THE SCENT OF DANGER: TETRODOTOXIN (TTX) AS AN OLFACTORY CUE OF PREDATION RISK

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Abstract. Larvae of the California newt (Taricha torosa) exhibit striking predator-avoidance behavior, escaping to refuges in response to a chemical cue from cannibalistic adults. In laboratory flow-tank experiments, stream water collected near free-ranging adults induced hiding responses in 100% of the larvae tested. Solutions prepared by bathing adults (in field and laboratory) also evoked strong hiding behaviors. Insensitive to adult feeding status (fed or starved), and clearly not an excretory product, the chemical cue was released from adult skin (i.e., in swabs of adult backs, sides, and bellies). Tetrodotoxin (TTX) was found in skin swabs of adults and in bathwater at 1 × 10^{-7} mol/L using reversed-phase high-pressure liquid chromatography (HPLC). Concentrations of 1 × 10^{-7} to 1 × 10^{-5} mol/L TTX standard, and equivalent dilutions of bathwater, triggered hiding behaviors in larvae, with no subsequent sublethal toxicity. The presence of TTX-sensitive cells within larval olfactory epithelium was confirmed by behavioral experiments and electrophysiological recordings. In contrast, larvae did not hide in response to two other, structurally mimetic compounds (saxitoxin and μ-conotoxin GIIB). Ontogenetically, larval behavioral responses to TTX and bathwater were strongest during weeks 3–5, diminishing to nil during week 7. No longer susceptible to adult cannibalism, larval indifference to the cue coincided with their ability to climb out of water and onto land.

Thus, newt larvae escape cannibalism by detecting a poison (TTX) well known as a chemical defense for conspecific adults. Eliciting a behavioral response in one case and inhibiting neural activity in the other, this compound results in opposing physiological effects, with avoiding predation as the common goal. Accordingly, TTX joins a select group of keystone molecules, each having critical, but different, ecological consequences at multiple trophic levels. The unique combination of bioactive properties makes a compelling case for asymmetrical selection as a force driving the evolution of adult–larval trophic interactions.

Key words: adult–larval interaction; amphibian; cannibalism; chemical ecology; larva; newt; predator; predator avoidance; prey; Taricha torosa; tetrodotoxin; TTX.

INTRODUCTION

Chemical signaling and ecological interactions

Understanding the mechanisms by which environmental chemical signals, chemical defenses, and other chemical agents mediate various life-history processes can lead to valuable insights about the forces driving the ecology and evolution of aquatic and terrestrial organisms. Sensory perception of chemical signals, for example, strongly influences predation (Stowe et al. 1987, Weissburg and Zimmer-Faust 1993), courtship and mating (Painter et al. 1998, Roelofs et al. 2002), aggregation and school formation (Miller et al. 1989, Griffiths and Magurran 1999), and habitat selection (Zimmer-Faust and Tamburri 1994, Linn et al. 2003, Sorensen et al. 2005). Additionally, prey organisms (animals, plants, and microbes) often produce chemical defenses that render their tissues unpalatable, or toxic, to consumers (Gil-Turnes et al. 1989, Daly 1995, Steinberg et al. 1995, Baldwin et al. 2006).

Establishing the principles that mediate chemical signal production and release is critical for interpreting biological responses to these stimuli within appropriate natural, historical contexts. The structures, concentrations, and fluxes of dissolved cues must be identified to determine chemical distributions over time and in space (Zimmer et al. 1999). This information is required for analyzing constraints imposed by natural physicochemical phenomena on biological responses at individual, population, and community levels. Moreover, properties of chemical signals must be dynamically scaled in laboratory and field experiments in order to simulate natural conditions (Weissburg 2000, Zimmer and But-
man 2000). Otherwise, serious artifacts can be introduced that render results equivocal.

