Including planocerid flatworms in the diet effectively toxifies the pufferfish, *Takifugu niphobles*

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Beginning with the larval stages, marine pufferfish such as *Takifugu niphobles* contain tetrodotoxin (TTX), an extremely potent neurotoxin. Although highly concentrated TTX has been detected in adults and juveniles of these fish, the source of the toxin has remained unclear. Here we show that TTX in the flatworm *Planocera multitentaculata* contributes to the toxification of the pufferfish throughout the life cycle of the flatworm. A species-specific PCR method was developed for the flatworm, and the specific DNA fragment was detected in the digesta of wild pufferfish adults. Predation experiments showed that flatworm larvae were eaten by the pufferfish juveniles, and that the two-day postprandial TTX content in these pufferfish was 20–50 μg/g. Predation experiments additionally showed flatworm adults were also eaten by pufferfish young, and after two days of feeding, TTX accumulated in the skin, liver and intestine of the pufferfish.

Tetrodotoxin (TTX), named after the Piscean order Tetradontiformes, is also known as pufferfish toxin after one of the main group of fishes in the order. TTX is a non-peptidic potent neurotoxin that specifically binds to voltage-gated sodium channels on excitable membranes of muscle and nerve tissues1,2. Apart from pufferfish, it has been found in various taxonomic groups of animals, including amphibians (e.g., California newt *Taricha torosa*; Costa Rica frog *Atelopus* spp.3), fishes (e.g., goby *Yongeichthys criniger*4), cephalopods (e.g., blue-ringed octopus *Hapalochlaena maculosa*5), gastropods (e.g., the Japanese ivory shell *Babylonia japonica*6; the opisthobranch *Pleurobranchaea maculata*7,8), crustaceans (e.g., the xanthid crab *Atergatis floridus*9), starfishes (e.g., *Astropecten* spp.11,12), flatworms (e.g., *Planocera* spp.13–16; *Stylochoplana* sp.17) and ribbonworms (e.g., *Cephalothrix* sp.14,18). Furthermore, TTX production has been observed in several species of bacteria that are symbiotic with pufferfish and are obtained from their prey19–22.

It has generally been understood that the *Takifugu* pufferfish species accumulate TTX via the food web, consisting of several steps, starting with TTX producing marine bacteria22,23. This speculation has been supported by several studies that have shown that non-toxic pufferfish are obtained when they are artificially cultured after hatching and fed non-toxic diets, and that these cultured non-toxic pufferfish become toxic when administered TTX orally24–28. However, tracing the toxification via the food chain exclusively to marine bacteria is unlikely to account for the amount of TTX in the pufferfish body, as bacteria produce minute amounts of toxin21,29–32. However, TTX levels produced by bacterial cultures under potentially non-optimum conditions might be significantly less than those synthesized by bacteria colonizing pufferfish organs. Indeed, the issue remains unsettled, and the toxification process in pufferfish still remains unclear. Recently, our lab showed that the pufferfish *Takifugu niphobles* fed on the naturally TTX-laden eggs of another pufferfish *Takifugu pardalis*, suggesting that *T. niphobles* effectively increased its own toxicity by feeding on the toxic eggs from another toxic species33. That report also proposed that TTX is progressively concentrated in a “TTX loop” that includes TTX-bearing organisms at higher trophic levels in the food web33.

Other studies from our lab have shown that although the source of TTX is largely unidentified in wild-caught juveniles of the pufferfish *T. niphobles*, larval pufferfish do contain minute amounts of TTX provided by their mother34,35. We have also shown that the larvae of the planocerid flatworm *Planocera multitentaculata* possess highly concentrated TTX in the body, and eggs are laid May through July, coinciding with the spawning season.
of the pufferfish *T. niphobles* in rocky inshore waters. Therefore, in the present study, we investigated the contribution of the planocerid flatworm to the toxification of the pufferfish *T. niphobles* throughout its life history.

