Title: Green spotted puffer can detect an almost nontoxic TTX analog odor using crypt olfactory sensory neurons

Running title： Green spotted puffer smell TTX analog

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Keywords

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

Summary statement:

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 that.

Abstract

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 (*Takifugu 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 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. Such 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.

Introductions

Tetrodotoxin (TTX) is a well-known neurotoxin that functions as a defense substance for toxic puffers. TTX has been believed to be accumulated in toxic puffer's bodies through the food chain, originally produced by bacteria such as *Pseudoalteromonas, Pseudomonas*, and *Vibrio* (Noguchi et al., 2006). However, TTX has also been detected in various 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, and toxic puffers had been eating egg plates of toxic flatworm 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 attractions 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). However, these studies have only been conducted on toxic puffers of the genus *Takifugu*. Thus, it is unclear whether toxic puffers are commonly attracted to 5,6,11-trideoxyTTX and which types of olfactory sensory neuron (OSN)s respond to the 5,6,11-trideoxyTTX.

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

Materials and Methods

<u>Animals</u>

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 each 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 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 teleost (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 1–2 h. The test aquarium was surrounded by a cardboard partition (25-cm height) so that the experimenter was invisible to the fish.

A video 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 to a laptop computer (ThinkPad T410; Lenovo, Beijing, China) 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 to the center of the test aquarium through an injection needle (21G, TERUMO Corporation, Tokyo, Japan) connected to a 1-mL syringe via polyvinyl chloride tube (The estimated final odorant concentration would be 10^{-7} M if the administered solution were uniformly diffused in the test aquarium).

The numeric x-y coordinates representing the movement of the fish over time (one coordinate in a frame) were extracted from each video using a 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 the fish's 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 on the central (a 4 cm radius) area from the center of the test aquarium. This criterion was based on the preliminary diffusion experiment that 0.8% methylcellulose-ABW colored with methylene blue remained in that area even after 10 min of administration (Movie S1). We excluded the data that fish stopped swimming longer than 5 seconds during image acquisition.

Statistics

Data are expressed as 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 excised bilateral olfactory organs. The excised olfactory organs were halved by a fine blade. The olfactory epithelium （OE） was distributed on one surface of the halved olfactory organs and washed three times for 5 min in ACSF. Each OE was exposed to an odorant solution (Vehicle, L-Arg, TTX, or 5,6,11-trideoxyTTX; 10^{-6} M, 1 mL, diluted with ACSF) for 5 min in a 1.5 mL test tube. After odorant stimulation, OE was fixed for 1 h in 4% paraformaldehyde at

141 4ºC, rinsed in phosphate-buffered saline (PBS), and cryoprotected in 20% sucrose in PBS
142 overnight at 4ºC. OE was embedded in the Tissue-Tek® O.C.T. compound (Sakura Finetek
143 Japan Co. Ltd., Tokyo, Japan). Cross cryosection (4–6 μm) of OE mounted onto gelatin-coated
144 slides and dried. After rehydration in PBS, the sections were blocked in 2% normal goat serum
145 （NGS） in PBS with 0.05% Tween-20 (PBST) for 3h at 28ºC.

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

153 For double immunohistochemistry, sections were successively incubated with 1) a
154 mixture of primary antibodies, anti-pS6 mouse monoclonal antibody (Ser235, Ser236 anti-pS6,
155 62016S, Cell Signaling Technology, Danvers, MA; 1:5000) diluted with anti-S100 rabbit serum
156 solution (Envision FLEX-S-100, Agilent Technologies, Santa Clara, CA), that was used in
157 zebrafish crypt OSNs as validated in Germanà et al. (2004), for 2 days at 4°C; 2) a mixture of
158 secondary antibodies, Alexa Fluor® 555-conjugated donkey anti-mouse IgG (A-31570,
159 Thermo Fisher Scientific; 1:500) and biotinylated goat anti-rabbit IgG (BA1000, VECTOR
160 Laboratories, Burlingame, CA, USA; 1:500) for 1 day at 4°C; 3) an Alexa Fluor® 488-
161 conjugated streptavidin (S11223, Thermo Fisher Scientific; 1:500) for 2 h at 28 °C. The
162 sections were rinsed triples for 5 min in PBST after each step of incubation, mounted in a
163 mounting medium (10% Mowiol 4-88, 25% glycerol, 0.2 M Tris-HCl (pH 9.0), and 2.5%–3.0%
164 1,4-diazabicyclo [2,2,2] octane; diluted with ultrapure water), and coverslipped.

