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Larval pufferfish protected by maternal tetrodotoxin☆

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ABSTRACT

Marine pufferfish contain tetrodotoxin (TTX), an extremely potent neurotoxin. All species of the genus *Takifugu* accumulate TTX in the liver and ovaries, although the tissue(s) in which it is localized can differ among species. TTX is the major defense strategy the pufferfish appears to use against predators. TTX is also used as a male-attracting pheromone during spawning. Here we demonstrate an additional (and unexpected) use of maternal TTX in the early larval stages of the *Takifugu* pufferfish. Predation experiments demonstrated that juveniles of all the species of fish used as predators ingested pufferfish larvae, but spat them out promptly. Liquid Chromatography-Tandem Mass Spectrometry (LC-MSMS) analysis revealed that the pufferfish larvae contain a small quantity of TTX, which is not enough to be lethal to the predators. Immunohistochemical analysis with anti-TTX monoclonal antibody revealed that the TTX is primarily localized in the body surface of the larvae as a layer of protection. Our study showed the female parent of the *Takifugu* pufferfish vertically transfers TTX to the larvae through its accumulation in the ovaries, and subsequent localization on the body surface of the larvae.

1. Introduction

TTX, a specific blocker of voltage-gated sodium channels of excitable membranes of muscle and nerve tissues (Colquhon et al., 1972; Narahashi, 2001), and one of the most potent neurotoxins, was long believed to occur exclusively in pufferfish. Subsequently, it was detected in the eggs of the California newt *Taricha torosa* (Mosher et al., 1964) and discovered in other fish, such as gobies, and invertebrates including octopuses, crabs, shellfishes, flat worms and ribbon worms (Noguchi et al., 2006; Miyazawa and Noguchi, 2001). TTX is produced primarily by marine bacteria, and it appears that it finds its way into pufferfish through the food chain (Noguchi et al., 1986, 1987, 2006; Yasumoto et al., 1986; Narita et al., 1987; Simidu et al., 1987; Noguchi and Arakawa, 2008).

Tissue-specific distribution of the toxin in TTX-bearing pufferfish, mainly the genus *Takifugu*, has been widely investigated from the view point of food hygiene (Tani,

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1945; Kanoh, 1988; Fuchi et al., 1991; Khora et al., 1991), revealing that while TTX is commonly distributed in the liver and ovaries, the localization in other tissues is species-specific (Noguchi et al., 2006; Noguchi and Arakawa, 2008). For example, while TTX was detected only in the intestine besides the liver and ovaries in *Takifugu rubripes*, it was found to be concentrated in the skin and intestine and marginally present in the testes and skeletal muscle in *Takifugu niphobles* (Noguchi et al., 2006; Noguchi and Arakawa, 2008). Previously, we demonstrated that tissue-specific distribution and the amount of TTX in the mature pufferfish *T. niphobles* were sex-dependent; female gonads and male liver showed the highest concentrations of the toxin followed by male skin (Itoi et al., 2012). Species, sex, and tissue specific differences in the distribution and concentration of TTX render unclear the exact function of the toxin in pufferfish, although it has been suggested that TTX may function as a chemical defense against predators (Fuhrman, 1986; Kodama et al., 1985) and as pheromone during spawning (Matsumura, 1995). In this study, we conducted predation experiments, measurement, and immunohistochemical analysis to elucidate the effect of TTX as a chemical defense in pufferfish larvae.

2. Materials and methods

2.1. Pufferfish eggs and larvae

Adult *T. rubripes* females captured from Ise Bay (Supplementary data, Fig. S1) and adult males from Enshu-