### Results

#### Toxicity of the wild pufferfish juveniles

The concentration and total amount of TTX in the juveniles from Oiso (obtained at three different time periods) and Katase are shown in Table 1. These values correspond to 3.16 ± 1.37 mouse unit (MU)/g (2.65 ± 2.16 MU/individual) in August, 2010 (n = 12); 3.32 ± 1.35 MU/g (4.57 ± 2.14 MU/individual) in August, 2011 (n = 9); and 21.68 ± 21.67 MU/g (2.50 ± 4.92 MU/individual) in July, 2015 (n = 21) for the juveniles obtained from Oiso, while for those from Katase (n = 24) in July, 2016 and 2017, the numbers shown translate to 54.75 ± 50.25 MU/g (11.70 ± 15.61 MU/individual) and 64.85 ± 38.21 MU/g (14.47 ± 12.60 MU/individual), respectively.

#### High-throughput sequencing

Next generation sequencing (NGS) analysis against mitochondrial cytochrome c oxidase subunit I (COI) showed that a sequence essentially identical to that of the flatworm *P. multitentaculata* was detected in the intestinal contents from the wild juvenile pufferfish *T. niphobles* captured from waters off Katase, Kanagawa, Japan in July 2016 (all of the three individuals analyzed in this study, Tables 2, S1 and S2) and 2017 (three of nine individuals analyzed in this study, Tables 3, S3 and S4). This sequence constituted 0.04–10.2% (all of three individuals), and 0.5–1.8% (three of nine individuals), of all the orthologous sequences from other TTX-bearing and non-toxic organisms found in the gut contents of *T. niphobles*, in 2016 and 2017, respectively. The nucleotide sequences of the partial COI gene obtained from the intestinal tract of *T. niphobles* are included in supplementary file “NGS_seq(2016).docx” and “NGS_seq(2017).docx”, and OTU ID are represented in Tables S2 and S4.

#### Planocerid-specific PCR and detection of planocerids from pufferfish gut contents

A PCR-based method with *P. multitentaculata*-specific primers was developed for detection of the flatworm from the intestinal contents of pufferfish: 28S ribosomal RNA (rRNA) gene amplified by PCR with universal primers from all flatworm samples, whereas DNA fragments encoding the mitochondrial COI gene were detected only in *P. multitentaculata*, with length 429 bp (Fig. 1). No fragment was observed in the related species, *P. reticulata*.

PCR with *P. multitentaculata*-specific primers amplified the 429 bp long mtDNA fragment from the intestinal content of the pufferfish *T. niphobles* young, two days after being fed with *P. multitentaculata* adults in the aquarium. Similarly, a *P. multitentaculata*-specific band was observed from the intestinal contents from the adult pufferfish captured off Hayama, in July, 2016 (Fig. 2).

#### Toxicity of the planocerid flatworm

The amount of TTX in the planocerid flatworm *P. multitentaculata* (n = 9; body weight, 3.49 ± 0.51 g) captured in April 2016 was 91.9 ± 0.51 µg/g (334.0 ± 201.9 µg/individual), corresponding to 418 ± 4 MU/g (1518 ± 918 MU/individual), whereas those captured in May, 2016 (n = 9; body weight, 3.47 ± 0.49 g) were 610.8 ± 2.1 µg/g (2091.4 ± 469.3 µg/individual), corresponding to 2776 ± 10 MU/g (9506 ± 2133 MU/individual). A statistically significant difference was observed between these two sample groups (P < 0.05, Fig. 3, Table S5).

The amount of TTX in the planocerid eggs obtained in 2015 was 1867 ± 1589 µg/g, corresponding to 8486 ± 7223 MU/g, whereas those obtained in 2016 contained 2673 ± 1214 µg/g, corresponding to 12150 ± 5518 MU/g (Fig. 3, Table S5). The amount of TTX in the planocerid larvae in 2015 and 2016 was 69 ± 21 ng/individual, corresponding to 0.314 ± 0.095 MU/individual, and 120 ± 46 ng/individual, corresponding to 0.545 ± 0.209 MU/individual, respectively (Fig. 3, Table S5).

