Title: Almost nontoxic tetrodotoxin analog, 5,6,11-trideoxytetrodotoxin,
as an olfactory chemoattractant for the grass puffer

Running title: Grass puffer can smell TTX analog

Authors: Yoshihisa Noguchi¹, Takehisa Suzuki¹, Keigo Matsutani¹, Ryo Sakakibara², Ryota
Nakahigashi², Masaatsu Adachi^{2†}, Toshio Nishikawa², and Hideki Abe^{1*}

Affiliations

1. Laboratory of Fish Biology, Graduate School of Bioagricultural Sciences, Nagoya
University, Furo-Cho, Chikusa-Ku, Nagoya, Aichi 464-8601, Japan

2. Laboratory of Organic Chemistry, Graduate School of Bioagricultural Sciences, Nagoya
University, Furo-Cho, Chikusa-Ku, Nagoya, Aichi 464-8601, Japan

[†] Present address: Laboratory of Heterocyclic Chemistry, Graduate School of
Pharmaceutical Sciences, Tohoku University, 6-3 Aoba, Aramaki, Aoba-ku, Sendai
980-8578, Japan

Corresponding author's email address

habe@agr.nagoya-u.ac.jp (Hideki Abe)

Keywords

Tetrodotoxin (TTX), TTX analog, chemoattractant, olfactory sensory neurons, EOG

Summary statement:

Electroolfactogram, behavioral experiments, and activity-dependent labeling of olfactory
sensory neurons suggest that an almost nontoxic TTX analog, 5,6,11-trideoxyTTX, acts as an
olfactory chemoattractant for grass puffers.

Abstract (217 words; max 250 words)

Toxic puffers contain the potent neurotoxin, tetrodotoxin (TTX). Although TTX is considered to serve as a defense substance, previous behavioral studies have demonstrated that TTX (extracted from the ovary) acts as an attractive pheromone for some toxic puffers. To determine the putative pheromonal action of TTX, we examined whether grass puffers (*Takifugu alboplumbeus*) can detect TTX using electrophysiological, morphological, and behavioral experiments.

Electroolfactogram results suggest that the olfactory epithelium of grass puffers responded in a dose-dependent manner to a type of TTX analog (5,6,11-trideoxyTTX), although it did not respond to TTX. We also examined the attractive action of 5,6,11-trideoxyTTX on grass puffers by recording their swimming behavior under dark conditions. Grass puffers preferred to stay on the side of the aquarium where 5,6,11-trideoxyTTX was administered, and their swimming speed decreased. Additionally, odorant-induced labeling of olfactory sensory neurons using a fluorescent dextran conjugate or immunohistochemistry against phosphorylated extracellular signal regulated kinase (pERK) revealed that labeled olfactory sensory neurons were localized in the region surrounding “islets” where there was abundant cilia on the olfactory lamella.

5,6,11-trideoxyTTX has been known to accumulate in grass puffers, but its toxicity is much lower (almost nontoxic) than TTX. Our results suggest that grass puffers can detect 5,6,11-trideoxyTTX using their nose and may positively use this functionally unknown TTX analog as an olfactory chemoattractant.

Introduction

Toxic puffers accumulate the potent neurotoxin, tetrodotoxin (TTX), which is a well-known antagonist of voltage-gated sodium channels. TTX was first believed to be present only in pufferfish from the family Tetraodontidae. However, it was later detected in taxonomically diverse animals (e.g., teleosts, amphibians, echinoderms, crustaceans, mollusks, and worms) inhabiting terrestrial, marine, freshwater, and brackish water environments (Noguchi and Arakawa, 2008).

Regardless of the abundant studies, the biological origin of TTX and its ecological functions remain unclear. However, the presence of TTX in such a variety of organisms suggests that the ultimate origin of TTX is exogenous or that it is symbiotically produced by marine bacteria (Magarlamov et al., 2017; Noguchi et al., 2006a). TTX and its analogs were detected in potential pufferfish prey, including starfish, gastropods, crustaceans, flatworms, and ribbonworms, in addition to several species of bacteria that are free-living or symbiotic with pufferfish or their potential food organisms (Noguchi and Arakawa, 2008). Several shell fragments of the TTX-bearing gastropod *Zeuxis siquijorensis* were detected in the digestive tract of the toxic puffer *Takifugu pardalis* (Noguchi et al., 2011). Additionally, juveniles of the cultivated tiger puffer *T. rubripes* became nontoxic in aquaria or cages that were suspended above the seafloor with a TTX-free diet (Honda et al., 2005; Noguchi et al., 2006b). These results suggest that TTX in pufferfish is a result of its accumulation through the food chain.

Why do toxic puffers accumulate TTX in their bodies? Some toxic puffers have TTX-secreting glands on the skin (Kodama et al., 1986), and electrical stimulation induced TTX secretion from the body surface of these puffers (Kodama et al., 1985). TTX induces repulsive or avoidance reactions in predatory fish (Sakakura et al., 2017); predators that ingested *Takifugu* larvae spat them out promptly (Itoi et al., 2014, 2018a). Thus, in toxic puffers, TTX functions as a biological defense substance against predators.

Although the main function of TTX in pufferfish appears to be defensive, several studies have reported that pufferfish can exploit TTX as a source of communication. Sexually active male grass puffers (*T. alboplumbeus*) were attracted to TTX (Matsumura, 1995). Artificially raised nontoxic tiger puffer (*T. rubripes*) juveniles were also attracted to TTX (Saito et al. 1997; 2000; Okita et al. 2013). These studies suggest that pufferfish may sense TTX. However, the TTX-sensing mechanism in these puffers remains unclear, although the

attractive effect of TTX on *T. rubripes* juveniles disappeared after nose ablation (Okita et al., 2013).

In this study, we found that a TTX analog, 5,6,11-trideoxyTTX, is an olfactory chemoattractant for the grass puffer. We demonstrated the presence of the olfactory sensing ability, specific olfactory sensory neurons (OSNs), and the attraction behavior of grass puffers in response to 5,6,11-trideoxyTTX. To the best of our knowledge, this is the first report that pufferfish positively use functionally unknown TTX analogs for chemical communication.

Materials and Methods

Animals

One hundred fifty-nine grass puffers (body length, 5–12 cm; bodyweight, 6–32 g) were collected from the coastal waters of Ise-bay in Minamichita-Cho on the Pacific coast of central Japan by line-fishing. Grass puffers were fed commercial diets (Tetra Krill-E; Spectrum Brands, Madison, WI, USA) in aerated 300 L tanks (10–25°C, approximately 50 fish per tank) filled with artificial seawater (ASW; Instant Oceans® Sea Salt, Cl⁻ 543 mM, Na⁺ 471 mM, Mg²⁺ 54.3 mM, K⁺ 10.7 mM, Ca²⁺ 10 mM Br⁻ 0.7 mM, Sr²⁺ 0.1 mM, F⁻ 0.05 mM, I⁻ 0.002 mM, CO₃²⁻/HCO₃⁻ 3.3 mM, pH 8.2; Instant Ocean, Blacksburg, VA, USA) under a 12-h light/12-h dark photoperiod. In this study, both males and females were used without distinction. All animals were maintained and used in accordance with the Nagoya university regulations on animal care and use in research.

