Ecological functions of tetrodotoxin in a deadly polyclad flatworm

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The deadly neurotoxin tetrodotoxin (TTX) is found in a variety of animal phyla and, because of its toxicity, is most often assumed to deter predation. On the tropical Pacific island of Guam, we found an undescribed flatworm (planocerid sp. 1) that contains high levels of TTX and its analogs. Through ecological experiments, we show that TTXs do not protect these flatworms from some predators but instead are used to capture mobile prey. TTX is known to have multiple ecological functions, which has probably led to its widespread presence among prokaryotes and at least 10 metazoan phyla.

prey-capture | chemical ecology

The ecological functions of tetrodotoxin (TTX, Fig. 1*a*) and its analogs have rarely been tested even though it has been isolated and characterized since 1965 (1; reviewed in ref. 2). The cellular mechanism of TTX paralysis is well understood (blocking sodium ion channels that control nerve impulses; ref. 3) but its functions for organisms are just beginning to be understood. Because of the potent toxicity of TTX, it is often assumed to protect marine and terrestrial organisms from predators (4–6). In terrestrial amphibians [*Taricha granulosa* (7) and *Atelopus* spp. (8)], and marine pufferfish (*Takifugu* spp.; ref. 9) TTX is found in the skin, where it could be tasted by potential predators. The ecological significance of TTX as a defensive compound was recently shown in the evolutionary ecology of newts (*Taricha granulosa*) and their snake predators (*Thamnophis sirtalis*) (10, 11). Alternative ecological functions of TTX are known from pufferfish, which use TTX as a pheromone to attract males to gravid females (12), and in the blue-ringed octopus *Hapalochlaena maculosa* and six species of arrowworms, all of which contain TTX in their venom glands (13–16).

On the tropical Pacific island of Guam, we found a flatworm (undescribed, but vouchered as planocerid sp. 1; ref. 17 and Fig. 1*b*) that eats gastropods. Flatworms in Planoceridae are poorly studied, and fundamental questions such as what and how they eat remain unknown for most species. The flatworm *Planocera multitentaculata* contains TTX (18), but its ecological role in this flatworm was never tested. In this study, we describe the feeding ecology of planocerid sp. 1, and through ecological experiments, test whether TTX is used for defense or prey capture.

Results and Discussion

Planocerid sp. 1 rapidly killed and ate a wide variety of gastropod molluscs from at least 11 different families and even another flatworm (Table 1). Of the animals tested, only *Conus pulicarius* and *Elysia rufescens* were not eaten. The feeding behavior of planocerid sp. 1 eating the cowry *Cypraea punctata* was recorded (Movie 1, which is published as supporting information on the PNAS web site); after 23 min, the flatworm had enveloped and killed the cowry, removed its body, and moved away from the empty shell. The speed at which planocerid sp. 1 consumed the cowry *Cypraea moneta* was significantly ($n = 6$, $P = 0.0369$) related to the relative size of the cowry (Fig. 2*a*); however, the size of the prey did not determine whether it was eaten (Fig. 2*b*). Many flatworms prey on sessile benthic organisms (19), but the consumption of such a variety of mobile prey is not known. On coral reefs, fish are often assumed to be the most important predators, but this study shows that small cryptic fauna such as flatworms are capable of killing a wide variety of animals found in "rubble" habitat.

How does this "primitive" metazoan capture and kill mobile prey? Using HPLC and MS chemical analysis (20) we found that planocerid sp. 1 contains TTX and some of its analogs (Fig. 3 *a* and *b*). Individual flatworms were dissected into three regions (Fig. 1*b*), which were then analyzed for toxin concentration. The two most concentrated toxins in these flatworms were TTX and