#### Toxification of pufferfish juveniles fed on flatworm larvae

In the predation experiments, the pufferfish juveniles fed heavily on planocerid larval and lost equilibrium in approximately 20 min, but then recovered. Multiple reaction monitoring (MRM) patterns showed that the peak in the liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis corresponding to TTX was detected in the planocerid fed-pufferfish juveniles (Fig. S1). The amount of TTX in the whole body of the pufferfish juveniles (18, 39 and 51 dph; days post hatch) was calculated to be 407 ± 102 to 1064 ± 123 ng/individual (19.5 ± 5.4 to 54.8 ± 17.1 µg/g) (Table 4). The peak corresponding to TTX was not detected in the cultured juveniles (18 and 39 dph) of the pufferfish *T. niphobles*, which fed on non-toxic feeds (Fig. S1).

#### Toxification of pufferfish young fed on adult flatworms

In these predation experiments, the pufferfish young fed on half of an adult flatworm and appeared to become toxified. MRM patterns showed that the peak in the LC-MS/MS analysis corresponding to TTX was detected in several tissues including intestine, liver and skin.

Table 1. Toxicity (concentration) and amount of TTX in wild pufferfish *Takifugu niphobles* juveniles. Data are represented mean ± standard deviation.

| Date            | Locality       | No. of individuals | Total length (mm) | Body weight (g) | Toxicity (ng/g) | TTX amount (ng/ind.) |
|-----------------|----------------|--------------------|-------------------|-----------------|-----------------|----------------------|
| 2010 August     | Oiso (35°18′N, 139°19′E) | 12                 | 27.6 ± 6.2         | 0.8 ± 0.5       | 694 ± 301       | 583 ± 475           |
| 2011 August     | Oiso (35°18′N, 139°19′E) | 9                  | 34.1 ± 1.8         | 1.4 ± 0.2       | 731 ± 298       | 1006 ± 471          |
| 2015 July       | Oiso (35°18′N, 139°19′E) | 21                 | 19.8 ± 6.0         | 0.2 ± 0.2       | 4770 ± 4768     | 550 ± 1082          |
| 2016 July       | Katase (35°18′N, 139°28′E) | 38                | 20.0 ± 2.4         | 0.2 ± 0.1       | 12044 ± 11054   | 2573 ± 3435         |
| 2017 July       | Katase (35°18′N, 139°28′E) | 9                  | 17.9 ± 5.8         | 0.2 ± 0.1       | 14267 ± 8406    | 3183 ± 2773         |
of the pufferfish (Fig. S1). The amount of TTX in the body of the toxified pufferfish young (12 months old), fed with a weakly toxic planocerid (152 ± 97 μg) was calculated to be 212 ± 227 (52–819) μg/individual, which corresponds to 964 ± 1032 MU/individual, demonstrating that almost all the TTX (129 ± 60%) in the flatworm was ingested by the pufferfish young (Fig. 4, Table S6). Similarly, the amount of TTX in the whole body of the toxified pufferfish young with a strongly toxic planocerid (1005 ± 305 μg) was calculated to be 181 ± 200 (9–543) μg/individual, which corresponds to 823 ± 909 MU/individual, demonstrating that some of the TTX (19 ± 21%) in the flatworm was ingested by the pufferfish young (Fig. 4, Table S6). TTX in the toxified pufferfish was localized in the liver (59.2 ± 12.7 to 62.0 ± 16.4%), intestine (14.7 ± 7.9 to 16.3 ± 13.9%) and skin (14.0 ± 11.2 to 22.1 ± 15.3%) (Table S7). MRM patterns showed that the peak in the LC-MS/MS analysis corresponding to TTX was not detected in any tissues from the cultured young (12 months old) T. niphobles that were raised on non-toxic feed (Fig. S1).