165

166 Confocal microscopy

167 Optical sections of the immunohistochemistry and propidium iodide-stained olfactory organs
168 were acquired using a FV1000D IX81 confocal laser-scanning microscope (Olympus, Tokyo,
169 Japan). All microscopic images used in this study were processed to pseudocolor stacks and
170 were adjusted for brightness and contrast using Fiji (https://fiji.sc/). Some confocal microscopy
171 images were analyzed using FluoRender (http://www.sci.utah.edu/software/fluorender.html;

172 Wan et al., 2012) for three-dimensional (3D) reconstructions and visualization.

173

174 **Results**

175 5,6,11-trideoxyTTX, an almost nontoxic TTX analog, attracts green spotted puffers.

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

191 Then, we next examined the changes in swimming behavior when 5,6,11-trideoxyTTX
192 (final concentration: 10^{-7} M) or its Vehicle (acetic acid in ABW; final concentration: $1.25 \times 10^{-}$
193 ^{7} N) was administered. Before Vehicle or 5,6,11-trideoxyTTX administration (-4 min, -2 min),
194 green spotted puffers swam around the peripherally and did not invade the central area of the
195 test aquarium (Fig. 2C and D, respectively). However, after the 5,6,11-trideoxyTTX
196 administrations (+2 min, +4 min), the fish actively swam in the central area (Fig. 2D, Movie
197 S3). In contrast, the fish remained swimming peripherally after the Vehicle administrations and
198 rarely invaded the central area (Fig. 2C). The cumulative spending time in the central area (1
199 min period) were calculated, and their time changes are plotted in Fig. 3B. Cumulative
200 spending time increased after the 5,6,11-trideoxyTTX administration. The mean cumulative
201 spending time of the 5,6,11-trideoxyTTX group (25.8 ± 13.0 s, n = 11) was longer than that of
202 the Vehicle group (9.4 ± 7.8 s, n = 12) at the end of the experimental period ($p = 0.003$; Fig.3B).

203 By contrast, when TTX was administered, the fish continued to swim around

204 peripherally and rarely invaded the central area (Fig. 2E, Movie S4). The cumulative spending
205 time in the central area was calculated, and their time changes are plotted in Fig. 3C. The mean
206 cumulative spending time in the TTX group (13.3 ± 8.7 s, n = 8) was longer than that in the
207 Vehicle group (9.4 ± 7.8 s, n = 12), but the Welch's *t*-test showed no significant difference
208 between the two groups (p = 0.345). These results suggested that 5,6,11-trideoxyTTX acts as
209 an olfactory chemoattractant in green spotted puffers as well as in grass puffers.

210

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

212 The olfactory organs of teleost have nasal cavities under the body surface, and their OE is
213 located on the floor of the nasal cavity (Olivares & Schmachtenberg, 2019). Green spotted
214 puffer also has olfactory organs, but their olfactory organs protrude from the dorsal head
215 surface with stalks and are exposed to the external environment (Fig. 4A, arrows). The stalk is
216 split into two fleshy flaps (Fig. 4B). The sensory epithelium (SE) is located on the inside (arrow
217 in Fig. 4B), and the non-sensory epithelium (NSE) is located on the outside of the flap
218 (arrowhead in Fig. 4B). Fig. 4C is a 3D reconstructed confocal microscopy image of the whole
219 mount OE stained with propidium iodide. The inside of the flap was shallowly curved, and SE
220 was surrounded and separated by the NSE at the edge of the flap. OSNs are densely distributed
221 throughout SE. The x-z confocal section shows the cross-sectional structure of SE (Fig. 4D).
222 Most OSNs in SE had spindle-shaped soma (Fig.4 D, arrow), although some large oval-shaped
223 OSNs (minor axis diameter = c.a. 10 μm) were scattered in SE (Fig. 4C and D, arrowheads).
224 To identify the 5,6,11-trideoxyTTX-sensitive OSN, we immunohistochemically labeled
225 odorant-activated OSNs using a pS6 antibody. Excised OE from green spotted puffer was
226 exposed to Vehicle (ACSF), L-Arg, 5,6,11-trideoxyTTX, or TTX. L-Arg activated spindle-
227 shaped OSNs (Fig. 5B, arrow). The cilia-like structure was frequently observed on the L-Arg
228 activated OSNs (Fig. 5B inset, arrowhead). 5,6,11-trideoxyTTX activated large oval-shaped
229 OSNs without cilia (Fig. 5C, arrow). The apical ends of 5,6,11-trideoxyTTX-sensitive OSNs
230 were invaginated, and their cell bodies were located in the superficial layer of OE. TTX
231 administration weakly labeled tiny numbers of oval-shaped OSNs (Fig. 5D, arrow). However,
232 Vehicle-treatment did not activate any OSNs (Fig. 5A). Besides, a small number of 5,6,11-
233 trideoxyTTX -sensitive OSNs were scattered over the OE, some of which were present as cell
234 clusters (Fig. 5E, arrows).