**Cannibalism and chemically mediated prey behavior**

Cannibalism, or intraspecific predation, can play an integral role in regulating population dynamics (Fox 1975, Polis 1981, Elgar and Crespi 1992). Although kinship recognition and sibling cannibalism have received considerable attention elsewhere (Pomeroy 1981, Walls and Roudebush 1991, Pfennig et al. 1993, Walls and Blaustein 1995), here we focus only on intraspecific predation by adults. To avoid being eaten, larvae and juveniles often live in spatially discrete habitats, and have activity patterns that are temporally distinct from adults (Schultz 1981, Werner et al. 1983, Sih 1992). Even when forced to reside in poor quality environments, the benefit of reduced predation risk can balance the cost of limited resources (Sih 1980, Lima and Dill 1990). A lack of hospitable habitats may, however, severely constrain larval choice. Thus, physical separation between larva and adults is not always the best or only solution. Alternatively, larvae may avoid adults via incise behavioral reactions to stimuli produced by their cannibalistic elders. By taking strong evasive actions and by rapidly seeking refuge in response to adults, larvae can maximize resource use while minimizing predation risk.

Chemical signaling processes can mediate behavioral interactions between prey and predators (Zimmer and Butman 2000, Rohr et al. 2002). In some cases, the selective pressures leading to evolution of anti-predator behavior are known (see review of Kats and Dill 1998). For example, individuals of chemically or morphologically defended species often ignore predator scents, and thus continue fitness-enhancing activities (e.g., feeding and mating) in the presence of enemies. In contrast, animals lacking chemical or morphological defenses typically exhibit rapid behavioral (e.g., escape or hiding) reactions to predators upon detection of olfactory cues (Semlitsch and Gavasso 1992, Wisenden 2000, Rohr et al. 2002). Moreover, frightened or damaged prey may release compounds upon predatory attack. These “alarm” substances are often species specific in warning other prey of impending danger (see review of Chivers and Smith 1998), but also can attract secondary consumers that eat the primary attackers (Mathis et al. 1995, Chivers et al. 1996).

**Molecular identities of waterborne predator-avoidance and alarm cues**

Predator-avoidance and alarm reactions are expressed by individuals of hundreds of aquatic metazoan species (Chivers and Smith 1998, Kats and Dill 1998). The quaternary ammonium base, anthopleurine, and hypoxanthine-3-N-oxide act as strong alarm signals for sea anemones and ostariophysan fish, respectively (Howe and Sheikh 1975, Brown et al. 2000, 2003). Ammonium has been reported as a predator-avoidance (or disturbed-conspecific) cue for red-legged frogs, crayfish, and Antarctic krill (Hazlett 1990, Strand and Hamner 1990, Kiesecker et al. 1999). This compound is, however, a common product of nitrogen catabolism, and is excreted by all aquatic heterotrophic organisms including predators and prey (Randall et al. 1998). Consequently, ammonium may lack the required specificity to perform as an unmistakable signal of predation risk.

Other compounds have been assigned predator-avoidance or alarm properties. For example, the free-amino acid, l-serine, causes fleeing in salmon. Initially, effects of this substance were ascribed to its association with the skin of mammalian piscivores (seals and sea lions; Idler et al. 1956). Subsequent research showed that l-serine, as well as other free-amino acids, are avoided by salmon irrespective of the biological source (Rehberg and Schreck 1986). Amino sugars have been proposed as predator-avoidance cues to marine copepods and larval crabs (Cohen and Forward 2003, 2005), but bioassay-guided fractionations have not yet identified the natural products causing evasive reactions. Thus, although predator-avoidance and alarm behaviors have received considerable attention, molecular identities of waterborne cues have been unequivocally determined for only a few species. This, despite the fact that signal molecule identification is requisite for establishing the chemosensory basis for predator-prey interactions. Previous research was performed mostly with ill-defined chemical cues at unknown concentrations and fluxes. In general the ecological and evolutionary significance of such findings therefore is difficult to interpret.

**Olfactory basis and sensory ecology of cannibal avoidance**

Many young salamanders exhibit avoidance reactions or increased refuge use in response to dissolved chemical signals released from stream predators, such as fish, insect larvae, snakes, and cannibals (Petranka et al. 1987, Skelly and Werner 1990, Sih et al. 1992, Kiesecker et al. 1996). Similarly, larvae of the California newt, Taricha torosa, show particularly strong refuge use in response to adult-cannibal waterborne cues (Elliott et al. 1993). For this species, olfaction and anti-predator behavior play pivotal roles in larval survival and life-history evolution (Elliott et al. 1993, Kerby and Kats 1998). Here, we established the chemical signal molecule evoking an avoidance reaction of larval newts to their cannibalistic elders. Analytical measurements of chemical signal molecules were direct and definitive. This research facilitates future studies where concentrations and fluxes can be dynamically scaled to natural levels. It also builds a strong foundation toward understanding the olfactory basis of predator-avoidance behavior, and thus, provides critical linkages among physiological, ecological, and evolutionary mechanisms that mediate cannibalism.
MATERIALS AND METHODS