Odorant chemicals

TTX (for biochemistry, citrate buffered, cat. No. 206–11071, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in ultra-pure water at 1.5×10^{-3} M. Two types of TTX analogs, 4,9-anhydroTTX and 5,6,11-trideoxyTTX, were synthesized and purified according to the methods described by Adachi et al. (2014a) and Adachi et al. (2014b), respectively. Synthesized TTX analogs were dissolved in 2.5×10^{-2} N acetic acid at 10^{-3} M and stored at –30°C until use. L-Arginine (Arg; a food odor cue for teleosts; FUJIFILM Wako) was prepared as a 10^{-2} M stock in artificial cerebrospinal fluid (ACSF; NaCl 231 mM, KCl 8.1 mM, MgCl₂ 1.3 mM, CaCl₂ 2.4 mM, D-glucose 10 mM, 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM; pH adjusted 7.4 with NaOH). For testing, test odorant solutions were prepared fresh as required by diluting aliquots of concentrated stock in ACSF or in ASW. L-Arginine was always tested at 10^{-5} M. When testing a series of solutions with different concentrations, the lowest dose was tested first, followed by solutions with concentrations that increased by a factor of 10 to 10^{-4} M. A vehicle solution was prepared in an identical manner by diluting 2.5×10^{-2} N acetic acid in ACSF or ASW (final concentration, 2.5×10^{-4} N).

Electroolfactogram recordings

Electroolfactogram (EOG) recordings were performed on secured grass puffers on a holding apparatus that was placed into a plastic experimental trough. Before recording, the fish was anesthetized by immersion in tricaine methanesulfonate (MS-222; 0.02%). The anesthetized grass puffer was mounted on the center groove of a hand-made plastic holder by wrapping it in a paper towel and aluminum foil after attaching an Ag–AgCl plate (1×1 cm) with the body wall as a reference electrode. The gills were perfused with ASW containing 0.01% MS-222 through a tube inserted into the mouth throughout the experiments (Flow rate: 2.5 mL/min). The skin and cartilage covering the right olfactory cavity were opened, and the exposed olfactory cavity was continuously perfused with ACSF using an 18 G stainless needle (NN-1838S; Terumo, Tokyo, Japan). The perfusion rate of ACSF was 1.0 mL/min. A computer-controlled solenoid valve (UMG1-T1; CKD, Komaki, Japan) delivered 10-s pulses of test solutions by switching between the ACSF and test solutions (diluted appropriately in ACSF) at 3-min intervals.

EOG responses were recorded using a saline–agar (0.6%)-filled glass capillary (tip diameter, 100 μ m) that was bridged with an Ag–AgCl wire filled with ACSF. The indifference electrode was placed lightly on the head skin surface near the perfused olfactory cavity, and the recording electrode was positioned over the olfactory rosette using micromanipulators (M-152 and MM-3; Narishige, Tokyo, Japan). The electrodes were insulated with a paraffin–Vaseline paste that was placed on the surface of the fish's head. Electrical signals were differentially amplified (gain 1,000 $\times$; Band-pass 0.8–100 Hz) using a DC preamplifier (AVH-11 in VC-11 digital oscilloscope; Nihon-Kohden, Tokyo, Japan) and notch filtered through a 60 Hz noise eliminator (Humbug; Quest Scientific, North Vancouver, Canada). The amplified EOG signal was digitized (10 kHz) and stored on a laptop computer

(ThinkPad T61; Lenovo, Beijing, China) using a USB-6251 data acquisition device (National Instruments, Austin, TX, USA) and WinEDR V3.2.7 software (http://spider.science.strath.ac.uk/sipbs/software_ses.htm; Strathclyde University, Glasgow, UK). In this study, we showed negative potential changes in the EOG as upward deflections.

Behavioral experiments

Behavioral experiments were performed using adult male and female grass puffers. For the behavioral assay, a grass puffer was transferred to a test aquarium ($50 \times 20 \times 20$ cm) that was filled with 5 L of fresh artificial seawater. The test aquarium was covered with a black curtain and illuminated by an infrared LED-array illuminator (940 nm, ca. 2 W; AE-LED56V2, Akizuki Denshi Tsusho Co., Tokyo, Japan) so that the experimenter was invisible to the fish. A video 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. The fish was allowed to acclimate to a dark environment for 90–180 min.

Time-lapse image acquisitions from the top of the test aquarium were started (1 s intervals). The time-lapse images were acquired on a laptop computer (ThinkPad T410; Lenovo, Beijing, China) using image acquisition software (Micro-Manager 1.4.22; <https://micro-manager.org>) and a USB-video capture unit (PCA-DAV, Princeton, Tokyo, Japan) at a resolution of 640×480 pixels. After 6 min of image acquisition, 5 mL of 10^{-5} M 5,6,11-trideoxyTTX or vehicle (5 mL of 2.5×10^{-4} N acetic acid) was gently applied to one side of the test aquarium through a silicone tube attached to a 10 mL syringe. Then, image acquisitions were continued for up to 6 min. The numeric x–y coordinates representing the movement of the fish over time (one coordinate in a frame) were extracted from each video using the UMA tracker software (<http://ymnk13.github.io/UMATracker/>; Yamanaka and Takeuchi, 2018). We first binarized the grayscale images using appropriate filters that were produced by the UMATracker. We then tracked the fish's position using the “Group tracker GMM” algorithm and exported the coordinates as a text file.

Activity-dependent labeling of ORNs

To study the ligand specificity of olfactory receptor neurons, we used odor-induced uptake of fluorescent dye-conjugated dextran (Døving et al., 2011). The fish was anesthetized using

Grass puffer can smell TTX analog

0.02% MS-222 and secured on the holding apparatus used in EOG experiments. The gills were continuously perfused with artificial seawater containing 0.01% MS-222 through a tube inserted into the mouth throughout the procedure. The skin and cartilage covering the olfactory cavity were opened, and the exposed olfactory cavity was continuously perfused with ACSF using an 18G stainless needle. The olfactory cavity of anesthetized fish was perfused with L-Arginine (Arg; 10^{-5} M) or 5,6,11-TrideoxyTTX (10^{-5} M) together with a dextran conjugated with Alexa Fluor® 555 (M.W. 10,000, 10^{-6} M; D34679, Thermo Fisher Scientific, MA, USA) for 5 min. In the control experiments, the olfactory cavity of anesthetized fish was exposed to only Alexa Fluor 555-dextran (10^{-6} M) for 5 min. The flow rate was approximately 1.0 mL/min. After the odorant application, the olfactory cavity was rinsed with a continuous flow of ACSF for at least 5 min. Fish were kept alive for 3 to 4 h in aerated artificial seawater at room temperature. Then, the fish was re-anesthetized using 0.1% MS-222 and decapitated. The decapitated head was immersed in 4% paraformaldehyde in 0.1 M PBS (pH 7.2, 4°C, 1 h). After fixation, the olfactory lamellae were dissected and rinsed with PBS. The olfactory lamellae were then counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1 ng/mL, 5 min) and mounted on a hole glass slide with mounting medium (10% Mowiol 4-88, 25% glycerol, 0.2 M Tris-HCl (pH 9.0), and 2.5%-3.0% 1,4-diazabicyclo [2,2,2] octane; diluted with ultrapure water).