235

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

237 5,6,11-trideoxyTTX-sensitive OSNs were large oval-shaped OSNs and scattered over the OE.
238 To identify the cellular subtype of 5,6,11-trideoxyTTX-sensitive OSNs, we performed double
239 immunohistochemistry of 5,6,11-trideoxyTTX-treated OE with a pS6 antibody and an S100
240 antibody. S100 protein is a specific marker for crypt OSNs (Germanà et al., 2004). The results
241 showed that both pS6 and S100 antibodies labeled oval-shaped OSNs with apical invagination.
242 (Fig. 6A and B). The nuclei of oval-shaped OSNs were large and located in the superficial layer
243 of OE (Fig. 6C, arrows). Merged confocal microscopy image indicated that 5,6,11-
244 trideoxyTTX-activated pS6 immunopositive OSNs are overlapped with S100 immunopositive
245 crypt OSNs (Fig. 6D, arrow). However, Vehicle-treatment did not activate any OSNs (Fig. 6E).

246

247 **Discussion**

248 We demonstrated that green spotted puffers are attracted to 5,6,11-trideoxyTTX, and crypt-type
249 OSNs detect 5,6,11-trideoxyTTX.

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

262 In teleosts, there are four types of OSNs intermingled in OE: Ciliated, microvillous,
263 crypt, and Kappe OSNs; the latter has hitherto not been described in teleost other than zebrafish
264 (Olivares and Schmachtenberg, 2019). These OSNs are different in morphology, location of
265 the cell bodies in OE, and responsiveness to the odorants (Hamdani and Døving, 2007). Ciliated
266 OSNs are bipolar cells with a long and fine apical dendrite with a few cilia, and the cell bodies
267 are in the deeper layer of OE. Microvillous OSNs are also spindle-shaped bipolar cells with a

268 short and thick apical dendrite with microvilli, and their cell bodies are in the middle layer of
269 OE. Crypt OSNs are oval-shaped with few cilia and microvilli, and their cell bodies are in the
270 superficial layer of OE. The most striking feature of crypt OSNs is apical invaginations
271 (Hansen and Finger, 2000). Although ciliated OSNs and microvillous OSNs are numerously
272 distributed, crypt OSNs are few and scattered in OE.

273 5,6,11-trideoxyTTX-activated pS6-immunopositive OSNs were oval-shaped with
274 apical invagination, but L-Arg-activated pS6-immunopositive OSNs were bipolar cells with
275 cilia; thus, the shapes of each odorant-activated OSNs are different. Additionally, a small
276 number of 5,6,11-trideoxyTTX-activated pS6-immunopositive OSNs are scattered in OE.
277 These morphological and locational features suggest that 5,6,11-trideoxyTTX-activated pS6-
278 immunopositive OSNs are crypt OSNs, so we performed double immunohistochemistry for
279 pS6 and S100 on OE. Double immunohistochemistry demonstrated that 5,6,11-trideoxyTTX-
280 activated pS6-immunopositive OSNs were also S100-immunopositive and had an oval-shape
281 with apical invagination, which is a characteristic of crypt OSNs. These findings suggest that
282 5,6,11-trideoxyTTX-sensitive OSNs are crypt OSNs.

