genus *Takifugu*, however. Thus, it is unclear if toxic puffers are commonly attracted to 5,6,11-trideoxyTTX and which types of olfactory sensory neurons (OSNs) respond to 5,6,11-trideoxyTTX.

In this study, we demonstrated that green spotted puffers are attracted to 5,6,11-trideoxyTTX, and that crypt-type OSNs are specifically activated by 5,6,11-trideoxyTTX. These findings may suggest that attraction to 5,6,11-trideoxyTTX is a common feature of toxic puffers that may be involved in reproduction, parental care, or effective toxification.

Materials and Methods

Animals

Green spotted puffers (*Dichotomyctere nigroviridis*; standard body length: 1.5–3.0 cm, bodyweight: 0.5–1.5 g) were purchased from a local dealer and maintained in 60 L tanks (28ºC; 20–30 fish per tank) filled with artificial brackish water (ABW; Instant Oceans®

Sea-Salt, Cl^- 543 mM, Na^+ 471 mM, Mg^{2+} 54.3 mM, K^+ 10.7 mM, Ca^{2+} 10 mM Br^- 0.7 mM, Sr^{2+} 0.1 mM, F^- 0.05 mM, I^- 0.002 mM, CO_3^{2-}/HCO_3^- 3.3 mM, pH 8.2; Instant Ocean, Blacksburg, VA) with a specific gravity of 1.010 under a 12 h light/12 h dark photoperiod. Fish were fed dried shrimp once a day. In this study, both males and females were used without distinction. All fish were maintained and used in accordance with the Nagoya University regulations on animal care and use in research.

Odorant solutions

L-Arginine (L-Arg, Sigma-Aldrich, St. Louis, MO, USA) and TTX (for biochemistry, citrate buffered, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were dissolved in ultrapure water and preserved as a stock solution at -30ºC. 5,6,11-trideoxyTTX was synthesized and purified according to the methods of Adachi et al. (2014). 5,6,11-trideoxyTTX was dissolved in a 0.025 N acetic acid solution (for behavioral assay) or ultrapure water (for immunohistochemistry) at 10^{-3} M and stored as a stock solution at -30ºC until use.

For the behavioral experiment, stock odorant solutions were diluted with ABW containing 0.8% methylcellulose. For immunohistochemistry, all stock solutions were diluted with artificial cerebro-spinal fluid for teleosts (ACSF; NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 2.4 mM, HEPES 10 mM, glucose 10 mM; pH 7.4).

Behavioral experiments

An overview of the system used in the experiment is shown in Fig. 1. All behavioral experiments were performed using three green spotted puffers at a time. First, three fish were transferred to a circular test aquarium (radius = 12.5 cm, depth = 8 cm) filled with ABW (2 L) prepared from deionized water and Instant Ocean® Sea-Salt and allowed to acclimate to the experimental environment for 1 to 2 h. The test aquarium was surrounded by a cardboard partition (25 cm height) so that the experimenter was invisible to the fish.

A video charge coupled device (CCD) camera (XC-ST50, Sony, Tokyo, Japan) equipped with a low-distortion C-mount lens (f = 4.4 mm; LM4NC1M, Kowa, Nagoya, Japan) was mounted vertically above the aquarium, and the camera system was adjusted so that the aquarium filled the camera frame. Time-lapse images (2 frames/s, 640 × 480 pixels)

from the top of the test aquarium were acquired on a laptop computer (ThinkPad T410; Lenovo, Beijing, China) for up to 10 min using image acquisition software (Micro-Manager 1.4.22; https://micro-manager.org) and a USB video capture unit (GV-USB2, I-O Data Device, Inc., Ishikawa, Japan). Five minutes after the start of time-lapse image acquisition, 1 mL of 2×10^{-4} M odorant solution (vehicle [ABW or 2.5×10^{-4} N acetic acid in ABW], L-Arg, TTX, or 5,6,11-trideoxyTTX; diluted with ABW containing 0.8% methylcellulose) was gently administered into the center of the test aquarium using an injection needle (21G, Terumo Corporation, Tokyo, Japan) connected to a 1 mL syringe via a polyvinyl chloride tube. The estimated final odorant concentration would be 10^{-7} M if the administered solution was uniformly diffused in the test aquarium.

The numeric x-y coordinates representing the movement of the fish over time (1 coordinate per frame) were extracted from each video using UMA tracker software (http://ymnk13.github.io/UMATracker/; Yamanaka and Takeuchi, 2018). We first binarized the grayscale images using appropriate filters that the UMATracker produced. We then tracked fish position using the "Group tracker GMM" algorithm and exported the coordinates to a text file. To quantify the degree of attraction, we calculated the time spent in the central area (within a 4 cm radius of the center of the test aquarium). This criterion was based on the preliminary diffusion experiment that showed that 0.8% methylcellulose-ABW colored with methylene blue remained in that area 10 min after administration (Movie S1). We excluded the data for fish that stopped swimming for longer than 5 s during image acquisition.

Statistics

Data are expressed as the mean ± standard error of the mean (SEM) and represented as a dot plot and a box-whisker plot (box: median and quartiles; whisker: 1.5 × interquartile range). Parametric data were analyzed using Welch's *t*-test. Outliers were detected and excluded using Grubb's test. Statistical analysis and graph preparations in this study were performed using R (version 4.0.4; https://www.r-project.org).