For immunohistochemistry, we excised the olfactory lamellae from anesthetized fish. The exposed olfactory lamella was washed three times for 5 min in ACSF and stimulated with odorant solutions (vehicle, arginine, TTX, or 5,6,11-trideoxyTTX; 10^{-6} M, 1 mL, diluted with ACSF) for 5 min in a 1.5 mL tube. After odor stimulation, olfactory epithelium (OE) was fixed in 4% paraformaldehyde and 15% picric acid in 0.1 M PBS overnight at 4°C, rinsed in PBS, and cryoprotected in 30% sucrose in PBS overnight at 4°C. OE was embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan). Cross cryosections (14–20 μ m) of OE mount onto gelatin-coated slides and dried. After rehydration in PBS, the sections were blocked in 2% normal goat serum (NGS) in PBS with 0.05% Triton-X100 (PBST) for 3 h at 28°C.

The sections were then incubated with anti-phosphorylated-p44/42 mitogen-activated protein kinase (MAPK; extracellular regulated kinase, ERK1/2) rabbit monoclonal antibody (#4370, Cell Signaling Technology, Danvers, MA, USA; 1:500), which was used in zebrafish olfactory epithelium as validated in Biechl et al. (2016), as a neural activity marker that was diluted with 0.2% NGS in PBST for 2 days at 4°C. The sections were washed three times for

5 min with 0.2% NGS in PBST. Sections were then incubated with Alexa Fluor® 488-conjugated secondary antibody (goat anti-rabbit IgG (H+L); A-11008, Thermo Fisher Scientific, 1:500) that was diluted with 0.2% NGS in PBST for 1 day at 4°C. The sections were washed three times for 5 min in PBST. Finally, sections were counterstained with DAPI (1:1000) for 5 min and mounted using mounting medium.

A confocal microscope (FluoVIEW 1000D, IX81, Olympus, Tokyo, Japan) was used to observe the details of olfactory lamellae. Pictures were taken in planes and were separated by 0.45 µm z-axis steps. Confocal microscopy images were analyzed using FluoRender (<http://www.sci.utah.edu/software/fluorender.html>; Wan et al., 2012) for three-dimensional (3D) reconstruction and visualization of dextran-labeled cells. Pre-processing of images before 3D reconstruction (median filtering [radius: 1 pixel] and trimming) was performed by Fiji (<https://fiji.sc/>).

Data analysis

All animal experiments were performed on fish of both sexes. Statistical analyses were performed using Kyplot5 (Kyence, Tokyo, Japan) and Igor Pro 6 software (WaveMetrics Inc., Lake Oswego, OR, USA). Data are presented as a dot plot or a box-whisker plot (box: median and quartiles; whiskers: 10%–90% range) or mean ± standard error of the mean (SEM). The statistical test that was used in each experiment is shown in the corresponding figure legend. No statistical methods were used to predetermine the sample size because many of the outcomes were unknown.

Results

EOG response of grass puffers to TTX

We first performed EOG recordings of adult grass puffers in response to a commercially available TTX. The EOG response to a 10-s stimulus with an amino acid solution (10^{-5} M L-Arg) was a slow monophasic potential with a steep rising phase followed by an exponential decay toward the baseline (Fig. 1A, bottom trace). However, TTX (10^{-7} and 10^{-5} M) did not induce significant changes in the EOG (Fig. 1A upper and middle traces, respectively). The EOG amplitude of 10^{-7} M TTX (0.027 ± 0.007 mV, $n = 4$; Fig. 1B○) and 10^{-5} M TTX (0.026

± 0.007 mV, $n = 7$; Fig. 1B●) were significantly lower than that of 10^{-5} M L-Arg (0.074 ± 0.008 mV, $n = 11$; Fig. 1B■).

EOG response to TTX analogs

We then examined the EOG responses of the grass puffer to the two synthesized intrinsic TTX analogs. A TTX analog, 4,9-anhydroTTX, did not induce significant changes in EOG traces (10^{-5} M; Fig. 2A, second trace) when the same concentration of L-Arg showed clear EOG responses (Fig. 2A, bottom trace). However, the same concentration of another TTX analog, 5,6,11-trideoxyTTX, induced a clear increase in EOG response (Fig. 2A, third trace). Vehicle control (2.5×10^{-5} N acetic acid solution in ASW) did not induce any changes in EOG traces (Fig. 2A, top trace). EOG amplitude of 4,9-anhydroTTX, 5,6,11-trideoxyTTX, and L-Arg (all 10^{-5} M) was 0.027 ± 0.004 mV [$n = 4$], 0.197 ± 0.029 mV [$n = 7$], and 0.128 ± 0.017 mV [$n = 7$], respectively. EOG amplitudes of 5,6,11-trideoxyTTX and L-Arg were significantly larger than those of the vehicle (0.032 ± 0.007 mV [$n = 5$]; Fig.2B).

We also examined the dose–response relationship of the EOG response to 5,6,11-trideoxyTTX. Threshold EOG response concentration for 5,6,11-trideoxyTTX ranged between 10^{-7} and 10^{-8} M (Fig. 3A). The response amplitude increased with a logarithmic increase in the concentration, and no response saturation was observed over the concentration range tested (Fig. 3B). These results suggest that the olfactory epithelium of the grass puffers can detect 5,6,11-trideoxyTTX.

Behavioral experiments

We next examined the behavioral response of adult grass puffers to 5,6,11-trideoxyTTX. One mature grass puffer was put in a test aquarium filled with 5 L of fresh artificial seawater ($50 \times 20 \times 20$ cm) and acclimated to the fish for the test environment in the dark (>1 h). Then, 5 mL of Vehicle or 5,6,11-trideoxyTTX (10^{-5} M) was administered to one side of the aquarium (the final estimated concentration of 5,6,11-trideoxy TTX was 10^{-8} M). Before administration, the fish intermittently swam left and right and occasionally stopped in one place in the aquarium (Fig.4 and 5, A–C). Vehicle administration did not change the swimming patterns of the fish (Fig. 4D–F). This swimming pattern was reflected in the time vs. X-axis position plot (Fig. 4G) and the time vs. swimming velocity plot (Fig. 4H). Conversely, administration of 5,6,11-trideoxyTTX caused the fish to approach and frequently

stop on the administered side (Fig. 5D and E, Movie S1). Although the time vs. x-axis plot did not show an apparent change in the swimming pattern (Fig. 5G), 5,6,11-trideoxyTTX administration decreased the swimming velocity of the fish (Fig. 5H).

To quantify the degree of attraction, we calculated the time spent on the administered sides and the mean swimming velocity of the individual grass puffers for every 1 min period. Time spent by the grass puffers on the administered side was longer with the 5,6,11-trideoxyTTX treatment than with the vehicle treatment ($p < 0.01$, two-way ANOVA; Fig. 6A and B), and the swimming velocity of grass puffers was decreased by 5,6,11-trideoxyTTX compared to the vehicle treatment ($p < 0.01$, two-way ANOVA; Fig. 6C and D). These results suggest that 5,6,11-trideoxyTTX worked as a chemoattractant for grass puffers.