283 The functional role of crypt OSN remains unclear. Crypt OSNs respond to the major
284 histocompatibility complex (MHC) peptides in zebrafish (*Danio rerio*; Bienchl et al., 2016)
285 and amino acids in mackerel (*Trachurus symmetricus*; Vielma et al., 2008). Crypt OSNs of the
286 juvenile rainbow trout respond either to amino acids, bile salts, and reproductive pheromones,
287 whereas those of the mature rainbow trout respond to gonadal extracts and hormones from the
288 opposite sex (Bazaes & Schmachtenberg, 2012). The number and the density of crypt OSNs
289 are sex-specific; moreover, the number and location of crypt OSNs in OE varies throughout
290 the year (Bettini et al., 2012; Hamdani et al., 2008). The studies in crucian carp showed that
291 the axons of crypt OSNs extend to the ventral part of the olfactory bulb that extends their axons
292 to the lateral part of the medial olfactory tract (lMOT) (Hamdani & Døving, 2006), and
293 disconnection of lMOT reduces male reproductive behavior (Weltzien et al., 2003). Axons in
294 the lMOT projected to the ventromedial telencephalon and the preoptic area in goldfish (Oka
295 et al., 1982; von Bartheld et al., 1984). Moreover, electrical stimulations of the ventral
296 telencephalon and preoptic area elicit spawning acts in male landlocked sockeye salmon, and
297 lesions of the same areas impair courtship behavior in male goldfish (Koyama et al., 1984;
298 Satou et al., 1984). These findings suggest that 5,6,11-trideoxyTTX-evoked signals from crypt
299 OSNs may be involved in the reproduction of puffers.

300 5,6,11-trideoxyTTX has almost no toxicity and does not act as a defense substance

301 against predators because the 5,6,11-trideoxyTTX has almost no affinity to voltage-gated
302 sodium channels (Yotsu-Yamashita et al., 1999). Toxic puffers accumulate TTX and its analogs
303 in their bodies, but their amounts and proportions vary by species and organs. 5,6,11-
304 trideoxyTTX is especially accumulated in the ovary, but the ratio of the amount of TTX and
305 5,6,11-trideoxyTTX in the ovary is double in grass puffer (Jang et al., 2010). Green spotted
306 puffers have TTX and its analogs in their liver and gonads (Chulanetra et al., 2011), and the
307 amount of 5,6,11-trideoxyTTX in the whole body is half the amount of TTX (Jang et al., 2010).
308 Why are toxic puffers having such a harmless toxin?

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

324 The second hypothesis is that toxic puffers use 5,6,11-trideoxyTTX as an olfactory cue
325 to find TTX-bearing organisms for feeding. Recently, toxic puffer (*Takifugu pardalis*) eggs and
326 toxic flatworms (*Planocera multitentaculata*) DNA had been detected in the intestinal contents
327 of grass puffers, and grass puffers eat egg plates of toxic flatworm containing highly
328 concentrated TTX (Itoi et al., 2015; Okabe et al., 2019). In another toxic puffer (*Chelonodon*
329 *patoca*), DNAs from the toxic flatworm and goby (*Yongeichthys criniger*) were detected in
330 intestinal contents (Itoi et al., 2020). Furthermore, some toxic puffers secreted TTX from the
331 glands in their skin when they were stimulated (Kodama et al., 1986), and predators ingested
332 toxic puffer larva or their eggs but spat them out promptly (Itoi et al., 2014). These studies
333 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 flatworm (*Prosthiostomum trilineatum*; Suo et al.,2021) have 5,6,11-trideoxyTTX as well as TTX. Green spotted puffer 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 TTX-bearing prey organisms and effectively accumulate TTX and its analogs in their body.

This study demonstrated that the green spotted puffers are attracted to 5,6,11-trideoxyTTX, and 5,6,11-trideoxyTTX activates crypt OSNs, which has been reported to sense odor information about 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.

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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., H.A.; Resources: 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.

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512 Figure Legends

513

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

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

518

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

521 Swimming trajectories of green spotted puffers before and after the administration of
522 Vehicle (ABW; A), L-Arg (10^{-7} M; B), Vehicle (1.25×10^{-7} N acetic acids in ABW; C),
523 5,6,11-trideoxyTTX (10^{-7} M; D), and TTX (10^{-7} M; E). Each panel represents the swimming
524 trajectories of three fish in 2 min under each condition, and the time scale added on the top
525 is the elapsed time with the start of odorant administration as time 0. The outer circle
526 represents the outer edge of the test aquarium, and the inner circle represents the central area
527 within a 4 cm radius from the center of the aquarium. The swimming trajectories in the
528 central area are shown as the bold line, and the trajectories in the other area are shown as
529 gray lines.