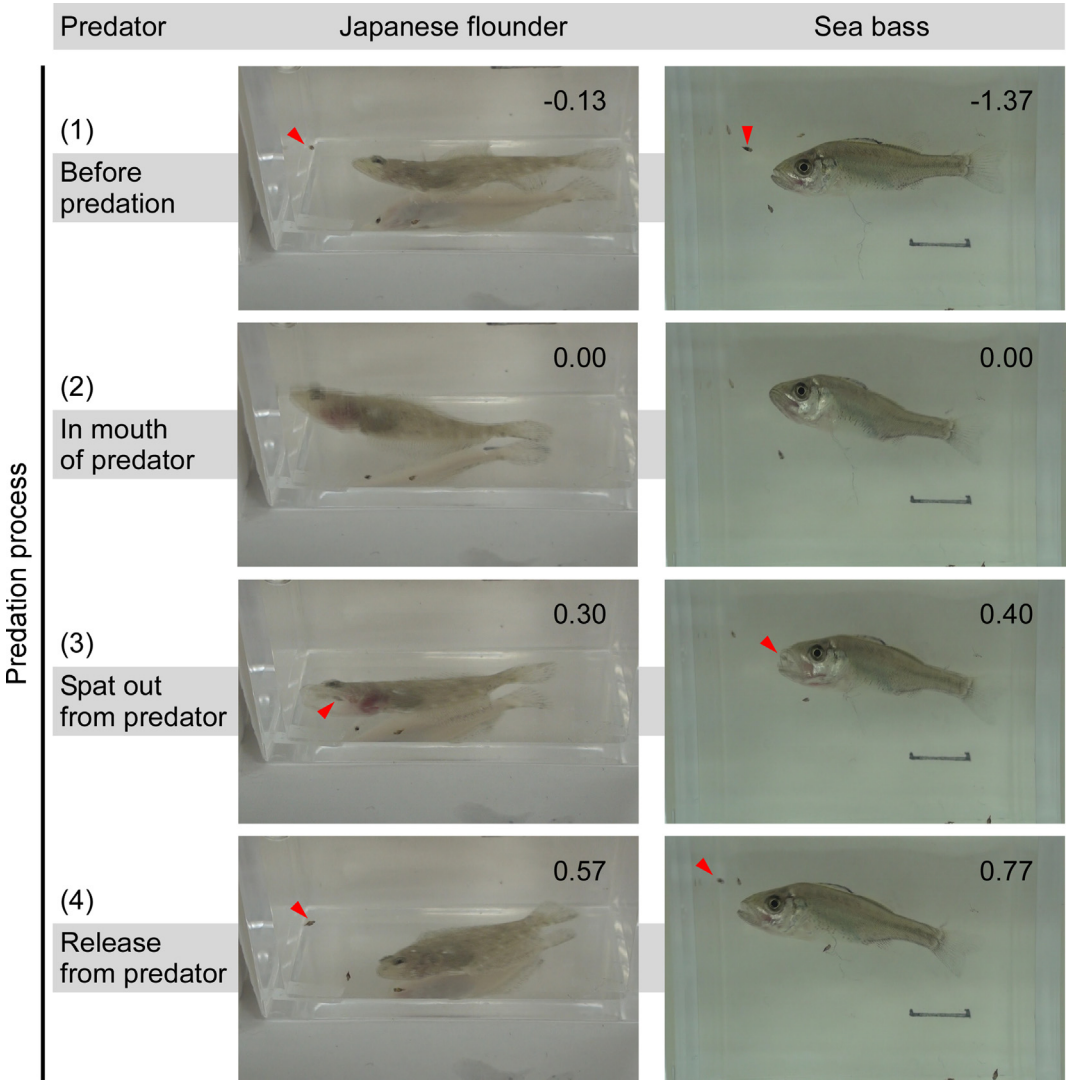

Fig. 1. Predation experiment using juvenile Japanese flounder and sea bass as predators and *T. rubripes* larvae as prey. The pictures represent, in order, the succession of events that lead to the rejection of the prey by the predators. (1): before ingestion of the prey; (2): *T. rubripes* larva (prey) just ingested by the predator; (3) and (4): an ingested prey larva has been spat out by a predator (indicated by the red arrow). Numerals in the panel indicate the time lapsed (in seconds) in the process of predation, with the clock at 0.00 s when the larva has just been ingested. The videos of the process are provided as Supplementary data: Videos S1 and S2 for *P. olivaceus* and *Lateolabrax* sp., respectively.

Nada Sea (Supplementary data, Fig. S1) were artificially bred, and the larvae subsequently grown in an aquaculture pond at Department of Sea-Farming, Aichi Fish Farming Institute. Fertilized *T. rubripes* eggs from wild specimens were also purchased from Marinetech (Aichi, Japan), and were hatched and grown in the aquarium at Department of Marine Science and Resources, Nihon University.