Activity-dependent labeling of 5,6,11-trideoxyTTX responding OSNs

To identify OSN that responded to 5,6,11-trideoxyTTX, we first observed the gross morphology of the olfactory epithelium from the grass puffer. Differential interference microscopy images of live olfactory lamellae showed that the surface of olfactory lamella had repeated structures in which the elliptical “islets” were bordered by oval cells and exposed on the surface (arrowheads in Fig. 7A). The cross-sectional structure of the olfactory lamellae showed that oval cells (arrowheads in Fig. 7B, C, and F) surrounded small islets where there were many cilia (SE in Fig. 7A and B).

Exposing the olfactory organ of the grass puffer to 5,6,11-trideoxyTTX (10^{-6} M) with 10^{-6} M of 10 kDa dextran conjugated with the fluorescent dye, Alexa Fluor 555, resulted in the labeling of several cells (arrows in Fig. 7G). The labeled cells were primarily found in the surrounding region of the islets (Fig. 7H, in the rectangular region). Application of Alexa Fluor 555-dextran without 5,6,11-trideoxyTTX did not lead to the labeling of any cells in the epithelium (Fig. 7D). Activity-dependent labeling of Alexa Fluor 555 was frequently seen as bright granular aggregations within the cell (Fig. 7I, J, and K). Additionally, an axon-like protrusion that extended from the basal membrane side of the labeled cell could be observed (arrow in Fig. 7K).

We also performed immunohistochemistry of OE with the pERK antibody as another activity-dependent labeling of 5,6,11-trideoxyTTX-sensitive OSNs. Excised OE from grass puffers was exposed to 5,6,11-trideoxyTTX (10^{-6} M), L-Arg (10^{-6} M), or vehicle for 1 min. Administration of 5,6,11-trideoxyTTX activated oval cells clustering on the surface of the

OE (Fig. 8A, Movie S2), whereas L-Arg administration labeled elongated cells with strongly labeled cilia whose somata were in the middle layer of the OEs (close to the basal membrane; Fig. 8B, Movie S3). Additionally, 5,6,11-trideoxyTTX-sensitive OSNs were localized in the NSE area of Fig. 7A. However, Vehicle administration did not activate any type of OSNs (Fig. 8C).

Discussions

In this paper, we electrophysiologically, morphologically, and behaviorally demonstrated that one of the TTX analogs, 5,6,11-trideoxyTTX, worked as an olfactory chemoattractant for grass puffers, *T. alboplumbeus*. To the best of our knowledge, this is the first report that shows that pufferfish positively use a functionally unknown, almost nontoxic TTX analog for chemical communications.

5,6,11-trideoxyTTX, but not TTX, is an odorant for grass puffers

We first examined whether commercially available TTX induces EOG responses in grass puffers. A previous behavioral study reported that TTX attracts male grass puffers during the spawning period (Matsumura 1995). TTX also attracted juvenile tiger puffers regardless of sex (Okita et al., 2013; Saito et al., 1997, 2000), and this attraction effect was blocked by nose ablation (Okita et al., 2013). However, TTX did not induce any EOG responses in our study. Previous studies used crude TTX that was extracted from the pufferfish ovary or liver. Okita et al. (2013) reported that they purified their crude TTX from the ovary of wild-caught adult tiger puffers, using the method of Ikeda et al. (2009). They reported that their crude TTX from pear puffer (*T. vermicularis*) ovaries contained both TTX and TTX analogs, including 5,6,11-trideoxyTTX, 4-epi TTX, and 4,9-anhydroTTX. We used a commercially available TTX in this study. The manufacturer's datasheet indicated that this TTX was also extracted from pufferfish ovary and liver, but the species were not specified. However, our electrospray ionization mass spectrometry analysis indicated that the commercially available TTX that we used did not contain detectable levels of these TTX analogs (except 4,9-anhydroTTX, a chemically equilibrium analog of TTX; data not shown). Thus, we hypothesized that the crude TTX extract from previous studies might contain TTX analogs, and some of these TTX analogs may act as olfactory chemoattractants for the pufferfish.

To investigate this hypothesis, we examined the effects of two types of synthesized TTX analogs (4,9-anhydroTTX and 5,6,11-trideoxyTTX) using EOG and a behavioral assay. Our EOG recordings showed that 5,6,11-trideoxyTTX induced an EOG response in grass puffers. In our recordings, the EOG amplitude of 10^{-5} M for 5,6,11-trideoxyTTX was significantly greater than that at the same concentration of L-Arg, which is a well-known odorant for many teleosts. Furthermore, 5,6,11-trideoxyTTX induced EOG responses that were lower than 10^{-8} M. Because the stimulation threshold of amino acids on the fish olfactory system is 10^{-9} M to 10^{-7} M (Hara, 1994), 5,6,11-trideoxyTTX is a potent, powerful odorant for grass puffers.

5,6,11-trideoxyTTX attracts grass puffers

We examined whether adult grass puffers are attracted to 5,6,11-trideoxyTTX. In response to 5,6,11-trideoxyTTX administration to one side of the aquarium, grass puffers were attracted to the administered side of the aquarium and showed the behavior of slowing down and swimming in that area. A preliminary test indicated that diffusion indicator dye reached half of the experimental aquarium within 2 min after administration and had spread throughout the aquarium at the end of the observation period. Thus, we estimated that the grass puffers could recognize 5,6,11-trideoxyTTX ranging 2×10^{-8} to 1×10^{-5} M within 2 minutes after administration. These estimated 5,6,11-trideoxyTTX concentrations correspond to the threshold concentration of the EOG response, although the concentration is much higher than those of the previous behavioral studies. Mature male grass puffers were attracted to 1.5×10^{-11} M TTX (Matsumura, 1995). Both male and female juvenile tiger puffers also could recognize 3.7×10^{-9} (Saito et al., 2000) and 2.6×10^{-8} M (Okita et al., 2013) of TTX, respectively. In these studies, they started their “choice” experiments after diffusion of TTX from agar or gelatin on one side of the Y-maze aquarium. These differences in experimental paradigms seem to contribute to the difference in the effective TTX concentration. Alternatively, TTX analogs other than 5,6,11-trideoxyTTX may also involve chemoreception, and multiple forms of TTX analogs other than TTX and 4,9-anhydroTTX may act synergistically. In olfaction, a combination of several compounds can be perceived as a specific odor, or a series of structurally similar compounds can be detected by a single olfactory receptor. Most pheromone specificity is achieved using a combination of compounds (Wyatt, 2014). For example, female sex pheromones in moths usually consist of multicomponent blends of five to six hydrocarbons including unbranched fatty acids,

alcohols, acetates, or aldehydes in particular combinations and ratios (de Bruyne and Baker, 2008). In goldfish and carp, a combination of species-specific metabolites of prostaglandin F₂ α (PGF₂ α), a well-known releaser pheromone that induces sexual behavior, strongly induces species-specific sexual behavioral responses (Lim and Sorensen, 2011). Thus, it is possible that other metabolites in pufferfish may play a synergistic action with 5,6,11-trideoxyTTX as a chemical signal to attract the opposite sex.