530

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

532 A: Cumulative spending time of individual puffers in the central area of the test aquarium
533 (1 min period) before and after the administration of L-Arg (10^{-7} M; gray box; n=6) or
534 its Vehicle (ABW; open box; n=8).

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

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

540 All the X-axis represent the elapsed time with the start of the odor administration as 0.

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

543

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

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

546 bar: 2 mm.
547 B: Magnified view of the olfactory organ. Arrow: Sensory epithelium, Arrowhead: Non-
548 sensory epithelium. Scale bar: 0.5 mm.
549 C: Reconstructed confocal microscopy image of the whole mount olfactory organ stained
550 by propidium iodide. SE: sensory epithelium, NSE: Non-sensory epithelium. Scale bar:
551 20 μm.
552 D: x-z confocal section of the dashed rectangular region in (C). Arrowhead: Large oval-
553 shaped OSNs. Arrow: spindle-shaped OSNs. Scale bar: 10 μm.
554
555 Figure 5: **5,6,11-trideoxyTTX activates oval-shaped OSNs.**
556 A~D: pS6 immunohistochemistry of OE cross-sections exposed to Vehicle (ACSF; A), L-
557 Arg (10^{-6} M; B), 5,6,11-trideoxyTTX (10^{-6} M; C), or TTX (10^{-6} M; D). Arrow:
558 activated OSNs. Scale bar: 10 μm. Insets in B and C: Magnified view of the pS6-
559 immunopositive OSNs. Arrowhead: Cilia of activated OSN. Scale bar: 2.5 μm.
560 E: Lower magnification confocal microscopy image of pS6-immunopositive OSNs in
561 OE cross-section exposed to 5,6,11-trideoxyTTX. Arrow: 5,6,11-trideoxyTTX-
562 activated OSNs. Scale bar: 50 μm.
563
564 Figure 6: **5,6,11-trideoxyTTX activates crypt OSNs.**
565 A~C: Reconstructed confocal microscopy images of 5,6,11-trideoxyTTX-administered OE
566 displaying pS6-immunopositive OSNs (A) or S100-immunopositive OSNs (B) or
567 stained with DAPI (C).
568 D: A merged image of A ~ C (pS6: magenta, S100: green, DAPI: blue). Each
569 immunopositive OSNs had apical invagination, and their cell bodies were located
570 superficial layer in OE. Arrow: Nucleus of 5,6,11-trideoxyTTX-activated OSNs. Scale
571 bar: 10μm.
572 E: No pS6-immunopositive OSN was seen in Vehicle (ACSF)-treated OE. Scale bar:
573 10μm.
574
575 Movie S1: **Diffusion test of odorant solution.**
576 0.8% methylcellulose-ABW solution (1 mL) colored with methylene blue, which was used
577 to dissolve the odorant solution, was administered from the center of the test aquarium filled
578 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**.

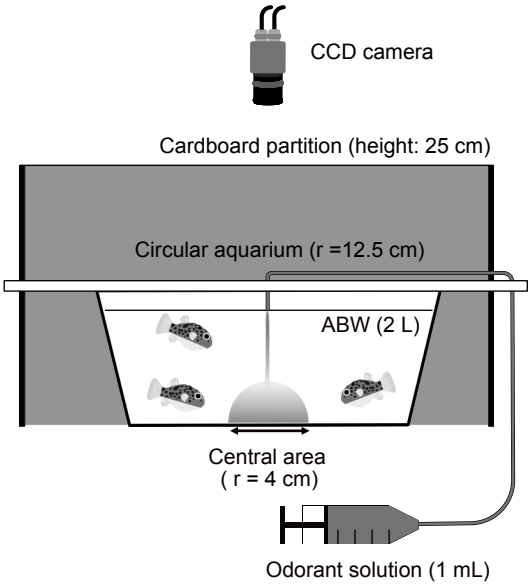
Vehicle or L-Arg solutions were administered from the center of the test aquarium at time 0 and remained in the aquarium. As a result, the fish frequently swam into the central area (purple circle) after L-Arg administration.