Immunohistochemistry

Fish were anesthetized by tricaine methanesulfonate (MS–222; 0.02%, Sigma-Aldrich) and the bilateral olfactory organs were excised. The excised olfactory organs were halved using a fine blade. The olfactory epithelium (OE) was distributed on one surface of the halved

142 olfactory organs and washed three times for 5 min in ACSF. Each OE was exposed to an
143 odorant solution (vehicle, L-Arg, TTX, or 5,6,11-trideoxyTTX; 10^{-6} M, 1 mL, diluted with
144 ACSF) for 5 min in a 1.5 mL test tube. After odorant stimulation, OE was fixed for 1 h in 4%
145 paraformaldehyde at 4ºC, rinsed in phosphate-buffered saline (PBS), and cryoprotected in
146 20% sucrose in PBS overnight at 4ºC. OE was embedded in the Tissue-Tek® O.C.T.
147 compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan). Cross cryosections (4–6 μm) of
148 OE were mounted onto gelatin-coated slides and dried. After rehydration in PBS, the sections
149 were blocked in 2% normal goat serum (NGS) in PBS with 0.05% Tween-20 (PBST) for 3 h
150 at 28ºC.

151 For single immunohistochemistry, sections were incubated successively in PBST with
152 1) an anti-phosphorylated ribosomal protein S6 (pS6) rabbit polyclonal antibody (Ser244,
153 Ser247 anti-pS6, 44-923G, Thermo Fisher Scientific, MA, USA; 1:5000) that was used in
154 mouse OSNs as validated in Sharma et al. (2017) as a neural activity marker for 2 days at
155 4°C; 2) an Alexa Fluor® 488-conjugated secondary antibody (goat anti-rabbit IgG (H+L),
156 A-11008, Thermo Fisher Scientific, 1:500) for 1 day at 4°C; and 3)
157 4',6-diamidino-2-phenylindole (DAPI; 1:1000 dilution) for 5 min at room temperature.

158 For double immunohistochemistry, sections were successively incubated with 1) a
159 mixture of primary antibodies, anti-pS6 mouse monoclonal antibody (Ser235, Ser236
160 anti-pS6, 62016S, Cell Signaling Technology, Danvers, MA; 1:5000) diluted with anti-S100
161 rabbit serum solution (Envision FLEX-S-100, Agilent Technologies, Santa Clara, CA) that
162 was used in zebrafish crypt OSNs as validated in Germanà et al. (2004) for 2 days at 4°C; 2)
163 a mixture of secondary antibodies, Alexa Fluor® 555-conjugated donkey anti-mouse IgG
164 (A-31570, Thermo Fisher Scientific; 1:500) and biotinylated goat anti-rabbit IgG (BA1000,
165 VECTOR Laboratories, Burlingame, CA, USA; 1:500) for 1 day at 4°C; and 3) an Alexa
166 Fluor® 488-conjugated streptavidin (S11223, Thermo Fisher Scientific; 1:500) for 2 h at
167 28°C. The sections were rinsed for 5 min three times in PBST after each incubation step,
168 mounted in a mounting medium (10% Mowiol 4-88, 25% glycerol, 0.2 M Tris-HCl (pH 9.0),
169 and 2.5%–3.0% 1,4-diazabicyclo [2,2,2] octane; diluted with ultrapure water), and
170 cover-slipped.

171

172 Confocal microscopy

173 Optical sections of the immunohistochemistry and propidium iodide–stained olfactory organs

were acquired using a FV1000D IX81 confocal laser-scanning microscope (Olympus, Tokyo, Japan). All microscopic images used in this study were processed to pseudocolor stacks and were adjusted for brightness and contrast using Fiji (https://fiji.sc/). Some confocal microscopy images were analyzed using FluoRender (http://www.sci.utah.edu/software/fluorender.html; Wan et al., 2012) for three-dimensional reconstructions and visualization.

Results

5,6,11-trideoxyTTX, an almost nontoxic TTX analog, attracts green spotted puffers

To investigate the behavioral response of green spotted puffers, we first created an odorant-induced behavior assay system indexed by the attraction to odorant stimuli administered to the center of a circular test aquarium. Three individuals were placed in the test aquarium and, following acclimation, L-Arg (a food odor for teleost) was administered to the center of the test aquarium (final concentration: 10^{-7} M). Before L-Arg administration, fish swam around the periphery. However, after L-Arg administration, they frequently swam into the center of the test aquarium (Fig. 2B). Vehicle administration did not change the swimming pattern of the fish (Fig. 2A). These changes in swimming patterns are shown in Movie S2. Thus, in addition to our preliminary diffusion test using the colored odorant solution, we considered the invasion to the test aquarium's central area (within a 4 cm radius: gray circles in Fig. 2) as an indicator of attraction toward the odorant. To quantify the attractant activity, we measured the time spent on the central area every minute and compared this cumulative spending time at 5 min after L-Arg administration with that for the vehicle control (ABW). The mean cumulative spending time in the L-Arg group (23.7 ± 11.8 s, n = 6) was longer than that in the vehicle group (7.8 ± 6.5 s, n = 8) at the end of the experiment (p = 0.029; Fig. 3A).

Next, we examined the changes in swimming behavior when 5,6,11-trideoxyTTX (final concentration: 10^{-7} M) or its vehicle (acetic acid in ABW; final concentration: 1.25×10^{-7} N) was administered. Before vehicle or 5,6,11-trideoxyTTX administration (-4 min, -2 min), green spotted puffers swam around the periphery and did not enter the central area of the test aquarium (Fig. 2C and D, respectively). However, after the 5,6,11-trideoxyTTX administrations (+2 min, +4 min), the fish actively swam in the central area (Fig. 2D, Movie S3). In contrast, the fish remained swimming peripherally after the vehicle administration and

206 rarely invaded the central area (Fig. 2C). The cumulative spending time in the central area (1
207 min period) was calculated, and the time changes are plotted in Fig. 3B. Cumulative spending
208 time increased after the 5,6,11-trideoxyTTX administration. The mean cumulative spending
209 time of the 5,6,11-trideoxyTTX group (25.8 ± 13.0 s, n = 11) was longer than that of the
210 vehicle group (9.4 ± 7.8 s, n = 12) at the end of the experimental period (p = 0.003; Fig.3B).