5,6,11-trideoxyTTX sensitive OSNs on the olfactory epithelium of grass puffers

The fluorescent dextran-labeled OSNs were sparsely distributed in the border area around the ciliary “islets” on the olfactory lamella of grass puffers by the co-administration of 5,6,11-trideoxyTTX. Comparative histological studies reported that the sensory epithelium of grass puffers was dispersed in many discrete islets on the surface of the olfactory lamella, and the border area of the sensory islet was classified as undifferentiated epithelium (Yamamoto and Ueda, 1979). However, our fluorescent dextran-labeling experiments suggested that oval cells that were activated by 5,6,11-trideoxyTTX were present in the border area around the sensory islets. These oval cells are also labeled with pERK antibody, a neuronal activity marker, after the exposure of 5,6,11-trideoxyTTX.

It is widely known that teleost fish are equipped with three types of OSNs that are intermingled in the OE but show distinct morphology (Bazáes et al., 2013). First, there are the ciliated cells with long dendrites and a few cilia, and second, there are microvillous cells with shorter dendrites than the ciliated cells, which have microvilli extending from their apical surface. The third type is the crypt cells. Crypt cells have an oval shape, are located close to the epithelial surface, and have few cilia and microvilli (Hansen and Eckart, 1998). Cells that were labeled with fluorescent dextran by 5,6,11-trideoxyTTX co-administration had no cilia or microvilli and were located on the epithelial surface around the islets of ciliated epithelium. Based on these morphological characteristics, the 5,6,11-trideoxyTTX-sensitive OSNs are presumed to be crypt or another unknown type of OSN.

Biological significance of 5,6,11-trideoxyTTX for grass puffers

On the basis of these experimental data, we concluded that adult grass puffers can detect 5,6,11-trideoxyTTX by olfaction. 5,6,11-trideoxyTTX has been reported to be present in the body of toxic pufferfish. The distribution of TTX and its analogs throughout the body of

Grass puffer can smell TTX analog

grass puffers was reported, and 5,6,11-trideoxyTTX accumulated mainly in the ovary (Jang et al., 2010). Remarkably, 5,6,11-trideoxyTTX accumulates more in the ovary than TTX. A receptor binding assay study of voltage-gated sodium channels in rat brain suspensions reported that the dissociation equilibrium constant of 5,6,11-trideoxyTTX (> 5000 nM) was extremely high compared with that of TTX (1.8 ± 0.1 nM; Yotsu-Yamashita et al., 1999). These studies suggest that 5,6,11-trideoxyTTX is almost nontoxic and that it does not act as a defense substance against predators. Kono et al. (2008) reported that the transformation from TTX to its deoxy analogs or oxidized analogs does not occur in the body of grass puffers. This result suggests that 5,6,11-trideoxy-TTX is a precursor to TTX, but it remains unclear whether 5,6,11-trideoxy-TTX is converted to TTX inside the toxic puffers or other organisms (Ueyama et al., 2018; Yotsu-Yamashita et al., 2013). Therefore, the biological significance of why toxic puffers accumulate such nontoxic TTX analogs remains unclear.

In the previous study, TTX was treated as an attractive sex pheromone for toxic puffers. Pheromones are defined as follows: “an odor or mixture of odorous substances, released by an individual (the sender) and evoking in conspecifics (the receivers) adaptive, specific, and species-typical response(s), the expression of which needs not require prior experience or learning” (Sorensen and Stacey, 2004) or “molecules that are evolved signals, in defined ratios in the case of multiple component pheromones, which are emitted by an individual and received by a second individual of the same species, in which they cause a specific reaction, for example, a stereotyped behavior or a developmental process” (Wyatt, 2014). In fish, 17,20 β -dihydroxy-4-pregnen-3-one and prostaglandin F₂ α (goldfish), a type of sex steroid that is found in ovarian cavity fluid and urine, and a bile acid that is released from gills, urine, and feces (jawless sea lamprey, rainbow trout, and lake char) have been reported to act as attractive sex pheromones (Sorensen and Stacey, 2004). TTX is secreted from the secretory glands on the skin of *T. pardalis*, *T. poecilonotus*, *T. vermicularis*, and grass puffers (Kodama et al., 1985) and is released from ovulated oocytes in the grass puffers (Matsumura 1995). Because 5,6,11-trideoxyTTX is particularly abundant in pufferfish ovaries (Puilingi et al., 2015), 5,6,11-trideoxyTTX may also be released from the ovarian luminal fluid during spawning periods.

These chemicals, known as fish pheromones, are synthesized by conspecific fish. However, current circumstantial evidence suggests that 5,6,11-trideoxyTTX, unlike other pheromones, is acquired from TTX-bearing organisms by eating them (Lago et al., 2015; Noguchi et al., 2011). In some animals, molecules made by other organisms are harvested

and used as pheromones. For example, male neotropical orchid bees can unsuccessfully display and attract females without a species-specific pheromone mix, such as limonenes, from the right kinds of orchid flowers (Zimmermann et al., 2009). Animals may also use molecules that are produced by bacteria as pheromones. Phase-change pheromones of the locust are guaiacol (2-methoxyphenol) and phenol, and they are produced by locust gut bacteria (Pener and Simpson, 2009).

Noguchi et al. (2006a) stated that wild toxic puffers tend to feed on benthic organisms, such as flatworms, starfish, small gastropods, and skeleton shrimp that are known to possess TTX. A recent study reported that a TTX-bearing planoceric flatworm was found in the gut content of grass puffers (Itoi et al., 2018b) and *Chelodactylus patoca* (Itoi et al., 2020). Furthermore, some carnivorous gastropods have 5,6,11-trideoxyTTX in addition to TTX (*Charonia lampas lampas*, Rodriguez et al., 2008; *Nassarius spp.*, Luo et al., 2012), and toxic snails (*Polinices didyma*, *Natica lineata*, *Natica vitellus*, *Zeuxis sufflatus*, *Niotha clathrata*, *Oliva miniacea*, *Oliva mustelina*, and *Oliva hirasei*) have been reported to be attracted to crude TTX extracts (Hwang et al., 2004). Additionally, grass puffers ingested toxic eggs of another pufferfish, *T. pardalis*, which contained 5,6,11-trideoxyTTX (Itoi et al., 2015). All these studies support that the pufferfish seems to be attracted by 5,6,11-trideoxyTTX and that it ingest TTX-bearing organisms as food, resulting in accumulating TTX as a possible defense mechanism and attractive pheromone.

In conclusion, the grass puffers seem to use 5,6,11-trideoxyTTX as an olfactory cue to capture TTX-bearing organisms and use the accumulated 5,6,11-trideoxyTTX as an attractive pheromone to attract other conspecific puffers. Thus, this study revealed that pufferfish might actively use functionally unknown TTX analogs for chemical communication. Further studies are needed to determine whether other TTX analogs can also be detected by olfaction and whether multiple TTX analogs synergistically induce such effects. However, we believe that our results provide a new perspective on the use of TTX by toxic puffers.

Acknowledgment

We would like to express our gratitude to Prof. Naoyuki Yamamoto for his advice during experimentation.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

Funding

This work was supported by Grant-in-Aids for Scientific Research (16K07435 and 19K06762 to H.A., 17K19195 to T.N. and H.A., and 17H06406 to T.N.).