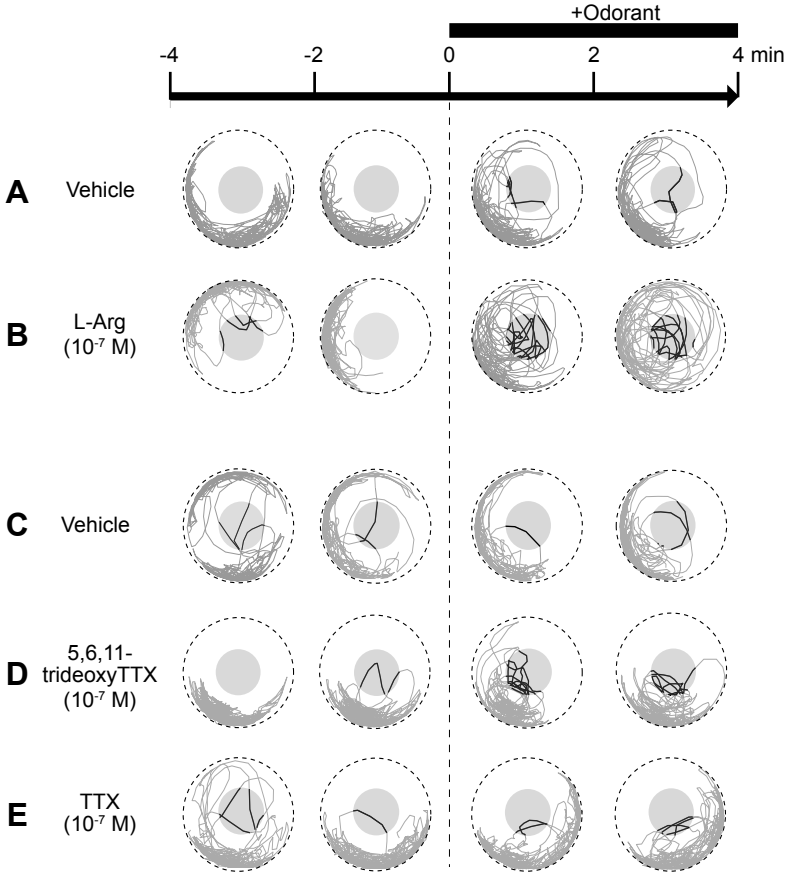
Movie S3: **Attractive swimming behavior of green spotted puffers toward 5,6,11-trideoxyTTX.**

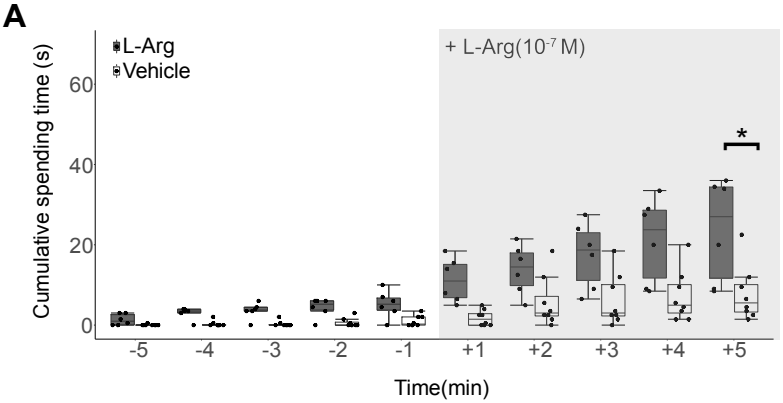
The 5,6,11-trideoxyTTX solution was added from the center of the test aquarium at time 0 and remained in the aquarium. As a result, the fish frequently swam into the central area (purple circle).