Fertilized eggs of *T. niphobles* were collected from the coastal waters off Enoshima Island (35°17′N, 139°28′E) in the summer months (May–July) of 2009–2013, and the larvae subsequently grown in an aquarium at Department of Marine Science and Resources, Nihon University.

2.2. Observation of predation behavior

Predation behavior was observed using *T. rubripes* larvae of 0–4 days post-hatch (dph) as the prey and several predator species in small aquaria and beakers. Juveniles of Japanese flounder *Paralichthys olivaceus* and sea bass *Lateolabrax* sp. purchased from Marinetech (Aichi, Japan) and Marua Suisan (Ehime, Japan), respectively, were used as the predatory fish against *T. rubripes* larvae (Supplementary data, Table S1). Juveniles of *Parablennius yatabei*, *Girella punctata*, *Chaenogobius annularis*, *Hypodytes rubripinnis*, *Omobranchus elegans* and *Tridentiger trigonocephalus* were collected from tidal pools in Enoshima Island (35°17′N, 139°28′E), and used as the predators against *T. niphobles* larvae (Supplementary data, Table S1). Juveniles of *G. punctata* were collected from tidal pools in Tanoura inlet (34°39′N, 138°58′E), and used as predators against *T. niphobles* eggs (Supplementarydata, Table S1). Adult *Artemia* and medaka larvae cultured in laboratory aquariums were used as negative control for the prey.

2.3. TTX quantification

Approximately 60–100 specimens of pufferfish larvae were pooled and the TTX was extracted from specimens with 0.1% acetic acid. Referring to a protocol (Shinno et al., 2007), quantification of TTX was performed using a Quattro Premier XE (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source coupled to an Acquity UPLC system (Waters). Chromatographic separation was achieved using an Atlantis HILIC Silica column (2.1 × 150 mm, 5 μm; Waters), coupled to an Atlantis HILIC Silica pre-column (2.1 × 10 mm, 5 μm; Waters). The mass spectrometer was operated in MRM, detecting in positive mode, analyzing two product ions at *m*/*z* 162 for quantification of TTX and *m*/*z* 302 for confirmation of the compound from the precursor ion at *m*/*z* 320.

2.4. Immunohistochemistry

Whole pufferfish larvae were fixed in 4% paraformaldehyde and embedded in paraffin, followed by sectioning, as described previously (Itoi et al., 2012). Sections were incubated with anti-TTX monoclonal antibody (Mouse IgG2a-κ, Nacalai Tesque inc., Kyoto, Japan), followed by reaction with fluorescent labeled secondary antibody (goat anti-mouse IgG2a (γ2a), Invitrogen, OR, USA). Observation of immunoreactivity image stitching was done with a BZ-9000 HS all-in-one fluorescence microscope (Keyence, Osaka, Japan). Sections were also treated with mouse IgG as negative controls instead of the primary antibody.

Table 1
Comparative predation levels against pufferfish larvae, eggs and non-toxic prey.[a]

Predator species	*n*	Percentage of prey surviving				
		TTX-bearing fish			Non-toxic organism	
		T. rubripes	*T. niphobles*		Larval medaka	Adult artemia
		Larva	Egg	Larva		
P. olivaceus[b]	25	100	–[c]	–	–	–
Lateolabrax sp.[b]	45	100	–	–	–	–
P. yatabei	5	–	–	100	0	0
G. punctata	6	–	–	100	0	0
C. annularis	6	–	–	100	0	0
G. punctata	14	–	100	–	–	–

[a] All data of the predation experiments are available in Supplementary data Table S1.
[b] Cultured specimens.
[c] Not applied.

2.5. Statistics

Difference in responses of predators (expelling vs swallowing) to TTX-bearing fish (pufferfish) and to non-toxic organisms (medaka and *Artemia*) was tested by the Pearson's Chi-square test with Yates' continuity correction using the statistical program called R, and the significant difference between groups was found (df = 1, chi-square = 110.0298, $P < 0.0001$). The data of *H. rubripinnis*, *O. elegans* and *T. trigonocephalus* used as predator species were removed from statistical analysis, because a specimen was collected in each species.