Table 1. Species eaten/not eaten by planocerid sp. 1

Family	Genus and species
Arcidae	*Barbatia tenella* (2)
Buccinidae	*Cantharus undosus* (1)
Trochidae	*Clanculus atropurpureus* (2)
Cypraeidae	*Cypraea annulus* (1)
Cypraeidae	*Cypraea caputserpentis* (3)
Cypraeidae	*Cypraea carneola* (1)
Cypraeidae	*Cypraea cribraria* (1)
Cypraeidae	*Cypraea erosa* (2)
Cypraeidae	*Cypraea fimbriata* (5)
Cypraeidae	*Cypraea helvola* (4)
Cypraeidae	*Cypraea isabella* (3)
Cypraeidae	*Cypraea moneta* (10)
Cypraeidae	*Cypraea punctata* (3)
Cypraeidae	*Cypraea talpa* (2)
Muricidae	*Drupella ochrostoma* (1)
Mitridae	*Imbricaria olivaeformis* (1)
Fasciolariidae	*Latirus barclayi* (1)
Mitridae	*Mitra cucumerina* (1)
Trochidae	*Monilea philippiana* (1)
Fasciolariidae	*Peristernia nassatula* (1)
Columbellidae	*Pyrene punctata* (6)
Mitridae	*Strigatella acuminata* (1)
Tellinidae	*Tellina robusta* (1)
Terebridae	*Terebra babylonia* (1)
Terebridae	*Terebra felina* (1)
Trochidae	*Trochus histrio* (6)
Turbinidae	*Turbo argyrostomus* (1)
Pseudocerotidae	*Thysanozoon* sp. (1)
Conidae	*Conus pulicarius* (1)*
Elysiidae	*Elysia rufescens* (1)*

The number in parentheses is the number of individual flatworms that ate this species.
*Species not eaten by planocerid sp. 1.

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Abbreviation: TTX, tetrodotoxin.

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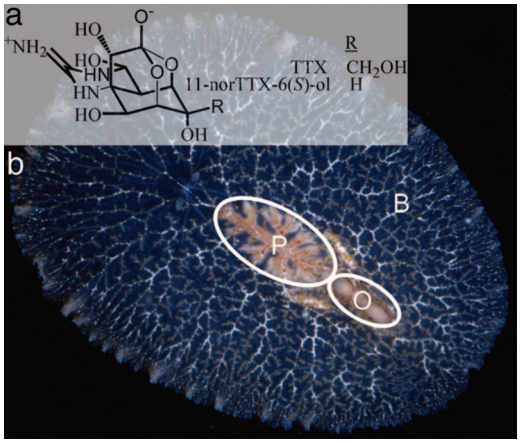

Fig. 1. Planocerid sp. 1. (*a*) The chemical structures of tetrodotoxin (TTX) and 11-norTTX-6(*S*)-ol. (*b*) A photo of planocerid sp. 1 indicating the dissected regions pharynx (P), organs (O), and body (B).

its analog 11-nortetrodotoxin-6(*S*)-ol (Fig. 3*a*). The highest concentrations of 11-norTTX-6(*S*)-ol were in the pharynx (the feeding organ) (one-way ANOVA, $P < 0.01$). The highest concentrations of TTX were found in the flatworms' egg masses (one-way ANOVA, $P < 0.01$) (Fig. 4*a*). Both the rough-skinned newt and the blue-ringed octopus also have high levels of TTX in their egg masses (21, 22), but whether this compound protects the eggs remains untested.

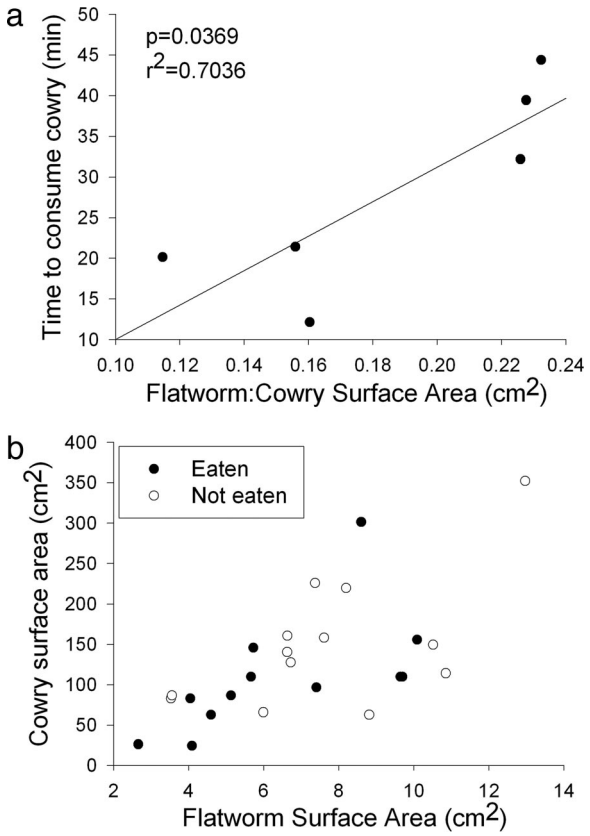

Fig. 2. The relationship of the size of the flatworm and its prey. (*a*) The speed that *C. moneta* was consumed by planocerid sp. 1. (*b*) The relationship of a cowry surface area and planocerid sp. 1 surface area and whether that cowry was eaten (filled circles) or not eaten (open circles).