Collection of egg masses and larval culture

Egg masses of the California newt (Taricha torosa) were collected from stream pools during March–May (1998–2005) in Arroyo Sequit Canyon, Cold Creek Canyon, Tuna Creek Canyon, and Zuma Canyon (Malibu, California, USA), and brought to the laboratory. For 1–2 weeks prior to hatching, eggs were incubated in dechlorinated tap water and maintained on a 12:12 dark:light cycle (light on, 0700 hours) at 17–20°C. Water was filtered to 0.45 μm, aerated constantly, and changed on a daily basis. After hatching, small groups of 10–15 larvae were placed in plastic trays (150 mL capacity) containing rocks as potential refuges. The larvae were fed ad libitum on water fleas (Daphnia pulex) and black worms (Lumbriculus variegatus). Total body length and survivorship were determined for each animal on alternate days through the end of week 7 (post-hatch).

General procedures

Behavioral bioassays of larvae to test and control solutions were performed in a large flume and in small chambers. These two types of flow tanks have complementary characteristics. The flume (1.5 m long × 0.20 m wide channel; 0.04-m water depth) was designed to represent qualitatively the local flow regime upstream of a newt larva. It was intended for illustrative studies. Use of the flume was limited, however, by insufficient quantities of test chemicals. In contrast, there was ample aqueous material for replicated experiments in the much smaller chambers (0.07 m long × 0.03 m wide channel; 0.01-m water depth), which mimicked the proximal flow approaching a larva. Moreover, distributions and concentrations of chemical stimuli could be controlled precisely in the chambers. Delivery of scent on the scale of an individual larva was simulated in these small flow tanks. No individual larva was used in more than one experiment, or in more than one trial within an experiment. For each test or control treatment, we drew larvae from a minimum of three egg masses.

Incandescent bulbs (Daylight Ultra, General Electric, Cleveland, Ohio, USA) were placed within baffled housings to provide diffuse overhead lighting. A spectroradiometer (model S2000, Ocean Optics, Dunedin, Florida, USA) was connected to a miniature fiber optic probe, and light was measured underwater in flumes/chambers at the same positions as video observations of larvae. The mean photon flux (40 μmol/m²/s), spectral composition (intensity peaks between 430 and 540 nm), and angular distribution (sharp decline in intensity between 40° and 50° relative to the zenith) in the flumes/chambers were similar to morning sunlight within natural stream habitats.

Flume.—The flow-through channel of the flume consisted of a section of polyvinylchloride pipe, sliced in half horizontally to create a free surface. Flow was driven by gravity via an upstream head tank. From this tank, dechlorinated, 5-μm-filtered tap water, at stream (17–20°C) temperature, entered an expanded section of pipe and then passed through two custom fabricated honeycomb baffles (0.5-cm cell diameter × 10 cm length) to straighten the flow. Flume hydrodynamic regimes represented average conditions at the field sites. The mean flume flow speed (3.5 cm/s) and turbulence intensity (0.32) at 2 cm above bottom were similar to mean currents measured near larval refuges in the field (D. W. Schar and R. K. Zimmer, unpublished data).

To mimic natural rock refuges, a small, black Plexiglas plate (8 cm long × 8 cm wide) was suspended horizontally above the bottom along the flume centerline, 1.2 m downstream of the channel entrance. In each trial, a single larva was introduced into the flume. It immediately swam under the refuge, where it remained for 15–30 min before emerging to meander about. No larva ventured farther than 5 cm from the plate. Larvae near refuges in the field showed essentially the same behaviors.

Replicate channels, side by side, allowed for simultaneous, blind testing of two solutions. A test or control solution was introduced only after an individual emerged. Isokinetich chemical release (at 0.5 cm above bottom) was performed using sterile polyethylene tubing (1.5 mm inside diameter [ID]) and a microprocessor-controlled syringe pump. The input site was 0.5 m upstream of the refuge, along the flume centerline. Maximum concentrations associated with stimulus delivery were 1.02 ± 0.73 × 10⁻² (mean ± sd) times concentrations of source solutions, as determined by fluorometric measurements. After substituting fluorescein dye for chemical stimuli, fluorescence was recorded with a fluorometer (model 10-AU-005, Turner Designs, Sunnyvale, California, USA) at the upstream edge of the refuge. Water was continuously evacuated (1 mL/min) through a custom-built flow-through detector cell (50-μL volume). Peak dye concentrations were attained 13.3 ± 4.7 s (mean ± sd) after initial dye input (N = 8 replicate trials).