References

- Adachi, M., Imazu, T., Sakakibara, R., Satake, Y., Isobe, M., and Nishikawa, T. (2014a). Total synthesis of Chiriquitoxin, an analogue of tetrodotoxin isolated from the skin of a dart frog. *Chem. – Eur. J.* **20**, 1247–1251. doi: 10.1002/chem.201304110
- Adachi, M., Sakakibara, R., Satake, Y., Isobe, M., and Nishikawa, T. (2014b). Synthesis of 5,6,11-trideoxytetrodotoxin. *Chem. Lett.* **43**, 1719–1721. doi: 10.1246/cl.140684
- Bazáes, A., Olivares, J., and Schmachtenberg, O. (2013). Properties, projections, and tuning of teleost olfactory receptor neurons. *J. Chem. Ecol.* **39**, 451–464. doi: 10.1007/s10886-013-0268-1
- Biechl, D., Tietje, K., Gerlach, G., and Wullmann, M.F. (2016) Crypt cells are involved in kin recognition in larval zebrafish. *Sci. Rep.* **6**, 24590. doi: 10.1038/srep24590.
- de Bruyne, M. and Baker, T. C. (2008) Odor detection in insects: volatile codes. *J. Chem. Ecol.* **34**, 882–897. doi: 10.1007/s10886-008-9485-4
- Døving, K., Hansson, K.-A., Backström, T., and Hamdani, H. (2011). Visualizing a set of olfactory sensory neurons responding to a bile salt. *J. Exp. Biol.* **214**, 80–87.

- doi: 10.1242/jeb.046607
- Hansen, A. and Eckart, Z.** (1998). The peripheral olfactory organ of the zebrafish, *Danio rerio*: An Ultrastructural Study. *Chem. Senses* **23**, 39–48.
doi: 10.1093/chemse/23.1.39
- Hara, T.** (1994). Olfaction and gustation in fish: an overview. *Acta Physiol Scand* **152**, 207–217. doi: 10.1111/j.1748-1716.1994.tb09800.x
- Honda, S., Arakawa, O., Takatani, T., Tachibana, K., Yagi, M., Tanigawa, A., and Noguchi, T.** (2005). Toxification of cultured puffer fish *Takifugu rubripes* by feeding on tetrodotoxin-containing diet. *Nippon Suisan Gakkaishi* **71**, 815–820.
doi:10.2331/suisan.71.815
- Hwang, P.-A., Noguchi, T., and Hwang, D.-F.** (2004). Neurotoxin tetrodotoxin as attractant for toxic snails. *Fish. Sci.* **70**, 1106–1112. doi: 10.1055/s-2005-917664
- Ikeda, K., Murakami, Y., Emoto, Y., Ngy, L., Taniyama, S., Yagi, M., Takatani, T., and Arakawa, O.** (2009). Transfer profile of intramuscularly administered tetrodotoxin to nontoxic cultured specimens of the pufferfish *Takifugu rubripes*. *Toxicon* **53**, 99–103.
doi: 10.1016/j.toxicon.2008.10.018
- Itoi, S., Kozaki, A., Komori, K., Tsunashima, T., Noguchi, S., Kawane, M., and Sugita, H.** (2015). Toxic *Takifugu pardalis* eggs found in *Takifugu niphobles* gut: Implications for TTX accumulation in the pufferfish. *Toxicon* **108**, 141–146.
doi: 10.1016/j.toxicon.2015.10.009
- Itoi, S., Sato, T., Takei, M., Yamada, R., Ogata, R., Oyama, H., Teranishi, S., Kishiki, A., Wada, T., Noguchi, K., et al.** (2020). The planoceric flatworm is a main supplier of toxin to tetrodotoxin-bearing fish juveniles. *Chemosphere* **249**, 126217.
doi: 10.1016/j.chemosphere.2020.126217
- Itoi, S., Suzuki, M., Asahina, K., Sawayama, E., Nishikubo, J., Oyama, H., Takei, M., Shiibashi, N., Takatani, T., Arakawa, O., et al.** (2018a). Role of maternal tetrodotoxin in survival of larval pufferfish. *Toxicon* **148**, 95–100.
doi: 10.1016/j.toxicon.2018.04.014
- Itoi, S., Ueda, H., Yamada, R., Takei, M., Sato, T., Oshikiri, S., Wajima, Y., Ogata, R., Oyama, H., Shitto, T. et al.** (2018b). Including planoceric flatworms in the diet effectively toxifies the pufferfish, *Takifugu niphobles*, *Sci. Rep.* **8**, 12302.

- 525 doi: 10.1038/s41598-018-30696-z
- 526 **Itoi, S., Yoshikawa, S., Asahina, K., Suzuki, M., Ishizuka, K., Takimoto, N., Mitsuoka,**
527 **R., Yokoyama, N., Detake, A., Takayanagi, C., et al. (2014).** Larval pufferfish
528 protected by maternal tetrodotoxin. *Toxicon* **78**, 35–40.
529 doi: 10.1016/j.toxicon.2013.11.003
- 530 **Jang, J.-H., Lee, J.-S., and Yotsu-Yamashita, M. (2010).** LC/MS Analysis of Tetrodotoxin
531 and Its Deoxy Analogs in the Marine Puffer Fish *Fugu niphobles* from the Southern
532 Coast of Korea, and in the Brackishwater Puffer Fishes *Tetraodon nigroviridis* and
533 *Tetraodon biocellatus* from Southeast Asia. *Mar. Drugs* **8**, 1049–1058.
534 doi: 10.3390/md8041049
- 535 **Kodama, M., Ogata, T., and Sato, S. (1985).** External secretion of tetrodotoxin from puffer
536 fishes stimulated by electric shock. *Mar. Biol.* **87**, 199–202.
537 doi: 10.1007/BF00539429
- 538 **Kodama, M., Sato, S., Ogata, T., Suzuki, Y., Kaneko, T., and Aida, K. (1986).**
539 Tetrodotoxin secreting glands in the skin of puffer fishes. *Toxicon* **24**, 819–829.
540 doi: 10.1016/0041-0101(86)90107-8
- 541 **Kono, M., Matsui, T., Furukawa, K., Takase, T., Yamamori, K., Kaneda, H., Aoki, D.,**
542 **Jang, J.-H., and Yotsu-Yamashita, M. (2008).** Examination of transformation
543 among tetrodotoxin and its analogs in the living cultured juvenile puffer fish,
544 kusafugu, *Fugu niphobles* by intramuscular administration. *Toxicon* **52**, 714–720.
545 doi: 10.1016/j.toxicon.2008.08.002
- 546 **Lago, J., Rodríguez, L., Blanco, L., Vieites, J., and Cabado, A. (2015).** Tetrodotoxin, an
547 extremely potent marine neurotoxin: Distribution, toxicity, origin and therapeutical
548 uses. *Mar. Drugs* **13**, 6384–6406. doi: 10.3390/md13106384
- 549 **Lim, H. and Sorensen, P. (2011).** Polar metabolites synergize the activity of prostaglandin
550 F2 α in a species-specific hormonal sex pheromone released by ovulated common
551 carp. *J. Chem. Ecol.* **37**, 695–704. doi: 10.1007/s10886-011-9976-6
- 552 **Luo, X., Yu, R., Wang, X.-J., and Zhou, M.-J. (2012).** Toxin composition and toxicity
553 dynamics of marine gastropod *Nassarius spp.* collected from Lianyungang, China.
554 *Food Addit. Contam. Part Chem. Anal. Control Expo. Risk Assess.* **29**, 117–27.
555 doi: 10.1080/19440049.2011.61506