**Discussion**

TTX in the offspring of toxic organisms such as pufferfish, octopus, newt and flatworm, appear to be obtained by means of a vertical maternal transfer. This maternally provided TTX provides even just-hatched larvae protection from predators. However, the protection provided by the maternal TTX, at least in pufferfish, which have microplanktonic stages in their life histories, decreases with age in the absence of further influx of TTX. The larvae and juveniles of pufferfish need to obtain TTX from food in order to continue to protect themselves from predators. Effective toxification of the larval and juvenile pufferfish appears to depend on their feeding on plankton that is laden with highly concentrated TTX. TTX-bearing organisms have been identified from various taxa, including ribbonworms and flatworms, suggesting that larval stages of the flatworm P. multietentaculata contain highly concentrated TTX (69–120 ng/larva), suggesting that larval stages of the flatworm could serve not merely as suitable food but also a source for the toxification of the pufferfish larvae/juveniles. Considering that toxic flatworm sequences were detected from intestinal contents of wild juveniles and young of the pufferfish T. niphobles, our results suggest that the flatworm contributes to the toxification of the pufferfish T. niphobles throughout its life (Fig. 5).

Kono et al. reported that dietary administrated TTX in the pufferfish T. niphobles was firstly accumulated into the liver, and then gradually transferred to the skin approximately 200 days after end of TTX administration: TTX content in the skin increased two-fold, while decreasing by 40% in the liver. These results suggest that apart from larval pufferfish, older individuals also use TTX as a chemical defense against predators. This inference is corroborated by the Sakakura et al. study, which found the survival rates of the TTX-bearing pufferfish T. rubripes juveniles were higher than those of the non-toxic individuals in a salt-pond mesocosm. In the predation experiments in this study, non-toxic T. niphobles juveniles and young were rapidly and effectively toxified after feeding on larvae and adult specimens of the flatworm P. multietentaculata, respectively, accumulating large quantities of TTX. Thus, the potential for pufferfish to accumulate large quantities of TTX exist; however, this potential does not

**Table 2.** DNA sequences from the intestinal contents of the pufferfish *Takifugu niphobles* juveniles collected at Katase in July 2016. The pufferfish juveniles were randomly selected from the specimens collected at Katase in July 2016, shown in Table 1. *The list of sequences from non-toxic organisms are shown in the supplementary data (Table S1).*

| Organism          | Acc. No. | Sequence identity (%) | Number of sequences from: |
|-------------------|----------|-----------------------|---------------------------|
|                   |          |                       | Pufferfish S1 | Pufferfish S2 | Pufferfish S3 |
| TTX-bearing organisms |         |                       |             |             |             |
| Flatworm Planocera multietentaculata | LC190986 | 99                     | 9873 | 70 | 35 |
| Newt Cypris pyrhogaster | EU880313 | 90                     | 54  | 0  | 0  |
| Goby Yorgieithys criniger | KT894736 | 99                     | 0   | 3  | 6  |
| Pufferfish Chelomodon patoca | KU692427 | 100                    | 3   | 0  | 2  |
| Non-toxic organisms* |         |                       |             |             |             |
| Annelida, Polychaeta   | 408      | 79                     | 65            |             |             |
| Annelida, Polychaeta   | 171      | 392                    | 1553          |             |             |
| Arthropoda, Arachnida  | 2875     | 0                      | 565           |             |             |
| Arthropoda, Crustacea  | 81406    | 11104                  | 21260         |             |             |
| Arthropoda, Crustacea, Amphipoda | 6 | 19416 | 68362 |             |             |
| Arthropoda, Crustacea, Copepoda | 853   | 70016                  | 280          |             |             |
| Arthropoda, Diplopoda  | 0        | 0                      | 1             |             |             |
| Arthropoda, Insecta    | 359      | 347                    | 681           |             |             |
| Bacillariophyta, Thalassiosirales | 251  | 0                      | 0             |             |             |
| Mollusca, Bivalvia     | 0        | 5                      | 0             |             |             |
| Mollusca, Gastropoda   | 918      | 240                    | 6             |             |             |
| Platyzelmintes, Polycladida | 0  | 0                      | 47            |             |             |
| Unidentified sequence  | 161      | 0                      | 1             |             |             |
| Total number of sequences | 97338 | 101672                  | 92864         |             |             |
| TTX amount (μg/individual) | 4.6     | 7.5                    | 15.5          |             |             |
always appear to be realized, even in laboratory-based predation experiments. Variation in individual toxicity is known to be high in wild Takifugu pufferfish populations\(^{23,24}\). In the present study too, large individual differences in toxicity were observed in *T. niphobles* young after the toxification experiments, suggesting that variation in individual toxicity might be one of the risk management in the survival strategies in the *Takifugu* pufferfish because of energy consumption for TTX-bearing in their body. The detection of TTX in young pufferfish at levels higher than the levels contained in the half flatworm they were fed, suggests that the difference might be due to the potential differences in TTX recovery or TTX extraction efficiencies for different tissues.