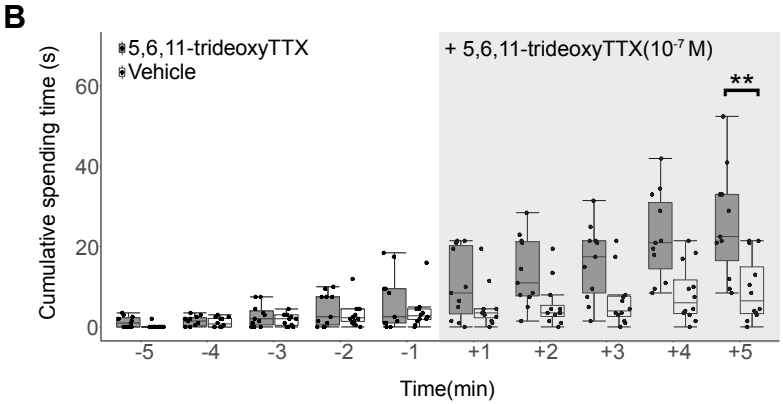
Movie S4: **No attractive swimming behavior of green spotted puffers toward TTX.**

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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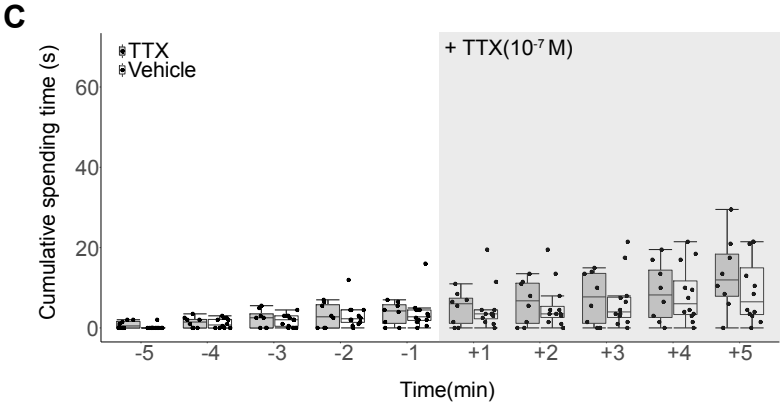
-5
-4
-3
-2
-1
+1
+2
+3
+4
+5
Time(min)


C
TTX
Vehicle
+ TTX(10-7 M)
Cumulative spending time (s)
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20
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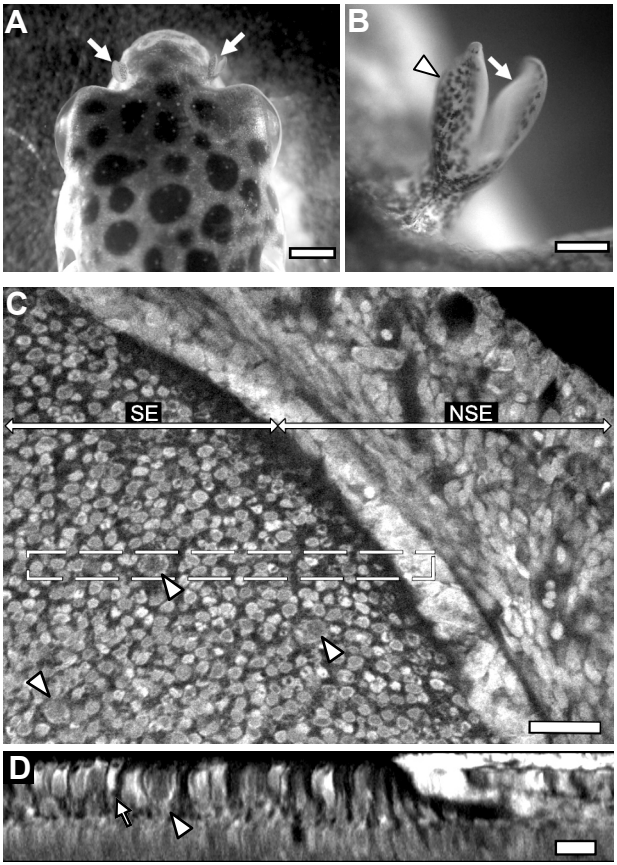
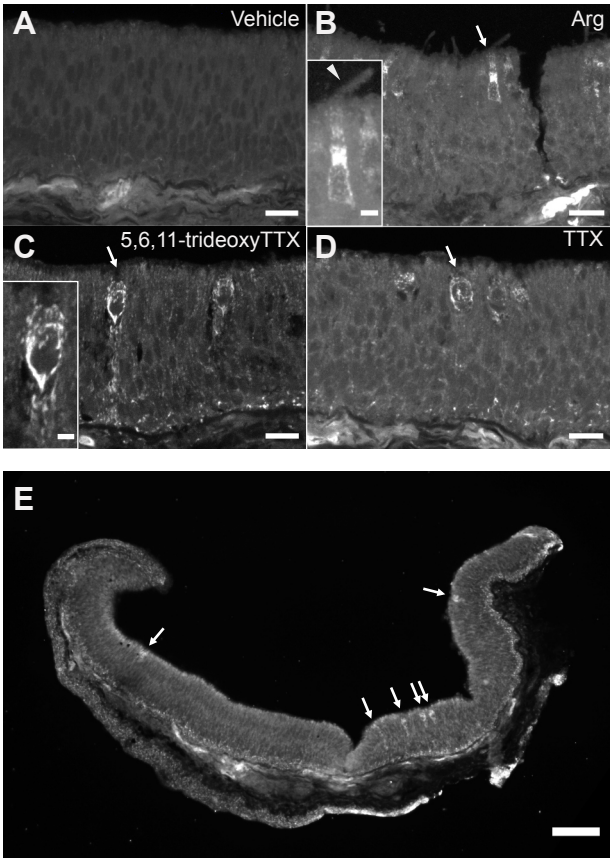
-3
-2
-1
+1
+2
+3
+4
+5
Time(min)