Using a high-resolution CCD camera placed 2 m above the channel, behavioral reactions of larvae were recorded over a 1-min interval following initiation of chemical release. Each larva was ultimately scored as either procuring refuge, or not, during this observation period. For select experiments, swimming speeds, angular velocities, and bearings of larvae exposed to test and control solutions were measured using computer-assisted video motion analysis (CAVMA; model VP 310, Motion Analysis, Santa Rosa, California, USA, ExpertVision, and custom software).

Chambers.—Like the flume, the chambers were flow-through systems, using dechlorinated, 5-μm-filtered, tap water at stream (17–20°C) temperature. Water was introduced, however, through a porous foam diffuser (porosity diameter = 100 μm) at the channel entrance. This technique greatly reduced the scales of motion and
evenly distributed momentum (mean velocity 0.8 cm/s) across the channel width. A thin (0.08 cm), black, Plexiglas plate (0.5 cm long × 3 cm wide) was suspended as refuge at 0.6 cm above bottom at the downstream end. Larvae never ventured farther than 4 cm from these plates. Bioassays were run simultaneously in 10 chambers, positioned side by side.

To begin each trial, a series of valves switched the flow from dechlorinated tap water to a test or control solution. This delivery system eliminated changes in pressure and air bubbles while switching lines. Upon introduction, chemical stimuli moved slowly downstream as a uniform front, with little mixing in the cross-channel direction. Throughout the chamber, stimulus intensity rapidly approached the source concentration. Fluorometric determinations indicated that maximum concentrations associated with stimulus delivery at the upstream edge of the refuge were 0.825 ± 0.015 times concentrations of source solutions (N = 12 replicate trials). Homogeneous distribution of the dye was established throughout the chamber within 30 s after initial delivery.

**Behavioral responses of larvae to adult-scented stream water**

Previous laboratory studies showed that adult newts release dissolved compounds and cause conspecific larvae to flee and hide (Elliott et al. 1993). Still undetermined was the extent to which water surrounding adults in natural stream habitats accumulated this dissolved chemical cue at behaviorally meaningful concentrations. In Arroyo Sequite Canyon, free-ranging adults were observed while they walked or swam in stream pools (20–40 cm depth). When an adult rested momentarily, local stream water was withdrawn gently (1 mL/s) via syringe pump through polyethylene tubing (1.0-mm ID). The tubing (4 m length) was attached to a thin (2-mm thick), stiff, stainless steel rod, and the opening positioned within ~5 cm of the adult torso. An observer maneuvered this hand-held assembly from shore without apparent disturbance to the animal. Over a two-month period, stream water (15–20 mL) was collected within the vicinity of each of 50 free-ranging adults. During this interval, control water (from adjacent pools without adults) was sampled using the same techniques. Following collection, each water sample was filtered immediately to 0.45 μm and put on dry ice for transport to the laboratory. Subsequently, water samples were combined in the laboratory to produce one test and one control solution. Bioassays were then performed in the flume on individual newt larvae (3–4 wk post-hatch).

**Source of adult chemical cue**

**Feeding status.**—To identify the source of the chemical cue, adult-conditioned water was made using adult newts collected by hand in Arroyo Sequite Canyon. Animals were used immediately on site or brought back to the laboratory. Adults were handled with sterile latex gloves and rinsed free of debris using dechlorinated tap water. In both field and laboratory assays, test solutions were prepared by bathing a total of 50 adult newts (~15 g of wet tissue mass per adult) for 1 h, each placed individually in 1 L of sterile tap water (same temperature as stream water at collection site). After 1 h, newts were removed, and each solution was membrane filtered to 0.45 μm, divided into 25 mL aliquots, and frozen for bioassays. Control solutions were prepared identically, but without the newts.