211 By contrast, when TTX was administered, the fish continued to swim around the
212 periphery and rarely invaded the central area (Fig. 2E, Movie S4). The cumulative spending
213 time in the central area was calculated, and the time changes are plotted in Fig. 3C. Although
214 the mean cumulative spending time in the TTX group (13.3 ± 8.7 s, n = 8) was longer than
215 that in the vehicle group (9.4 ± 7.8 s, n = 12), Welch's *t*-test showed no significant difference
216 between the two groups (p = 0.345). These results suggest that 5,6,11-trideoxyTTX acts as an
217 olfactory chemoattractant in green spotted puffers as well as in grass puffers.

218

219 <u>5,6,11-trideoxyTTX activated oval-shaped OSNs</u>

220 The olfactory organs of teleosts are located in nasal cavities under the body surface, and their
221 OE is located on the floor of the nasal cavity (Olivares & Schmachtenberg, 2019). Green
222 spotted puffers have olfactory organs that protrude from the dorsal head surface with stalks
223 and are exposed to the external environment (Fig. 4A, arrows). Each stalk is split into two
224 fleshy flaps (Fig. 4B). The sensory epithelium (SE) is located on the inside (arrow in Fig. 4B),
225 and the non-sensory epithelium (NSE) is located on the outside of the flap (arrowhead in Fig.
226 4B). Figure 4C shows a three-dimensional reconstructed confocal microscopy image of the
227 whole-mount OE stained with propidium iodide. The inside of the flap was shallowly curved,
228 and the SE was surrounded and separated by the NSE at the edge of the flap. OSNs are
229 densely distributed throughout the SE. The x-z confocal section shows the cross-sectional
230 structure of the SE (Fig. 4D). Most OSNs in the SE had spindle-shaped soma (Fig. 4D,
231 arrow), although some large oval-shaped OSNs (minor axis diameter = c.a. 10 μm) were
232 scattered in the SE (Fig. 4C and D, arrowheads). To identify the
233 5,6,11-trideoxyTTX-sensitive OSN, we immunohistochemically labeled odorant-activated
234 OSNs using a pS6 antibody. Excised OE from the green spotted puffer was exposed to
235 vehicle (ACSF), L-Arg, 5,6,11-trideoxyTTX, or TTX. L-Arg activated spindle-shaped OSNs
236 (Fig. 5B, arrow). The cilia-like structure was frequently observed on the L-Arg activated
237 OSNs (Fig. 5B inset, arrowhead). 5,6,11-trideoxyTTX activated large oval-shaped OSNs

without cilia (Fig. 5C, arrow). The apical ends of 5,6,11-trideoxyTTX-sensitive OSNs were invaginated, and their cell bodies were located in the superficial layer of the OE. TTX administration weakly labeled a small number of oval-shaped OSNs (Fig. 5D, arrow). Vehicle treatment did not activate any OSNs, however (Fig. 5A). A small number of 5,6,11-trideoxyTTX -sensitive OSNs were scattered over the OE, some of which were present as cell clusters (Fig. 5E, arrows).

Crypt OSNs were activated by 5,6,11-trideoxyTTX

5,6,11-trideoxyTTX-sensitive OSNs were large, oval-shaped, and scattered over the OE. To identify the cellular subtype of 5,6,11-trideoxyTTX-sensitive OSNs, we performed double immunohistochemistry of 5,6,11-trideoxyTTX-treated OE with a pS6 antibody and an S100 antibody. S100 protein is a specific marker for crypt OSNs (Germanà et al., 2004). The results showed that both pS6 and S100 antibodies labeled oval-shaped OSNs with apical invagination (Fig. 6A and B). The nuclei of oval-shaped OSNs were large and located in the superficial layer of the OE (Fig. 6C, arrows). Merged confocal microscopy imaging indicated that 5,6,11-trideoxyTTX-activated pS6 immunopositive OSNs are overlapped with S100 immunopositive crypt OSNs (Fig. 6D, arrow). However, vehicle treatment did not activate any OSNs (Fig. 6E).

Discussion

We demonstrated that green spotted puffers are attracted to 5,6,11-trideoxyTTX, and that crypt-type OSNs detect 5,6,11-trideoxyTTX. Previous studies reported that TTX attracts tiger puffers (*T. rubripes*, Okita et al., 2013; Saito et al., 2000) and grass puffers (*T. alboplumbeus*, Matsumura,1995; Noguchi et al., 2021). These puffers live in seawater, and the green spotted puffer lives in brackish water. Many brackish and marine Tetraodontidae species have TTX and its analogs in their body. The whole mitogenome sequence shows that Tetraodontidae can be phylogenetically divided into four clades (clade A~D; Yamanoue et al., 2011). All four clades contain both toxic and nontoxic puffers, and the genus *Takifugu* and the genus *Dichotomyctere* belong to different clades (clade C and D, respectively). Although further studies using pufferfish belonging to the other clades are needed, these phylogenic data suggest that the 5,6,11-trideoxyTTX-attracting nature appears to be a synapomorphy for marine or brackish toxic puffers belonging to these clades. The olfactory sensing ability of

270 5,6,11-trideoxyTTX is probably a common feature among toxic puffers.