A
B
C
SE
NSE
D


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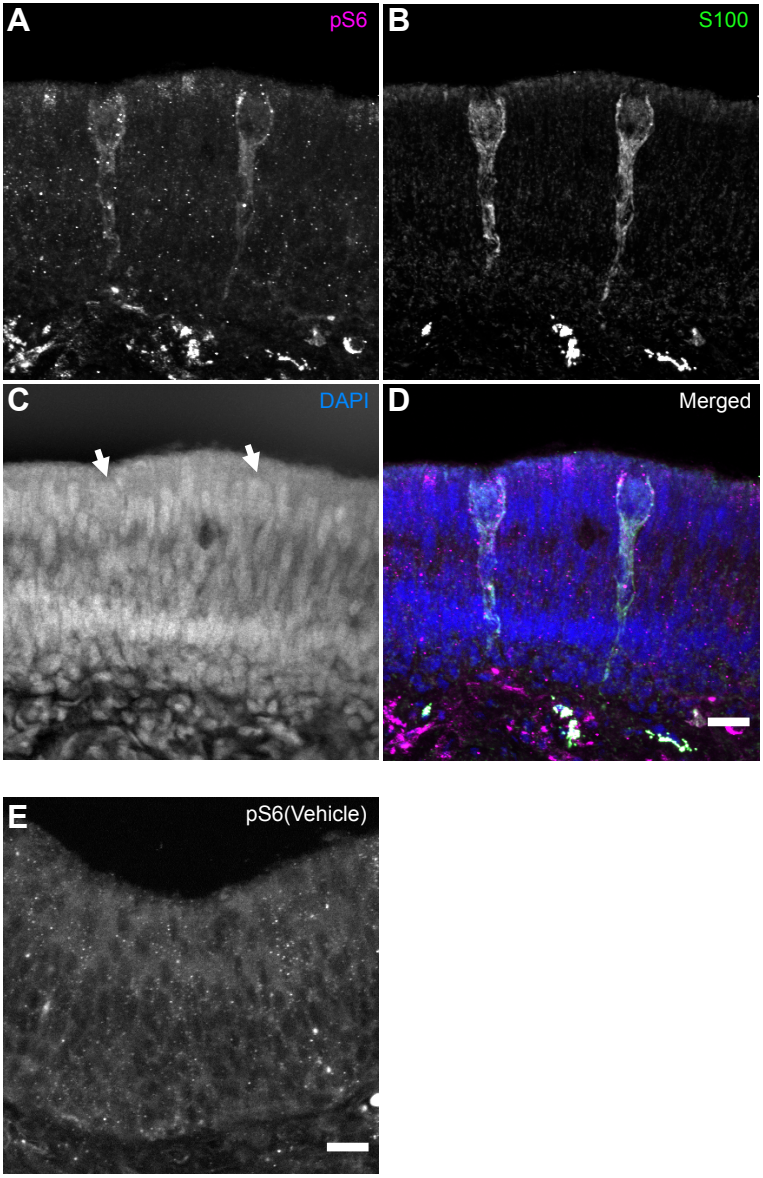
5,6,11-trideoxyTTX
D
TTX
E


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