Flatworms are known to contribute to the toxification of other animals as well; one study reported that dog neurotoxicosis occurred after consuming the side-gilled sea slug *Pleurobranchaea maculata*\(^9\), and a subsequent study revealed that the sea slugs were toxified by feeding on the flatworm *Stylochoplana* sp.\(^9,17\). The extent of pufferfish toxicity varies not only among individuals but also by habitat\(^23\), which might be associated with the habitat-specific population size of the planocerid flatworms\(^9\). In any cases, although planocerid flatworms contribute to the toxification of organisms at higher trophic levels, further investigation is needed to reveal the source of TTX in the flatworms for a better understanding of the TTX-loop in the marine environments. Our study shows that in the study area, the population size of the flatworm *P. multitetaculata* is much larger than that of the related species, *P. reticulata*. Indeed, *P. multitetaculata* appears to make a greater contribution to the toxification of the larvae and juveniles of the pufferfish, because toxic eggs of *P. multitetaculata*, and not of *P. reticulata*, have been observed in the area during the spawning period of the pufferfish\(^9,17\). The TTX source of the planktonic flatworms (excluding the maternal TTX) might be key to resolving the missing link to the TTX-loop proposed by our previous study\(^33\).

The toxification of the TTX-bearing organisms, including pufferfish, has been thought to be achieved through the classical food webs\(^22,23\), although the evidence thus far has largely only been the presence of organisms with indigestible tissues such as the starfish *Asteropeten polyacanthus*, identified from the gut-contents of TTX-bearing organisms, such as a trumpet shell *Charonia sauliae*\(^4\). There has been little evidence of the toxification of other toxic organisms by organisms without indigestible tissues except for the flatworm *Stylochoplana* sp., which is considered responsible for the toxification of the grey side-gilled sea slug *Pleurobranchaea maculata*\(^9,17\). In our study, in order to determine if wild *T. niphobles* fed on the flatworm *P. multitetaculata*, we developed PCR methods specific to the flatworm based on results from NGS analysis against a generic animal COI gene. Our results suggest that some wild pufferfish feed on the flatworm in the inshore waters around Hayama, Japan, and that future large scale metagenomics analyses of the intestinal contents of TTX-bearing organisms might reveal the mechanism of toxification.

| Organism | Acc. No. | Sequence identity (%) | Number of sequence from pufferfish individual: |
|----------|----------|-----------------------|-----------------------------------------------|
|          | No. 1 No. 2 No. 3 No. 4 No. 5 No. 6 No. 7 No. 8 No. 9 | |
| Flatworm *Planocera multitetaculata* | LC190986 | 100 | 0 0 0 936 0 272 833 0 0 |
| Ribbonworm *Cephalothrix simula* | GU726607 | 86 | 0 0 0 0 0 2 0 0 0 |
| Sea snail *Rapana venosa* | KP976378 | 100 | 0 0 0 101 0 2 0 0 0 |

Table 3. DNA sequences from the intestinal contents of the pufferfish *Takifugu niphobles* juveniles collected at Katase in July 2017. The pufferfish juveniles were randomly selected from the specimens collected at Katase in July 2017, shown in Table 1. The list of sequences from non-toxic organisms are shown in the supplementary data (Table S3).
In conclusion, we have shown, by means of TTX quantification, predation experiments, and tools of molecular biology, that the pufferfish *T. niphobles* can be toxified simply by feeding on the flatworm *P. multitentaculata*. The TTX content of the flatworm increased in association with increasing body weight, and flatworms carrying TTX were classified into planocerid and the related species. These reports and our results would contribute to the elucidation of the pufferfish toxification mechanism.