271 In teleosts, there are four types of OSNs intermingled in OE: ciliated, microvillus,
272 crypt, and kappe OSNs; the latter has not hitherto been described in teleosts other than
273 zebrafish (Olivares and Schmachtenberg, 2019). These OSNs differ in morphology, location
274 of cell bodies in the OE, and responsiveness to odorants (Hamdani and Døving, 2007).
275 Ciliated OSNs are bipolar cells with a long and fine apical dendrite with a few cilia, and their
276 cell bodies are located in the deeper layer of the OE. Microvillous OSNs are spindle-shaped
277 bipolar cells with a short and thick apical dendrite with microvilli, and their cell bodies are
278 located in the middle layer of the OE. Crypt OSNs are oval-shaped, have few cilia and
279 microvilli, and their cell bodies are in the superficial layer of OE. The most striking feature of
280 crypt OSNs is apical invaginations (Hansen and Finger, 2000). Although ciliated OSNs and
281 microvillous OSNs are abundantly distributed, crypt OSNs are minimal and scattered in the
282 OE.

283 5,6,11-trideoxyTTX-activated pS6-immunopositive OSNs were oval-shaped with
284 apical invagination, but L-Arg-activated pS6-immunopositive OSNs were bipolar cells with
285 cilia; thus, the shapes of each odorant-activated OSNs differ. Additionally, a small number of
286 5,6,11-trideoxyTTX-activated pS6-immunopositive OSNs are scattered in the OE. These
287 morphological and locational features suggest that 5,6,11-trideoxyTTX-activated
288 pS6-immunopositive OSNs are crypt OSNs. As such, we performed double
289 immunohistochemistry for pS6 and S100 on the OE. Double immunohistochemistry
290 demonstrated that 5,6,11-trideoxyTTX-activated pS6-immunopositive OSNs were
291 S100-immunopositive and had an oval shape with apical invagination, which is a
292 characteristic of crypt OSNs. These findings suggest that 5,6,11-trideoxyTTX-sensitive OSNs
293 are crypt OSNs.

294 The functional role of crypt OSNs remains unclear. Crypt OSNs respond to the major
295 histocompatibility complex (MHC) peptides in zebrafish (*Danio rerio*; Bienchl et al., 2016)
296 and amino acids in mackerel (*Trachurus symmetricus*; Vielma et al., 2008). Crypt OSNs of
297 juvenile rainbow trout respond to either amino acids, bile salts, or reproductive pheromones
298 whereas those of mature rainbow trout respond to gonadal extracts and hormones from the
299 opposite sex (Bazaes & Schmachtenberg, 2012). The number and density of crypt OSNs are
300 sex-specific; moreover, the number and location of crypt OSNs in the OE varies throughout
301 the year (Bettini et al., 2012; Hamdani et al., 2008). Studies on crucian carp showed that 1)
302 the axons of crypt OSNs extend to the ventral part of the olfactory bulb which extends their

303 axons to the lateral part of the medial olfactory tract (lMOT) (Hamdani & Døving, 2006), and
304 2) that disconnection of the lMOT reduces male reproductive behavior (Weltzien et al., 2003).
305 Axons in the lMOT projected to the ventromedial telencephalon and the preoptic area in
306 goldfish (Oka et al., 1982; von Bartheld et al., 1984). Moreover, electrical stimulations of the
307 ventral telencephalon and preoptic area elicit spawning acts in male landlocked sockeye
308 salmon, and lesions in the same areas impair courtship behavior in male goldfish (Koyama et
309 al., 1984; Satou et al., 1984). These findings suggest that 5,6,11-trideoxyTTX-evoked signals
310 from crypt OSNs may be involved in the reproduction of puffers.

311 5,6,11-trideoxyTTX has almost no toxicity and does not act as a defense substance
312 against predators because 5,6,11-trideoxyTTX has almost no affinity to voltage-gated sodium
313 channels (Yotsu-Yamashita et al., 1999). Toxic puffers accumulate TTX and its analogs in
314 their bodies, but the amounts and proportions vary among species and organs.
315 5,6,11-trideoxyTTX is particularly accumulated in the ovaries, but the ratio of the amount of
316 TTX and 5,6,11-trideoxyTTX in the ovaries is double in grass puffers (Jang et al., 2010).
317 Green spotted puffers have TTX and its analogs in their liver and gonads (Chulanetra et al.,
318 2011), and the amount of 5,6,11-trideoxyTTX in the whole body is half the amount of TTX
319 (Jang et al., 2010). Why do toxic puffers contain this harmless toxin?

320 The first hypothesis is that 5,6,11-trideoxyTTX acts as an olfactory chemoattractant
321 for conspecific puffers. Our study suggests that 5,6,11-trideoxyTTX is detected by crypt
322 OSNs, which are thought to detect the olfactory information involved in reproduction.
323 Matsumura (1995) reported that mature male grass puffers are attracted to the extremely low
324 concentration of TTX (1.5×10^{-11} M) during the spawning season. They gather and spawn in
325 early summer (Motohashi et al., 2010), and TTX levels in the ovaries increase during the
326 spawning periods (Itoi et al., 2016). In addition to grass puffers, male tiger puffers chase
327 females before spawning (Shibata et al., 2006). Although little is known about the
328 reproductive behavior of green spotted puffers, it has been observed that their eggs are
329 attached to hard and flat submerged surfaces in shallow water and are guarded by males until
330 hatching; moreover, adults protect fry schools by directing them to the roots of mangroves
331 (Pethiyagoda 1991; Rüdiger and Baensch, 1991). It has also been reported that the males of
332 some freshwater puffers protect their eggs and use their fins to provide fresh water until they
333 hatch. (Doi et al., 2014). These studies suggest that male green spotted puffers may use
334 5,6,11-trideoxyTTX to find a female for mating or to locate their eggs for parental behavior.