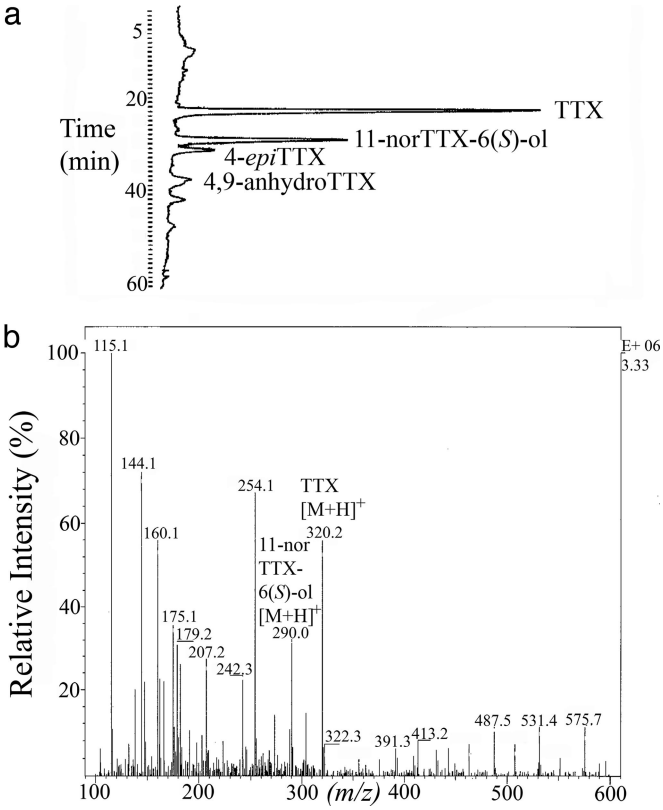

Fig. 3. The chemical identification of tetrodotoxin in planocerid sp. 1. (*a*) A HPLC-fluorescent detection system trace of planocerid sp. 1 (organ). (*b*) The electrospray ionization mass spectrum of tetrodotoxins from the pharynx of planocerid sp. 1.

Another species of planocerid flatworm was found with a high concentration of TTX, which was assumed to be defensive (18). If TTX and analogs (TTXs) serve a defensive function in these flatworms, we would have expected higher toxin concentrations distributed throughout the body. To test whether these flatworms are protected from potential predators, we offered whole, live flatworms to a natural assemblage of reef fish at two reefs on Guam. Three of five flatworms (Fingers Reef, $P = 0.44$) and eight of nine flatworms (Gun Beach, $P = 1$) were consumed by the fish. Tetrodotoxin and its analogs may be deterrent to other predators or at higher concentrations, but live planocerid sp. 1 did not deter feeding by reef fish.

Alternatively, if TTX is used for prey capture, we would expect the concentration of TTX to decrease immediately after feeding. We measured TTX and 11-norTTX-6(*S*)-ol concentrations 1, 4, and 8 days after feeding cowries to flatworms in the laboratory. There was no significant difference in the concentrations of TTX compared to 11-norTTX-6(*S*)-ol (two-way ANOVA, $P = 0.43$) and no significant interaction between toxin and time ($P = 0.52$). The concentration of both toxins immediately after feeding was significantly less than their concentrations 8 days after feeding ($P = 0.0011$) (Fig. 4*b*). The initial concentrations of the toxins were not significantly different from their concentrations immediately after feeding, but we could not control for the flatworms' feeding history before they were collected. To ensure that the flatworm mass did not affect the concentrations of TTX after feeding, we ran a one-way ANOVA on the wet weight (log transformed) of the flatworms. None of the treatment groups differed except the 4-day treatment, which had larger flatworms than the initial treatment ($P = 0.0356$, Tukey-Kramer post hoc test). The high levels of TTX and 11-norTTX-6(*S*)-ol in the