3. Results and discussion

In the predation experiments 0–4 dph *T. rubripes* larvae were used as the prey and juvenile Japanese flounder *P. olivaceus* and sea bass *Lateolabrax* sp. as predators. Both species of predators ingested the pufferfish larvae but spat them out immediately (Fig. 1, Table 1; Supplementary data, Table S1). A similar behavior was observed when *T. niphobles* larvae (0–10 dph) and fertilized eggs were used as prey

Table 2
Quantitative data of the TTX assays of the pufferfish larvae and eggs, and non-toxic organisms used in the predation experiments.

Sample	TTX amount (ng/individual)	TTX concentration (ng/g)
TTX-bearing fish		
T. rubripes larva	0.096	114
T. niphobles larva	0.107	471
T. niphobles egg	1.604	5475
Non-toxic organism		
Larval medaka	nd[a]	nd
Adult artemia	nd	nd

[a] Not detected.

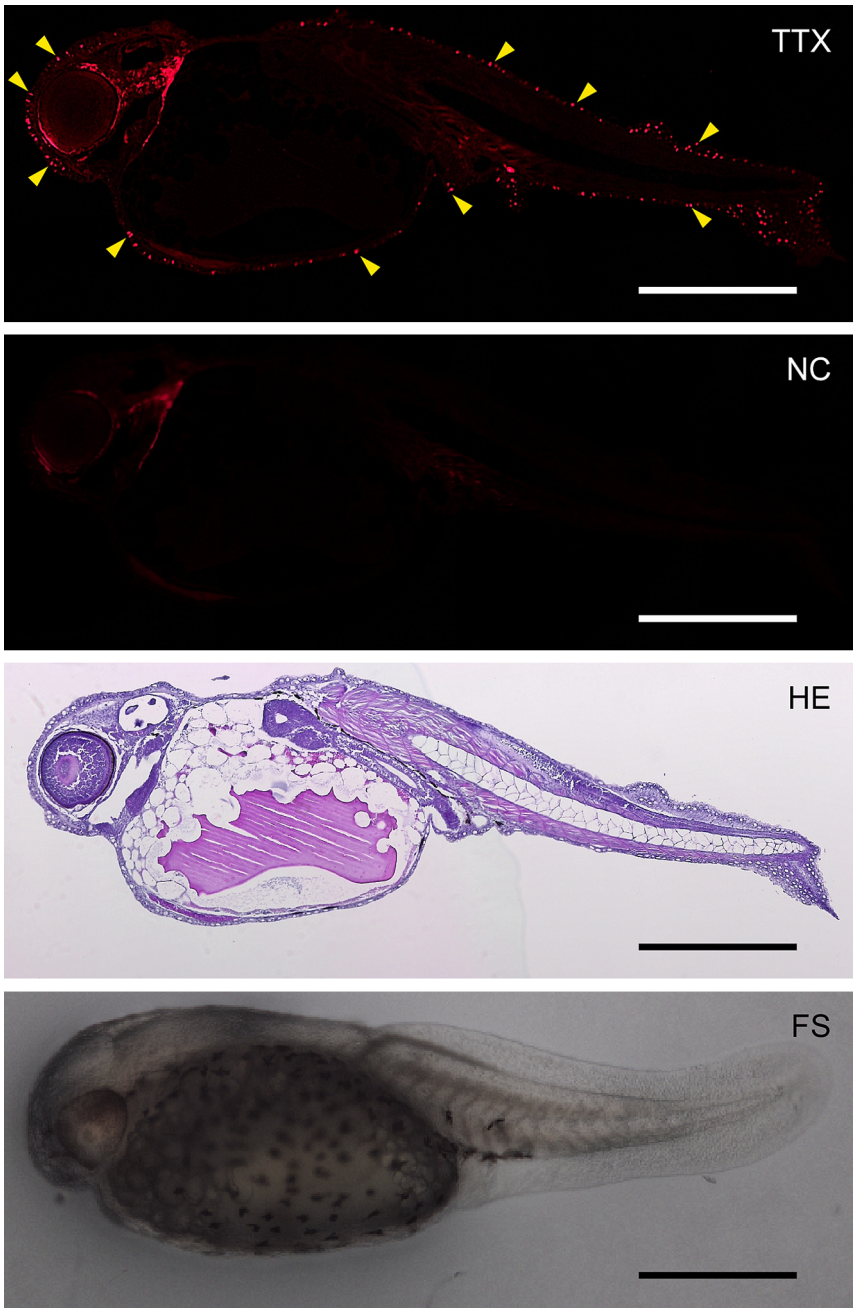

Fig. 2. Immunoreactivity for TTX in the larvae of *T. rubripes*. TTX, TTX-antibody treated larva; NC, negative control; HE, HE staining; FS, fixed larva. Positive signals to TTX-antibody are observed as red color (Yellow arrowheads). The reaction for the negative control was done with mouse IgG, and the tissue structure was observed by means of HE staining. Scale bars: 0.5 mm.