335 The second hypothesis is that toxic puffers use 5,6,11-trideoxyTTX as an olfactory

cue to find TTX-bearing organisms for feeding. Recently, toxic puffer (*T. pardalis*) eggs and toxic flatworm (*Planocera multitentaculata*) DNA was detected in the intestinal contents of grass puffers, which eat the egg plates of toxic flatworms containing highly concentrated TTX (Itoi et al., 2015; Okabe et al., 2019). In another toxic puffer (*Chelonodon patoca*), DNA from toxic flatworms and gobies (*Yongeichthys criniger*) was detected in intestinal contents (Itoi et al., 2020). Furthermore, some toxic puffers secreted TTX from the glands in their skin when stimulated (Kodama et al., 1986), and predators ingested toxic puffer larva or their eggs but spat them out promptly (Itoi et al., 2014). These studies suggested that toxic puffers accumulate TTX and use it as a defense substance against predators. Because it has been reported that TTX-bearing prey organisms, such as bivalves and gastropods (Biessy et al., 2019), ribbon worms (*Cephalothrix* cf. *simula*; Vlasenko and Magarlamov, 2020), and flatworms (*Prosthiostomum trilineatum*; Suo et al., 2021) have 5,6,11-trideoxyTTX as well as TTX. Green spotted puffers feed on mollusks, crustaceans, and other invertebrates (Rainboth, 1996), and these phyla appeared to contain various TTX-bearing organisms (Noguchi and Arakawa, 2008). Thus, green spotted puffers may use 5,6,11-trideoxyTTX as an olfactory cue to selectively feed on TTX-bearing prey organisms and effectively accumulate TTX and its analogs in their body.

This study demonstrated that green spotted puffers are attracted to 5,6,11-trideoxyTTX, and that 5,6,11-trideoxyTTX activates crypt OSNs, which have been reported to sense odor information related to reproduction. These results raise the possibility that the attraction to 5,6,11-trideoxyTTX is a common characteristic among toxic puffers. Additionally, recent studies on feeding behavior suggest that 5,6,11-trideoxyTTX, as an olfactory chemoattractant, may be involved in the reproduction, parental care, or effective toxification of these toxic puffers.

Acknowledgments

We would like to express our gratitude to Professor Naoyuki Yamamoto and Drs. Maki Goto and Hanako Hagio (Nagoya University) and Dr. Kazuya Fukuda (currently Hiroshima Univ.) for their suggestions during experimentation.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.S., H.A.; Methodology: T.S., H.A., R.N., M.A., T.N.; Validation: T.S., H.A.; Formal analysis: T.S.; Investigation: T.S., R.N., H.A.; Resources: T.N., H.A.; Data curation: T.S., H.A.; Writing - original draft: T.S., H.A.; Writing - review & editing: TS, T.N., H.A.; Visualization: T.S.; Supervision: T.N., H.A.; Project administration: H.A.; Funding acquisition: H.A., T.N.

Funding

This work was supported by Grants-in-Aid for Scientific Research (19K06762 to H.A., and 17K19195 to T.N. and H.A., and 17H06406 to T.N.).

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Figure Legends

Figure 1: **Schematic drawing of the test aquarium used in this study.**

Side view (left) and top view (right) of the test aquarium with odorant administration syringe. Three green spotted puffers were acclimatized in the aquarium until the odorant administration from the syringe. See the Materials and Methods section for details.

Figure 2: **Swimming trajectories of green spotted puffers in response to odorant administration.**

Swimming trajectories of green spotted puffers before and after the administration of Vehicle (ABW; A), L-Arg (10^{-7} M; B), Vehicle (1.25×10^{-7} N acetic acids in ABW; C), 5,6,11-trideoxyTTX (10^{-7} M; D), and TTX (10^{-7} M; E). Each panel represents the swimming trajectories of three fish in 2 min under each condition; the time scale at the top show the elapsed time with the start of odorant administration as time 0. The outer circle represents the outer edge of the test aquarium, and the inner circle represents the central area within a 4 cm radius from the center of the aquarium. The swimming trajectories in the central area are shown as the bold line, and the trajectories in the other area are shown as gray lines.

Figure 3: **Green spotted puffers were attracted to 5,6,11-trideoxyTTX.**

A: Cumulative spending time of individual puffers in the central area of the test aquarium (1 min period) before and after the administration of L-Arg (10^{-7} M; gray box; n=6) or its Vehicle (ABW; open box; n=8).

B: Cumulative spending time of puffers before and after the administration of 5,6,11-trideoxyTTX (10^{-7} M; gray box; n = 11) or its vehicle (1.25×10^{-7} N acetic acid; open box; n = 12).

C: Cumulative spending time of puffers before and after the administration of TTX (10^{-7} M; gray box; n = 8) or its Vehicle (1.25×10^{-7} N acetic acid; open box; n = 12).

The X-axis represents the elapsed time with the start of the odor administration as 0.

Box plots show the median, quartiles (boxes) and 1.5 × interquartile range (whiskers). Welch's t-test. *: $p < 0.05$, **: $p < 0.01$.

Figure 4: **Olfactory epithelium of the green spotted puffer.**

A: Dorsal view of the head region of a green spotted puffer. Arrows: Olfactory organ.