**Materials and Methods**

**Pufferfish and flatworm.** Wild (toxic) pufferfish *Takifugu niphobles* juveniles (15–34 mm total length, 0.10–0.67 g body weight) were captured in August 2010, August 2011 and July 2015 from coastal waters of Oiso, Japan (35°18′N, 139°19′E) and in July 2016 and July 2017 from coastal waters of Katase, Japan (35°18′N, 139°28′E). Wild adult and young specimens were captured during June–July of 2016, and June–July of 2017 off the coast of Hayama, Japan (35°15′N, 139°34′E). Adult specimens of the flatworm *Planocera multitentaculata* were captured during April–July of 2015 and 2016 also at the coast of Hayama, Japan, while (toxic) flatworm larvae hatched from eggs that were spawned in the laboratory aquaria, by wild parents derived from Hayama.

**LC-MS/MS analysis.** TTX was extracted from samples with 0.1% acetic acid, the extract was filtered through a membrane of pore size 0.45-μm (SupraPure Syringe Filter, PTEE-Hydrophilic, Recentec, Taipei, Taiwan) and subjected to analysis using a LC-MS/MS, following Itoi et al. Quantification was done using a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source coupled to an Acquity UPLC system (Waters), following Itoi et al. Chromatographic separation was done using an Atlantis HILIC Silica column (2.1 mm × 150 mm, 5 μm; Waters), coupled to an Atlantis HILIC Silica pre-column (2.1 mm × 10 mm, 5 μm; Waters), with gradient elution of formic acid/acetonitrile. 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. The calibration curve was

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**Figure 1.** Location of primers in the 28S rRNA gene from *Planocera multitentaculata* and various flatworm species. (a) A schematic of the 28S rRNA gene of *P. multitentaculata* and various flatworm species, and locations of oligonucleotides used as primers for PCR and DNA sequencing. Arrows represent the location and direction of primers. (b) Electrophoretic pattern of PCR products from *P. multitentaculata* and various flatworm species. White arrow indicates PCR products specific to *P. multitentaculata*, and black arrow indicates those common to various flatworm species. Lane M, molecular weight marker; lanes 1 and 2, *P. multitentaculata*; lane 3, *Planocera reticulata*; lane 4, *Planocera* sp.; lane 5, *Paraplanobera oligoglena*; lane 6, *Callioplanina marginata*; lane 7, *Discoplana gigas*; lane 8, *Pseudostylochus obscurus*; lane 9, *Notocomplana humilis*. Full-length gels are included in a Supplementary Information file (Fig. S3).
A high-throughput sequencing approach was used to analyze the intestinal contents of juvenile pufferfish *T. niphobles*. Genomic DNA was extracted from the intestinal contents using a Fast DNA spin kit for Soil (MO Bio Laboratories, Illkirch, France) according to the manufacturer's instructions. Fragments of the COI gene (approximately 500 bp) were amplified via PCR using universal COI primers: 1st-IntF (5′-Seq A-GCTCT TCCCA TCTGT GCCAG C GCCGT AA-3′) and 1st-HCoMr (5′-Seq B-CTCTT CCGAT CTTAH ACTTC NGGGT G KCCR AARAA TCA-3′), where Seq A (5′-ACACT CCTTC CCTAC ACGAC-3′) and Seq B (5′-GTGAC TGGAG TTCAG ACGTG TG-3′) represent nucleotide sequences targeted by the second PCR primers. A blocking primer (5′-TTACC CCCCT CCTAG AGGAA ATCTT G GCCACGCGAG G-Spacer C3-3′) was utilized to prevent amplification from the pufferfish *T. niphobles*. A Spacer C3 GpG in the 3′ end of the blocking primer was added to prevent amplification from the pufferfish *T. niphobles*. A Spacer C3 GpG in the 3′ end of the blocking primer was added.
to prevent elongation without affecting annealing properties, and minimizing predator DNA amplification. PCR amplification was done under the following conditions: an initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 67 °C for 15 sec and 52 °C for 30 sec, and extension at 72 °C for 30 sec, with a final extension step at 72 °C for 5 min. PCR products were amplified again using additional forward primer (5′-Adaptor C-Tag sequence-Seq A-3′) and reverse primer (5′-Adaptor D-Seq B-3′), where Adaptors C and D were used for the MiSeq sequencing reaction. Thermal cycling was done under the following conditions: an initial denaturation at 94 °C for 2 min followed by 12 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec, with a final extension step at 72 °C for 5 min. PCR amplicons from each sample were used for high-throughput sequencing on a MiSeq Genome Sequencer (Illumina, CA, USA). The sequences obtained for each sample were grouped based on tag sequences, and average read length of 320 bp was obtained. Negative controls (reactions with no template) were prepared for all steps of the process after DNA extraction to