Adult-conditioned tap-water solutions were made using both starved and fed animals. In the field, guts were flushed of each associated adult after preparing solutions (Hanson et al. 1994: Methods). Stomachs were partially filled, containing mostly insects, worms, and an occasional conspecific larva. In the laboratory, adults were either fed ad libitum on water fleas and black worms, or starved, for three weeks prior to preparing bathwater solutions. Bioassays performed in the large flume thus tested for effects of feeding status (fed vs. starved) and site (laboratory vs. field) on adult release of chemical cue.

**Cloaca.**—Newts release secretions, feces, urine, and other metabolites through a common orifice, called a cloaca. Any of these products might function as the adult chemical cue that causes larvae to hide (Simon and Madison 1984). To determine if release of dissolved or particulate compounds from the cloaca induces a larval response, the orifice was sealed shut with inert silicon gel (Corning vacuum grease, Dow Corning, Midland, Michigan, USA). The gel (1 mL) was applied with a sterile applicator while holding each newt in a damp paper towel. Newts with sealed cloacae were then used in preparing an adult-conditioned tap-water solution (described previously). As controls, solutions were made using newts with gel on their tails, or by bathing a gel-coated microscope slide.

The effectiveness of the gel seal on the cloaca was evaluated before performing bioassays. To each of eight adults, a small volume (0.1 mL) of concentrated (1g/L) fluorescein dye solution was inserted into the cloaca. The cloaca was then sealed with 1 mL of gel, and each adult was placed separately into a plastic container of 250-mL dechlorinated tap water. Over 1 h, water was continuously evacuated (1.0 mL/min) from each container and monitored for fluorescence. No measurable fluorescence was recorded from water sampled in any trial, indicating that the seal was indeed watertight.

**Skin.**—Newt skin is an important site of chemical exchange (Randall et al. 1998). To determine if skin was the location of cue release, solutions were prepared from the back, side, and belly of adults at Arroyo Sequite Canyon. In separate trials, sterile cotton swabs were dipped in dechlorinated tap water, then rubbed on the back, side, or belly of adult newts for 1 min. Swabs were immersed into 1.5 mL of dechlorinated tap water in sterile tubes, and placed on dry ice. Identical procedures
were also used in the laboratory to prepare solutions from skin swabs of 3–4 week old newt larvae. As a control for nonspecific compounds from amphibian skin, solutions were made on site from the tree frog, *Hyla cadaverina*, using the same methods. Additional control solutions were readied by immersing swabs in tap water, but without touching either frog or newt skin. Each swab rinse was ultimately diluted to 200 mL with tap water before bioassays in the small chambers.

**Identity of the adult chemical cue**

Adult newts produce tetrodotoxin (TTX) as a chemical defense against vertebrate predators (Brodie 1968). Highest concentrations of this water-soluble toxin are found on skin surfaces (Hanifin et al. 2004). In our bioassays, larvae fled and hid only in response to substances diffusing from adult newt skin (see **Results**: Adult skin is the source of a hiding cue). These findings called for additional experiments to determine if TTX functions as an adult chemical cue stimulating larval flight and concealment behaviors.

To establish bioactivity levels in all assays, TTX concentrations were measured for test and control solutions. Aqueous samples of swab rinses and adult-conditioned waters were reacted with o-phthalaldehyde (OPA) reagent to generate fluorescent TTX derivatives before separation by reversed-phase HPLC. A commercial standard (Sigma-Aldrich, Saint Louis, Missouri, USA) was used to identify the reaction time of a fluorescent TTX derivative. Fluorescent products were isolated on an ultrasphere octadecyl silane (ODS) column (4.6 × 25 cm, 5-μm particle size; Beckman Coulter, Fullerton, California, USA) using modified procedures of Roth (1971) and Onoue et al. (1983). The column was equilibrated in solvent A, and 1 min after injection, a linear gradient was run from 0% to 20% solvent B in solvent A for 15 min (solvent A, 1:19:80 tetrahydrofuran: methanol: 0.05 mol/L sodium acetate; solvent B, 80:20 methanol: 0.05 mol/L sodium acetate). Both solvent mixtures had a pH of 6.8. Derivatives were detected after elution (1 mL/min) from the column using a spectrofluorimeter (Jasco, Easton, Maryland, USA). Peaks were monitored at 453 nm using an excitation wavelength of 332 nm, and integrated by System Gold Nouveau software (Beckman Coulter).