559 Scale bar: 2 mm.
560 B: Magnified view of the olfactory organ. Arrow: Sensory epithelium,
561 Arrowhead: Non-sensory epithelium. Scale bar: 0.5 mm.
562 C: Reconstructed confocal microscopy image of the whole mount olfactory organ stained
563 by propidium iodide. SE: sensory epithelium, NSE: Non-sensory epithelium. Scale bar:
564 20 μm.
565 D: x-z confocal section of the dashed rectangular region in (C). Arrowhead: Large
566 oval-shaped OSNs. Arrow: spindle-shaped OSNs. Scale bar: 10 μm.
567
568 Figure 5: **5,6,11-trideoxyTTX activates oval-shaped OSNs.**
569 A~D: pS6 immunohistochemistry of OE cross-sections exposed to Vehicle (ACSF; A),
570 L-Arg (10^{-6} M; B), 5,6,11-trideoxyTTX (10^{-6} M; C), or TTX (10^{-6} M; D). Arrow:
571 activated OSNs. Scale bar: 10 μm. Insets in B and C: Magnified view of
572 pS6-immunopositive OSNs. Arrowhead: Cilia of activated OSNs. Scale bar: 2.5 μm.
573 E: Lower magnification confocal microscopy image of pS6-immunopositive OSNs in
574 OE cross section exposed to 5,6,11-trideoxyTTX. Arrow:
575 5,6,11-trideoxyTTX-activated OSNs. Scale bar: 50 μm.
576
577 Figure 6: **5,6,11-trideoxyTTX activates crypt OSNs.**
578 A~C: Reconstructed confocal microscopy images of 5,6,11-trideoxyTTX-administered OE
579 displaying pS6-immunopositive OSNs (A) or S100-immunopositive OSNs (B) or
580 stained with DAPI (C).
581 D: A merged image of A to C (pS6: magenta, S100: green, DAPI: blue). Each
582 immunopositive OSN had apical invagination, and their cell bodies were located in
583 the superficial layer of the OE. Arrow: Nucleus of 5,6,11-trideoxyTTX-activated
584 OSNs. Scale bar: 10μm.
585 E: No pS6-immunopositive OSN was observed in Vehicle (ACSF)-treated OE. Scale
586 bar: 10μm.
587
588 Movie S1: **Diffusion test of odorant solution.**
589 0.8% methylcellulose-ABW solution (1 mL) colored with methylene blue, which was
590 used to dissolve the odorant solution, was administered from the center of the test
591 aquarium filled with 2L ABW. The diffusion area of methylene blue remained within a 4

cm radius from the center of the aquarium (purple circle) even after 10 min.

Movie S2: **Attractive swimming behavior of green spotted puffers toward L-Arg**.

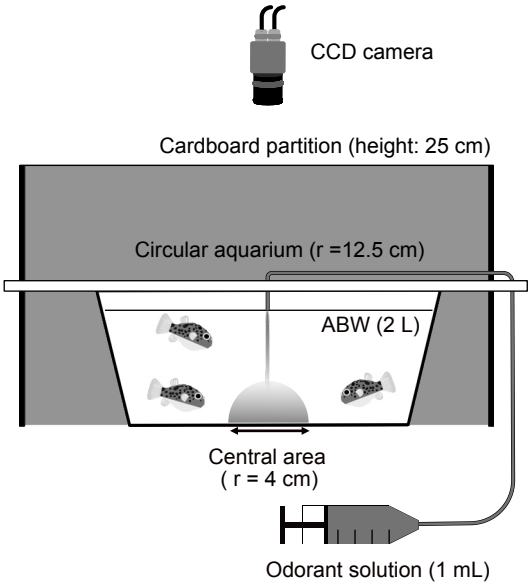
Vehicle or L-Arg solutions were administered from the center of the test aquarium at time 0 and remained in the aquarium. As a result, the fish frequently swam into the central area (purple circle) after L-Arg administration.

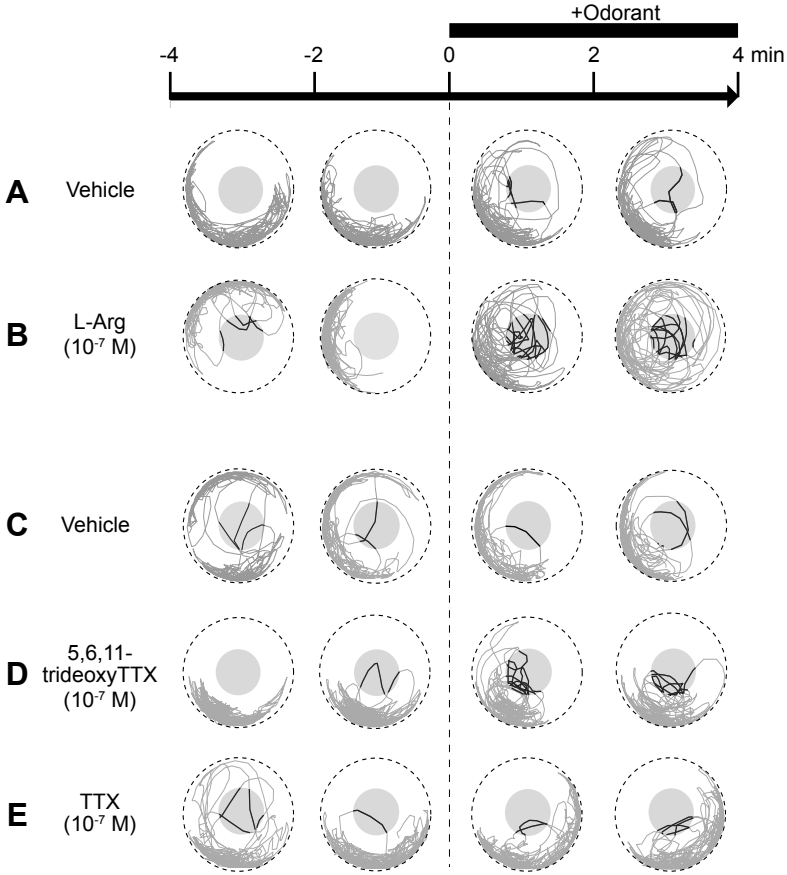
Movie S3: **Attractive swimming behavior of green spotted puffers toward 5,6,11-trideoxyTTX.**