### Table 4. Toxification of the juvenile pufferfish after feeding on the flatworm larvae.

| Pufferfish (year) | No. of individuals | Total length (mm) | Body weight (g) | TTX content in flatworm larvae (μg)a | TTX concentration in pufferfish (μg/g)b | TTX amount of pufferfish (ng/ind.)b | Ingestion rate (%) |
|-------------------|--------------------|-------------------|-----------------|--------------------------------------|-----------------------------------------|-------------------------------|-------------------|
| **Flatworm-fed individual** | | | | | | | |
| 18 dph (2016) | 28 (3 replicates) | 6.1 ± 0.2 | 0.006 ± 0.002 | 3.7 | 37.2 ± 21.7 | 116 ± 19 | 2.9 ± 0.5 |
| 39 dph (2015) | 26 (3 replicates) | 7.6 ± 0.8 | 0.008 ± 0.001 | 12.1 | 54.8 ± 17.1 | 407 ± 102 | 3.4 ± 0.8 |
| 51 dph (2015) | 30 (3 replicates) | 11.1 ± 1.6 | 0.022 ± 0.007 | 15.7 | 19.5 ± 5.4 | 474 ± 241 | 3.0 ± 1.5 |
| **Non-toxic feed-fed individual** | | | | | | | |
| 18 dph (2016) | 9 (1 replicate) | 6.0 ± 1.4 | 0.006 | N/Ac | N/A | N/Dd | N/A |
| 39 dph (2015) | 28 (3 replicates) | 8.0 ± 1.2 | 0.009 ± 0.002 | N/A | N/A | N/D | N/A |

| Table 4. Toxification of the juvenile pufferfish after feeding on the flatworm larvae. aTTX content in the flatworm larvae fed by a juvenile of the pufferfish was calculated. bPufferfish TTX values represent means of three independent replicate experiments. cN/A: not applicable. dN/D: not detected. |

**Figure 4.** Toxification of the young pufferfish Takifugu niphobles after feeding on the adult flatworm Planocera multitentaculata. Closed circles refer to TTX accumulation in the pufferfish after feeding on half of one flatworm, while the open circles refer to the accumulation in half of one flatworm. Non-toxic pufferfish were used as a predators, and the toxic flatworms were used as prey. The flatworms collected in April and May were subjected to trial I and trial II of the toxification experiment (see text), respectively. Gray circle refers to the rate of accumulation of TTX in the pufferfish after feeding on the flatworm. Values are the mean of nine independent replicate experiments. Bars represent means ± standard deviation. Student’s t-test was employed for statistical comparison (*P < 0.05).
check for contamination. Before analyzing the food organisms, we removed sequences if they met any of the following criteria: <40 bp in length, with a phred-equivalent quality score of <20, containing ambiguous characters, with an uncorrected barcode, or missing the primer sequence. The identities of the phylotypes were analyzed by comparing the sequences against the DDBJ/EMBL/GenBank databases using a BLAST search.