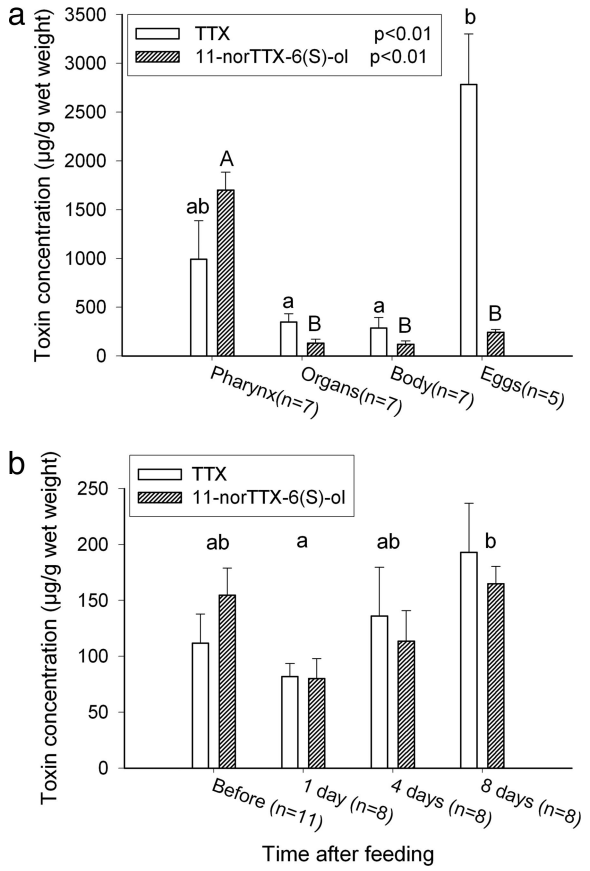

Fig. 4. Tetrodotoxin and 11-norTTX-6(*S*)-ol concentrations in planocerid sp. 1. (*a*) The concentrations of TTX (open bars) and 11-norTTX-6(*S*)-ol (hatched bars) in each dissected region of planocerid sp. 1 and their egg masses. A and B and a and b indicate statistically different groups for each toxin ($P < 0.01$, one-way ANOVA followed by the Tukey-Kramer post hoc test). (*b*) The concentrations of TTX and 11-norTTX-6(*S*)-ol in whole flatworms at different times after feeding. a and b indicate statistically different groups of time after feeding ($P < 0.01$, two-way ANOVA followed by the Tukey-Kramer post hoc test).

pharynx and low toxin concentration after feeding show that these toxins are used during prey capture. The source of TTX in organisms is often attributed to bioaccumulation (23–25). Our data show that TTXs concentrations did not increase immediately after feeding so they are not accumulated from the flatworm's prey. Because feeding is a fundamental function for survival, it is more likely that the flatworm or symbiotic bacteria produce TTXs endogenously.

TTX is a structurally complex natural product (Fig. 1*a*) that is found in a wide range of distantly related organisms in both terrestrial and marine ecosystems (26). Even though it is extensively used to study the function and structure of sodium ion channels (27), the evolutionary ecology of TTXs remains poorly understood. Whether TTX and its analogs are produced by multiple eukaryotic organisms, bioaccumulated through the food chain (23–25), or acquired from bacterial symbionts remains debated (28–30). In this study, we did not determine the source of TTXs; however, we show that TTX and 11-norTTX-6(*S*)-ol decrease in the process of feeding and accumulate over time. Other ecological studies of tetrodotoxin show its multiple uses for defense (10, 11) and pheromones (12), and now we show direct evidence for a prey capture function. Many basic ecological questions remain unstudied; i.e., do symbiotic bacteria produce TTX for their host, what is the function of TTX in bacteria, and what are the functions of the many TTX analogs? TTX remains an excellent compound for exploring the fundamental role of secondary metabolites in the ecology of both marine and terrestrial organisms.