and juveniles of six different non-toxic species that were caught in the spawning grounds of the prey fish were used as the predators (Table 1; Supplementary data, Table S1, Fig. S2). Medaka (*Oryzias latipes*) larvae (4–7 dph) acclimated to sea water and adult artemia *Artemia* sp. (4–7 mm) used as negative controls (i.e., non-toxic) for the prey (Fig. 1, Tables 1 and 2; Supplementary data, Table S1). Significantly difference was observed between the responses of predators to TTX-bearing fish and to non-toxic organisms ($P < 0.0001$). LC-MSMS analysis revealed very small amounts of TTX in the egg (1.604 ng/egg; 5.5 μg/g) and larvae of *T. niphobles* (0.107 ng/larva; 471 ng/g), and *T. rubripes* (0.015–0.096 ng/larva; 65–221 ng/g; Table 2; Supplementary data, Table S2, Fig. S3), suggesting that the

amount of TTX in the pufferfish larvae does not constitute a lethal dose to the juvenile predator fish. Minimum lethal dose of TTX was estimated by intraperitoneal injection: minimum lethal dose of TTX in the several non-toxic teleost species was 0.3–1.8 mouse unit/20 g body mass, corresponding to 3–18 ng/g (Noguchi et al., 2006). However, it is clear from these results that the predators can sense even the miniscule amount of TTX in the larval pufferfish.

Localization of maternal TTX in the pufferfish larvae (0–4 dph) was investigated using immunohistochemical techniques with an anti-TTX monoclonal antibody. Interestingly, positive immunoreactions were observed on the body surface of larval *T. rubripes* (the adult skin of which is nontoxic) (Noguchi et al., 2006; Tatsuno et al., 2013), and no specific reaction was observed in the internal organs (Fig. 2). A similar localization of TTX was observed in *T. niphobles* larvae (Supplementary data, Fig. S4), suggesting that the larvae of different species of the genus *Takifugu* localized TTX on their body surface (mucous). Obviously, localizing of TTX on larval body surface (as opposed to secreting it in an internal organ), form a reasonable survival strategy for pufferfish larvae that lacks other defenses. Many predatory fish appear to promptly sense TTX on the body surface of the prey larvae. For example, apart from those cited above, it has been reported that the gustatory organs of rainbow trout (*Oncorhynchus mykiss*) and arctic char (*Salvelinus alpinus*) can sense extremely low levels of TTX (Yamamori et al., 1988).

This study indicates that the pufferfish accumulate TTX in the ovary in order to pass it on the larvae as protection against predators. Indeed, TTX was detected in the eggs and larvae from already spawned *T. rubripes*, demonstrating that the female parent transfers TTX vertically to the eggs and larvae from the ovaries (Supplementary data, Table S3). TTX is also used for in the protection of fertilized eggs (Table 1) as it is seen on the surface of fertilized eggs of *T. niphobles* (Matsumura, 1995). As the amount of TTX per fertilized egg decreased after fertilization (Supplementary data, Fig. S5), it is possible that TTX might be released from the surface of the egg to signify the TTX against the predators.

In conclusion, the present results suggest that the maternal TTX in the pufferfish larvae would contribute to beneficial strategies for increasing the survival of egg and larvae. It is easy to imagine that the explosive speciation of *Takifugu* would benefit from the TTX. Recently, it was reported that the pufferfish of the genus *Takifugu* successfully diverged and radiated during a short period of the Pliocene (2.6–5.3 million years ago) in marine waters around East Asia (Yamanoue et al., 2009). Although TTX is a useful strategy of defense protecting the fish and its larvae from predation, overfishing may cause drastic decline of *Takifugu* species populations despite of the fish's toxicity. Therefore, these species need special protection from overfishing.

Ethical statement

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

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2013.11.003.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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