- 556 **Magarlamov, T. Y., Melnikova, D. I., and Chernyshev, A. V. (2017).** Tetrodotoxin-
557 producing bacteria: detection, Distribution and migration of the toxin in aquatic
558 systems. *Toxins* **9**, 166. doi: 10.3390/toxins9050166
- 559 **Matsumura, K. (1995).** Tetrodotoxin as a pheromone. *Nature* **378**, 563–564.
560 doi: 10.1038/378563b0
- 561 **Narahashi, T. (2001).** Pharmacology of tetrodotoxin. *J Toxicol* **20**, 67–84.
- 562 **Noguchi, T. and Arakawa, O. (2008).** Tetrodotoxin – Distribution and accumulation in
563 aquatic organisms, and cases of human intoxication. *Mar. Drugs* **6**, 220–242.
564 doi: 10.3390/md20080011
- 565 **Noguchi, T., Arakawa, O., and Takatani, T. (2006a).** TTX accumulation in pufferfish.
566 *Comp. Biochem. Physiol. Part D Genomics Proteomics* **1**, 145–152.
567 doi: 10.1016/j.cbd.2005.10.006
- 568 **Noguchi, T., Arakawa, O., and Takatani, T. (2006b).** Toxicity of pufferfish *Takifugu*
569 *rubripes* cultured in netcages at sea or aquaria on land. *Comp. Biochem. Physiol. Part*
570 *D Genomics Proteomics* **1**, 153–157. doi: 10.1016/j.cbd.2005.11.003
- 571 **Noguchi, T., Onuki, K., and Arakawa, O. (2011).** Tetrodotoxin poisoning due to pufferfish
572 and gastropods, and their intoxication mechanism. *ISRN Toxicol.* **2011**, e276939.
573 doi: 10.5402/2011/276939
- 574 **Okita, K., Yamazaki, H., Sakiyama, K., Yamane, H., Niina, S., Takatani, T., Arakawa,**
575 **O., and Sakakura, Y. (2013).** Puffer smells tetrodotoxin. *Ichthyol. Res.* **60**, 386–389.
576 doi: 10.1007/s10228-013-0353-z
- 577 **Pener, M. P. and Simpson, S.J. (2009).** Locust phase polyphenism: An update. *In Advances*
578 *in Insect Physiology, edited by S. J. Simpson and M. P. Pener*, **36**, 1–272. Academic
579 Press. doi: 10.1016/S0065-2806(08)36001-9.
- 580 **Puilingi, C., Kudo, Y., Cho, Y., Konoki, K., and Yotsu-Yamashita, M. (2015).**
581 Tetrodotoxin and its analogues in the pufferfish *Arothron hispidus* and *A.*
582 *nigropunctatus* from the Solomon Islands: A comparison of their toxin profiles with
583 the same species from Okinawa, Japan. *Toxins* **7**, 3436–3454.
584 doi: 10.3390/toxins7093436
- 585 **Rodriguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., and Botana, L.**
586 **(2008).** First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet

- 587 shell *Charonia lampas lampas* in Europe. *Anal. Chem.* **80**, 5622–5629.
- 588 doi: 10.1021/ac800769e
- 589 **Saito, T., Kageyu, K., Goto, H., Murakami, K., and Noguchi, T.** (1997). Tetrodotoxin
- 590 attracts pufferfish (‘torafugu’, *Takifugu rubripes*). *Toxicon* **35**, 489.
- 591 doi: 10.1016/S0041-0101(97)84745-9
- 592 **Saito, T., Kageyu, K., Goto, H., Murakami, K., and Noguchi, T.** (2000). Tetrodotoxin
- 593 attracts pufferfish (“torafugu” *Takifugu rubripes*). *Bull. Inst. Ocean. Res. Dev. Tokai*
- 594 *Univ.* **21**, 93–96.
- 595 **Sakakura, Y., Takatani, T., Nakayasu, J., Yamazaki, H., and Sakiyama, K.** (2017).
- 596 Administration of tetrodotoxin protects artificially raised juvenile tiger puffer
- 597 *Takifugu rubripes* from predators. *Fish. Sci.* **83**, 191–197.
- 598 doi: 10.1007/s12562-016-1046-0
- 599 **Sorensen, P. and Stacey, N.** (2004). Brief review of fish pheromones and discussion of their
- 600 possible uses in the control of non-indigenous teleost fishes. *N. Z. J. Mar. Freshw.*
- 601 *Res.* **38**, 399–417. doi: 10.1080/00288330.2004.9517248
- 602 **Ueyama, N., Sugimoto, K., Kudo, Onodera, K., Cho, Y., Konoki, K., Nishikawa, T., and**
- 603 **Yotsu-Yamashita, M.** (2018). Spiro bicyclic guanidino compounds from pufferfish:
- 604 possible biosynthetic intermediates of tetrodotoxin in marine environments. *Chem. –*
- 605 *Eur. J.* **24**, 7250–7258. doi: 10.1002/chem.201801006
- 606 **Wan, Y., Otsuna, H., Chien, C.-B., and Hansen, C.** (2012). FluoRender: An application of
- 607 2D image space methods for 3D and 4D confocal microscopy data visualization in
- 608 neurobiology research. In *2012 IEEE Pacific Visualization Symposium*, pp. 201–208.
- 609 doi: 10.1109/pacificvis.2012.6183592
- 610 **Wyatt, T. D.** (2014). Animals in a chemical world In: *Wyatt, T. D., Pheromones and Animal*
- 611 *Behavior: Chemical Signals and Signatures 2nd ed.*, Cambridge University Press,
- 612 New York, pp.1–48.
- 613 **Yamamoto, M. and Ueda, K.** (1979). Comparative morphology of fish olfactory epithelium,
- 614 IX. Tetraodontiformes. *Zool. Mag.* **88**, 210–218.
- 615 **Yamanaka, O. and Takeuchi, R.** (2018). UMATracker: an intuitive image-based tracking
- 616 platform. *J. Exp. Biol.* **221**, jeb182469. doi: 10.1242/jeb.182469
- 617 **Yotsu-Yamashita, M., Sugimoto, A., Takai, A., and Yasumoto, T.** (1999). Effects of

Grass puffer can smell TTX analog

specific modifications of several hydroxyls of tetrodotoxin on its affinity to rat brain membrane. *J. Pharmacol. Exp. Ther.* **289**, 1688–1696.

Yotsu-Yamashita, M., Abe, Y., Kudo, Y., Ritson-Williams, R., Paul, V., Konoki, K., Cho, Y., Adachi, M., Imazu, T., Nishikawa, T., et al. (2013). First identification of 5,11-dideoxytetrodotoxin in marine animals, and characterization of major fragment ions of tetrodotoxin and its analogs by high resolution ESI-MS/MS. *Mar. Drugs* **11**, 2799–2813. doi: 10.3390/md11082799

Zimmermann, Y., Ramírez, S., and Eltz, T. (2009). Chemical niche differentiation among sympatric species of orchid bees. *Ecology* **90**, 2994–3008. doi: 10.1890/08-1858.1

Figure 1: Grass puffer did not respond to TTX.