Materials and Methods

Feeding Experiments. All flatworms were individually maintained in plastic basins (10.8 liters). To determine the potential diet breadth of planocerid sp. 1, a single individual of each prey species was placed in a basin with an individual flatworm and left for 24 h. Most of the prey species used to determine potential diet breadth were common animals collected from the same habitat as the flatworms. If this species was not eaten, it was replaced with a gastropod that the flatworms were known to eat. If the second gastropod was not eaten, the flatworm was assumed to not be hungry and the first gastropod species was not included in the eaten/not eaten list. Six flatworms were watched as they ate *C. moneta*, and the time it took from touching the cowry to removing its body from the shell was recorded. To determine whether the size of the prey limited prey capture, 35 flatworms were offered one cowry of the following species: *Cypraea annulus*, *Cypraea caputserpentis*, *Cypraea cribraria*, *Cypraea helvola*, *Cypraea isabella*, *Cypraea labrolineata*, or *C. moneta*. Multiple cowry species were used to obtain a range of size classes. After 24 h, the cowry was recorded as eaten or not eaten. If the original cowry was not eaten, it was removed and replaced with a smaller cowry. If the flatworm did not eat the second cowry, it was considered not hungry and excluded from the analysis. The formula for the surface area of a cylinder ($2\pi r^2 + 2\pi rh$) was used to approximate the surface area of the cowry, where the radius (r) was the length from the aperture to the edge of the shell and the height (h) was the length of the aperture. Because of the inherent plasticity of these flatworms, two photos of each flatworm were taken by using a Sony Mavica digital camera set on high resolution. Each digital photo was analyzed by using the program IMAGEJ to determine the surface area of the flatworm (the flatworm was assumed to be a two-dimensional object). The reported surface area for each individual flatworm is the average of the values for the two photos.

Chemical Analysis. Live flatworms were patted dry with a paper towel and weighed. They were then placed in individual vials and frozen. Seven flatworms were dissected into three regions before being frozen (Fig. 1*b*). All of the frozen samples were freeze-dried and then extracted in 0.05 M acetic acid (2 ml/g of flatworm). Each specimen was centrifuged at 18,600 × *g* for 30 min after which the supernatant was filtered (Millipore, 30,000 nominal molecular weight limit). The filtrate was diluted 10-fold with 0.05 M acetic acid and 5 µl of this sample solution was injected onto a HPLC-fluorescent detection system to analyze for TTX and its analogs. HPLC conditions are described in Shoji *et al.* (20), with the column temperature changed to 4°C. For further identification of TTXs, a part of the sample solution (1 µl) of pharynx of one specimen was applied to electrospray ionization mass spectrometer (TSQ700, Finnigan-MAT, San Jose, CA) by flow injection with MeOH at a flow rate of 0.2 ml/min.

Fish Deterrence Assays. Whole, live flatworms were offered to a natural assemblage of reef fish at two different locations on Guam [Gun Beach (N 13°31.460, E 144°48.101) and Fingers Reef (N 13°26.695, E 144°38.198)] at a constant depth of 7 m. A similar feeding assay was used to determine whether sea hares were protected from fish predators (31). For each replicate the fish were offered a control food of catfish food pellets (Cargill Aquafeed) and then a live flatworm. The fish *Abudefduf sexfasciatus*, *Abudefduf vaigiensis*, *Cheilinus fasciatus*, *Thalassoma lutescens*, *Thalassoma hardwickii*, and *Naso vlamingii* were ob-

served feeding on the flatworms. The number of flatworms and control food eaten was recorded, and the data were analyzed by Fisher's exact test.

Change in Toxin Concentration After Feeding. Flatworms were collected from the local reefs around Guam and were held in the lab without feeding for at least 2 days. Eleven randomly selected flatworms were removed and immediately frozen, the remaining flatworms were fed one cowry of the following species: *C. annulus*, *C. caputserpentis*, *C. cribraria*, *C. helvola*, *C. isabella*, *C. labrolineata*, or *C. moneta*. After they ate, the flatworms were randomly selected to be in one of three groups, 1 day, 4 days, or 8 days after feeding. We waited 24 h after feeding to ensure the flatworms had adequate time to digest their food and the weight of the meal did not influence total TTXs concentrations. Individual flatworms were frozen and extracted as described above. The concentration of TTX and 11-norTTX-6(*S*)-ol over the four time periods were rank transformed and then statistically compared by using a two-way ANOVA, followed by the Tukey-Kramer post hoc test.

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