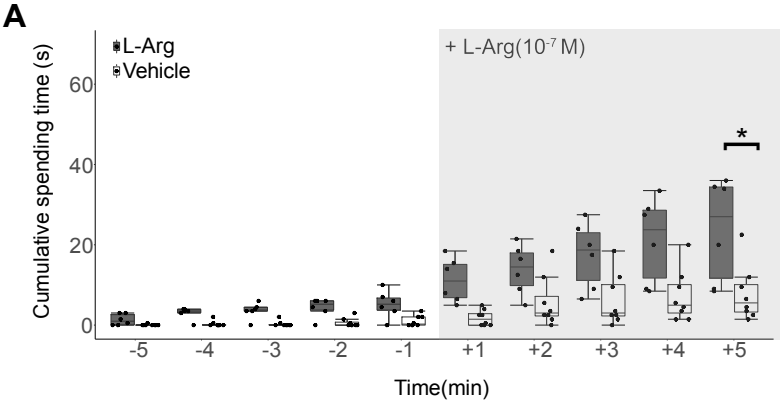
The 5,6,11-trideoxyTTX solution was added from the center of the test aquarium at time 0 and remained in the aquarium. As a result, the fish frequently swam into the central area (purple circle).

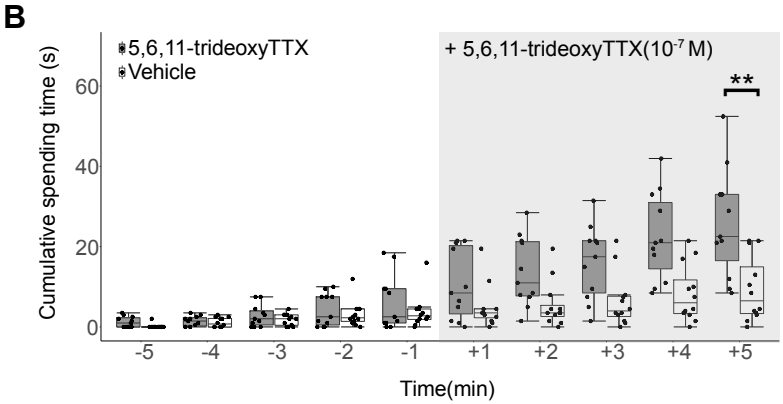
Movie S4: **No attractive swimming behavior of green spotted puffers toward TTX.**

TTX solution was added from the center of the test aquarium at time 0 and remained in the aquarium. The fish continued to swim around peripherally and rarely invaded the central area (purple circle).


CCD camera
Cardboard partition (height: 25 cm)
Circular aquarium (r =12.5 cm)
ABW (2 L)
Central area
(r = 4 cm)
Odorant solution (1 mL)


+Odorant
-4
-2
0
2
4 min
A
Vehicle
B
L-Arg
(10-7 M)
C
Vehicle
D
5,6,11-
trideoxyTTX
(10-7 M)
E
TTX
(10-7 M)


A
L-Arg
Vehicle
+ L-Arg(10-7 M)
*
Cumulative spending time (s)
0
20
40
60
-5
-4
-3
-2
-1
+1
+2
+3
+4
+5
Time(min)


B
5,6,11-trideoxyTTX
Vehicle
+ 5,6,11-trideoxyTTX(10-7 M)
**
Cumulative spending time (s)
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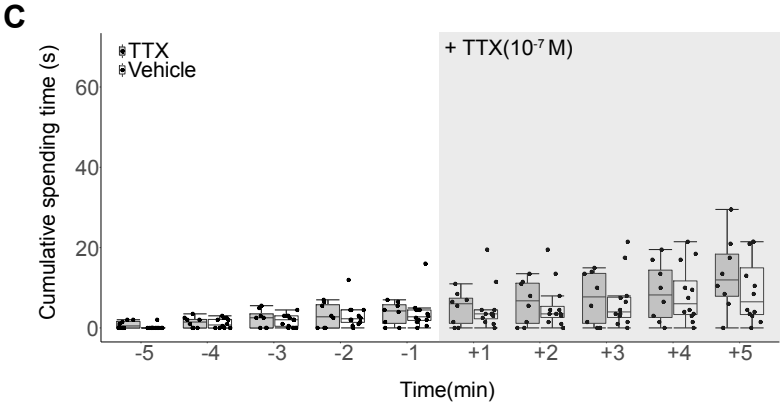
60
-5
-4
-3
-2
-1
+1
+2
+3
+4
+5
Time(min)