**DNA extraction and PCR amplification.** Small tissue samples from adult specimens of the flatworm *P. multitentaculata*, and intestinal contents from wild specimens of the pufferfish *T. niphobles* were collected. Total genomic DNA was extracted from the flatworm tissues and the intestinal contents of the pufferfish using the method of Noguchi et al. with some modification. Briefly, proteinase K-treated samples were subjected to phenol/chloroform extraction with Maxtract High Density (Qiagen, Germantown, MD, USA). Partial fragments of 28S rRNA gene were amplified by PCR using primers HRNT-F2 (5′-AGTTC AAGAG TACGT GAAAC C-3′) and HRNT-R2 (5′-AACAC CTTTT GTGGT ATCTG ATGA-3′), which were designed with universal primers for the 28S rRNA gene (approx. 1,000 bp) of various polyclads, whereas those of COI gene were amplified by PCR using *P. multitentaculata*-specific primers PMTF1 (5′-TTATT ATTGG GTTCA TTTGT GGTAG AG-3′) and PMTR2 (5′-AATCA TACCA AACCC CGGC-3′), which were designed based on the sequences of the COI gene (429 bp) from *P. multitentaculata* and other polyclads (Fig. 1). PCR amplification was done in a 20 μl reaction mixture containing genomic DNA as a template, 1 unit ExTaq DNA polymerase (Takara Bio, Shiga, Japan), 1.6 μl of 2.5 mM deoxynucleotide triphosphates (dNTP), 5 μl of 5 μM primers, and 2 μl of 10× ExTaq DNA polymerase buffer (Takara Bio). The thermal cycling program for the PCR consisted of an initial denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 45 s.

**Direct sequencing.** Prior to sequencing the amplified product, the DNA fragment was purified by chloroform extraction, followed by polyethylene glycol (PEG) 8000 precipitation and ethanol precipitation. Both strands were sequenced using a 3130xl genetic analyzer (Applied Biosystems, Foster, CA, USA) and a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The nucleotide sequences of the amplified products were aligned using Clustal Omega with those in the DDBJ/EMBL/GenBank databases obtained using a BLAST search.

**Toxification experiment.** *Pufferfish juveniles vs. planocerid larvae.* The toxification experiment was carried out using non-toxic juveniles (within 2 months old) of the pufferfish *T. niphobles* (8–10 individuals) as the predator and toxic planocerid larvae (2000–3000 larvae) as prey, in a 500-ml beaker. The treatment was repeated three times, except for the control sample in 2016. In the toxification experiment, the non-toxic juveniles of the pufferfish *T. niphobles* (standard length: 7.2–12.4 mm; body weight: 0.06–0.30 g) fed on the toxic planocerid larvae. After more than two days of feeding, the pufferfish juveniles (8–10 individuals pooled in a sample) and non-toxic control (8–10 individuals pooled in a sample) were subjected to the TTX extraction process followed by LC-MS/MS analysis.
Pufferfish young vs. planocerid adults. The toxification experiment was also carried out in a 50 L glass aquarium using non-toxic young (12 months old) of the pufferfish T. niphobles as the predator and adult flatworm F. multifenestrulata as the prey. Since no significant difference in the TTX distribution was observed in both halves of adult flatworm individual (Fig. S2), half of the body of a flatworm adult was subjected to LC-MS/MS analysis, and the remaining half fed to a non-toxic pufferfish young, which were kept in a 50 L glass aquaria with a circulating filtration system. The treatment was repeated nine times. After more than two days of feeding on the adult planocerid, the pufferfish young were subjected to TTX extraction followed by LC-MS/MS analysis.

Statistical analysis. The statistical significance of differences in the amount of toxin was analyzed by means of a student’s t-test. Data are given as mean ± standard deviation.

Ethical statement. All animal procedures comply with the Japanese Government Animal Protection and Management Law (No. 105) and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).

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Author Contributions

S.I. and H.S. designed research; S.I., H.U., R.Y., M.T., T. Sato, S.O., Y.W., R.O., H.O., T. Shirto, K.O. and T.T. collected samples, performed research, and analyzed data; E.S. prepared non-toxic pufferfish young; S.I. wrote the main manuscript text, prepared Table 1; H.U. and R.Y. prepared Figures 1 and 2, and the related supporting data; S.I., R.Y., M.T. and T.T. prepared Figures 3 and 4, and the related supporting data; S.I. and R.Y. prepared Figure 5; T. Sato prepared Table 2; R.O. prepared Table 3; S.I., R.Y. and K.O. prepared Table 4, and the related supporting data.

Additional Information

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