A: EOG responses of the olfactory epithelium of *T. alboplumbeus* to TTX (10^{-7} M, upper trace; 10^{-5} M, middle trace) and L-Arg (10^{-5} M, under trace).

B: EOG amplitudes of TTX (10^{-7} M, n = 6, open circle; 10^{-5} M, n = 7, filled circle) and L-Arg (10^{-5} M, n = 11, filled square). Box plots show the median, quartiles (boxes), and 10%–90% range (whiskers), and dot plots show raw EOG amplitudes. Steel's multiple comparison test: ** $p < 0.01$.

Figure 2: 5,6,11-trideoxyTTX induced EOG response of the grass puffer.

A: EOG responses to Vehicle (top trace), 4,9-anhydroTTX (10^{-5} M, second trace), 5,6,11-trideoxyTTX (10^{-5} M, third trace), and L-Arg (10^{-5} M, bottom trace) from same grass puffer.

B: EOG amplitudes of Vehicle (n = 5, open circles), 4,9-anhydroTTX (n = 4, filled circles), 5,6,11-trideoxyTTX (n = 7, filled triangles), and L-Arg (n = 7, filled square). Box plots show the median, quartiles (boxes), and 10%–90% range (whiskers) of EOG amplitudes, and dot plots show the raw value of EOG amplitudes. Steel's multiple comparison test: * $p < 0.05$.

Figure 3: EOG response to 5,6,11-trideoxyTTX was dose-dependent.

A: Representative EOG responses by a grass puffer were evoked by increasing the concentration of 5,6,11-trideoxyTTX. The period of 5,6,11-trideoxyTTX administration is shown by the black bar.

B: Dose-response relationship of 5,6,11-trideoxyTTX obtained from n = 5–7 fish. Peak EOG responses are plotted as a function of the concentrations that were delivered. Black circles show the individual EOG amplitudes and open circles show the mean EOG amplitude at given concentrations. Error bars represent the standard error. The dotted line represents a Hill equation fitted to the data (EC_{50} : 9.62×10^{-6} M, Hill coefficient: 1.21).

Figure 4: Swimming activity of a grass puffer before and after Vehicle administration.

Tracked location of a grass puffer (2-min periods) before (A–C) and after Vehicle administration (D–F). Glid: 100 mm.

Positions of the fish are plotted along the horizontal axis of the aquarium every 1 s.

Vehicle (5 mL of 2.5×10^{-4} N acetic acid solution) was applied at time 0 and remained in the aquarium thereafter (H). Swimming velocity of the fish is presented in G (H).

Figure 5: Swimming activity of a grass puffer before and after the 5,6,11-trideoxyTTX administration.

Tracked location of a grass puffer (2-min periods) before (A–C) and after 5,6,11-trideoxyTTX application (D–F). Glid: 100 mm.

Positions of the fish are plotted along the horizontal axis of the aquarium every 1 s. 5,6,11-trideoxyTTX (5 mL of 10^{-5} M dissolved in 2.5×10^{-4} N acetic acid solution) was applied at time 0 and remained in the aquarium thereafter (G). Swimming velocity of the fish is presented in G (H).

Figure 6: 5,6,11-trideoxyTTX attracts grass puffers.

A and B: Changes in the time (1-min period) spent by grass puffers in response to an odorant administered on one side of the experimental aquarium (A: Vehicle, B: 5,6,11-trideoxyTTX). The dotted line represents the chance level (= No odorant preference). Open or hatched boxes in the plot represent the time periods of the odorant administrations. Dot plots show the value from individual fish, and box plots show the median, quartiles (boxes), and 10%–90% range (whiskers).

C and D: Changes in the swimming speed (mm/s) in response to Vehicle (C) or 5,6,11-trideoxyTTX (D). The results of the statistical analysis are shown in the text.

Figure 7: Activity-dependent fluorescent dextran uptake labeled 5,6,11-trideoxyTTX responding olfactory sensory neurons on the olfactory epithelium of the grass puffer.

Differential interference contrast photomicrographs of the surface (A) and the edge (B) of the olfactory lamella of grass puffer. Arrowheads represent the non-ciliated epithelium

Grass puffer can smell TTX analog

that surrounds the “islets” of the sensory epithelium (SE). Sensory cilia are visible on the surface of the sensory epithelium. Scale bar = 20 μ m.

Confocal microscopy images of the olfactory lamella after Vehicle administration (C–E). Nuclear staining using DAPI (C) visualized the cells surrounding the islets whose nuclei were located on the surface of the olfactory lamella. Co-administration of Alexa Fluor555-dextran and Vehicle did not label any cell-like structures (D). Superimposed image of C and D (E). The image is a z-projection of 41 pictures (z-step, 0.45 μ m).

Confocal microscopy image of the olfactory lamella after 5,6,11-trideoxyTTX administration (F–H). Nuclear staining using DAPI (F), Alexa Fluor555-dextran (G), and their composite image (H). Co-administration of Alexa Fluor555-dextran labeled the large oval cells that were located in the surrounding region of the islets (arrows in G). Scale bar = 20 μ m. The image is a z-projection of 28 pictures (z-step, 0.45 μ m).

Enlarged image of the rectangular region in H (I). Alexa Fluor 555 labeling was frequently seen as bright granular aggregations within the cell. Scale bar = 5 μ m.

Another z-projection image (17 planes with a z-step of 0.48 μ m) of Alexa Fluor555-dextran labeled cells by 5,6,11-trideoxyTTX administration (surface side view, J; and basal membrane side view, K). The arrow indicates an axon-like structure that extends from the Alexa Fluor555-dextran-labeled cell. Scale bar = 5 μ m.

Figure 8: Activity-dependent labeling by pERK antibody labeled oval-shaped cells on the surface of the olfactory epithelium.

pERK-immunopositive OSNs after exposure to 5,6,11-trideoxyTTX (A) or L-Arg (B). No labeled cells were observed with the Vehicle control (C). Scale bar: 5 μ m.

Movie S1: Attractive swimming behavior of grass puffers toward 5,6,11-trideoxyTTX observed under infrared illumination.

Vehicle or 5,6,11-trideoxyTTX solutions were added from the right side of the test aquarium 6 minutes after the start of image acquisitions.

Grass puffer can smell TTX analog

719 **Movie S2: Reconstructed 3D view of the 5,6,11-trideoxyTTX-sensitive OSNs on the**
 720 **surface of olfactory epithelium shown in Fig. 8A.**

721 pERK-immunopositive OSNs after exposure to 5,6,11-trideoxyTTX. The same confocal
 722 microcopy data as in Fig.8A was used. Scale bar: 10 μ m.

723

724 **Movie S3: Reconstructed 3D view of the L-Arg-sensitive OSNs in the middle layer of**
 725 **olfactory epithelium shown in Fig. 8B.**

726 pERK-immunopositive OSNs after exposure to L-Arg. The same confocal microcopy data as
 727 in Fig.8B was used. Scale bar: 10 μ m.

A

B

A

B

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8