C
TTX
Vehicle
+ TTX(10-7 M)
Cumulative spending time (s)
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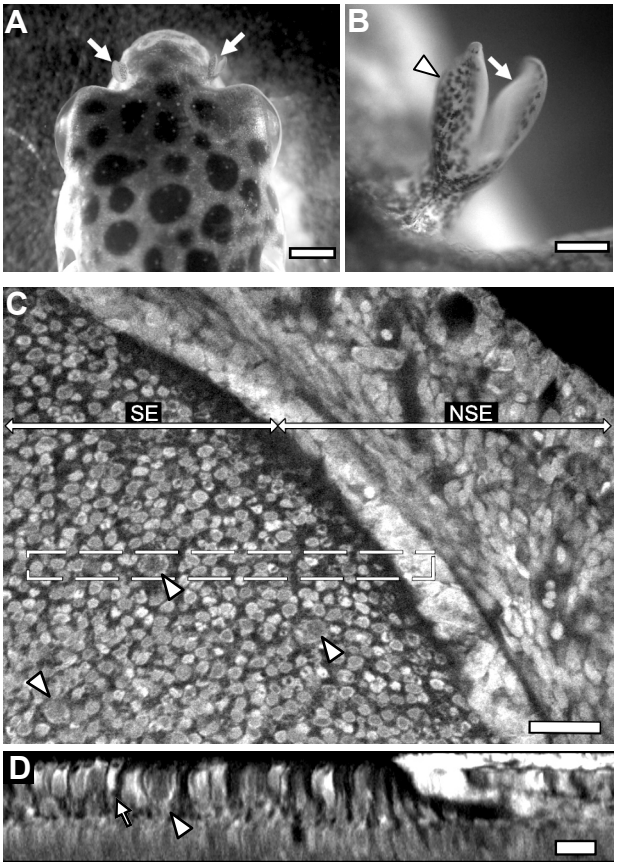
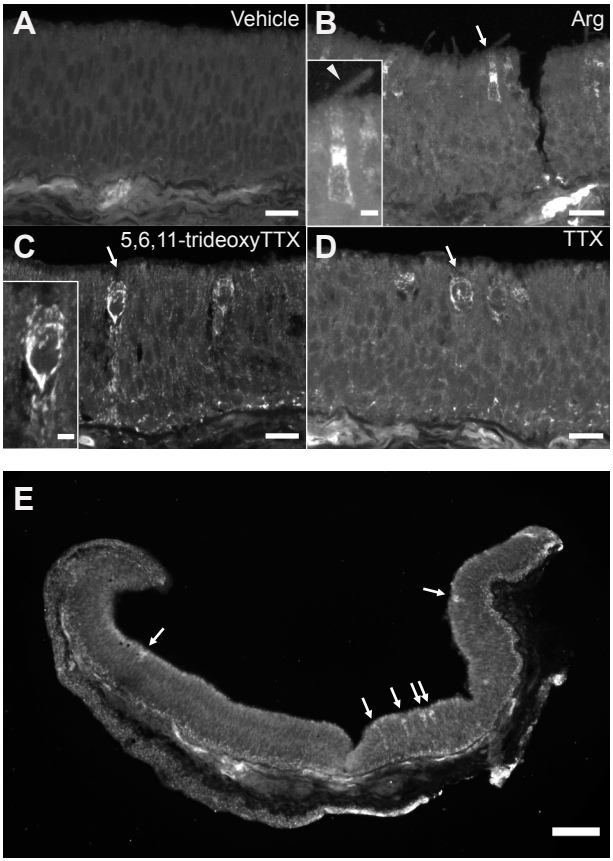
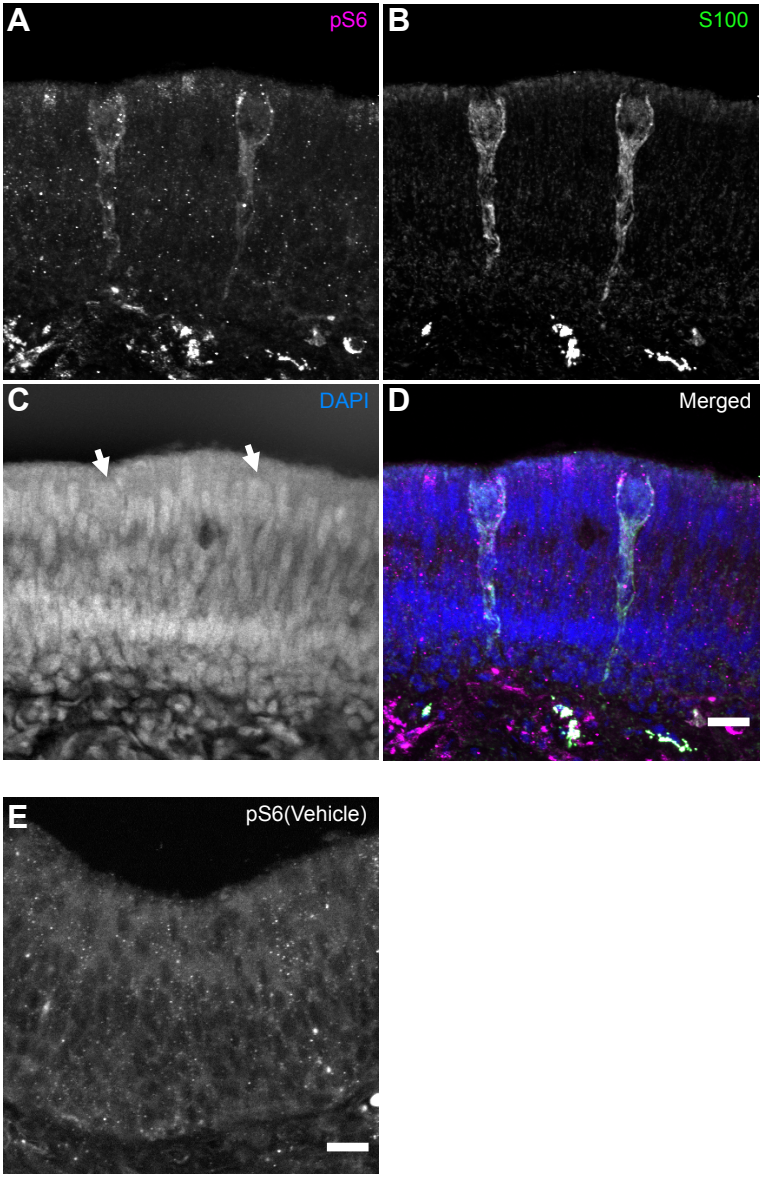
+3
+4
+5
Time(min)


A
B
C
SE
NSE
D


A
Vehicle
B
Arg
C
5,6,11-trideoxyTTX
D
TTX
E


A
pS6
B
S100
C
DAPI
D
Merged
E
pS6(Vehicle)