Three experiments were performed in the small chambers, with the application of dechlorinated tap water serving as the control in each case. (1) Pure TTX standard was bioassayed at concentrations from 1 × 10^-7 to 1 × 10^-3 mol/L, along with the same concentration of TTX in adult-conditioned water. Responses of larvae to standard and adult-scented waters would be similar if TTX is the cue. A positive result was succeeded by the following two experiments. (2) To test for an ontogenetic response to TTX, larvae were exposed weekly to 1 × 10^-7 mol/L TTX in standard and adult-conditioned water. Bioassays began 3 d after larvae hatched and continued through week 7. As before, each individual larva was used only in one trial. (3) Larvae were bioassayed with TTX, saxitoxin (STX), and μ-conotoxin GIIB (CTX) at 1 × 10^-7 mol/L. This final experiment tested the specificity of larval response to TTX, relative to other neurotoxins.

**Physiological correlates for behavioral responses**

Amphibians can detect chemical stimuli by either a sense of smell or taste (Kauer 2002). An experiment was therefore performed to distinguish between these two sensory modalities. The olfactory passageways of 30 larvae (2–3 weeks post-hatch) were blocked by applying inert silicon gel (0.1 mL) to the external openings, or nares, with a cotton swab. Each individual was then bioassayed (in a small chamber) with either 1 × 10^-7 mol/L TTX or dechlorinated tap water. Fifteen trials were conducted using each stimulus. To control for animal handling, a second group of 30 animals was tested in the same manner. This time, however, dechlorinated tap water was applied to their nares and gel to their foreheads prior to testing. As an additional control, neither tap water nor gel was applied to the heads of a third group of 30 larvae, which were bioassayed as before. If control animals from both groups showed strong hiding reactions to TTX, but larvae having blocked nares did not, then olfaction would be implicated as the principal sensory modality.

Electrophysiological recordings were made from the olfactory epithelium of newt larvae to complement behavioral bioassays. Each larva was prepared for extracellular recording by decapitating and removing the lower jaw. Then, the head was pinned to a Sylgard recording chamber filled with amphibian Ringers solution (in mmol/L: 140 NaCl, 10 KCl, 1.8 CaCl2, 2 MgCl2, 5 HEPES; pH 7.2), so that a recording electrode could be placed within the olfactory epithelium. A reference electrode was positioned on the head, and a ground electrode put into the bath underneath the head. Each of these 3 mol/L NaCl/agar-filled (1–3%) glass electrodes had a tip diameter of ≈10–20 μm and was made of silver/silver chloride wire. Responses of the epithelium to applied chemical stimuli were filtered at 1–2 kHz using a low-noise, differential, DC amplifier. The output was further amplified, then displayed on an oscilloscope and stored digitally (100 Hz; Digidata 1200 A/D board and Axotape software, Axon Instruments, Union City, California, USA).

Electrical activity was recorded in response to TTX, forskolin, and artificial freshwater (AFW), the latter two solutions serving as controls. Forskolin diffuses across olfactory receptor cell membranes, activates adenyl cyclase, and leads to the opening of ion channels and action potential generation. Its application thus controlled for the viability of preparations. Each test or control solution was introduced as a small-volume bolus (50 μL) into a continuous stream of AFW that bathed the olfactory epithelium. Following each application, a solution was rinsed free from a preparation by the AFW.
stream. The order of test or control presentation was determined for each preparation using a random numbers generator.

The California newt is registered as a U.S. Fish and Wildlife Service "species of concern," and native populations are at historical lows. To minimize impacts on wild populations, a maximum of four egg masses were collected from any given stream over each calendar year. Because each, successful, electrophysiological recording required the sacrifice of many larvae, we kept the number of replicates to a minimum. Short preparation viability (due to decapitation) further constrained the number of stimulus treatments, and precluded the generation of dose-response curves. It was exceedingly difficult to find and record from the tiny region (~200 µm length) of olfactory epithelium associated with the internal nares. Although preliminary, electrophysiological data were valuable in confirming behavioral results.

RESULTS

Newt larvae hide in response to adult-scented stream water

Newt larvae showed striking behavioral responses to adult-scented water. The test solutions (natural, adult-conditioned, or control stream water) were introduced only after a larva had emerged from underneath a shaded refuge. Control water evoked no response in 10/10 larvae. The larvae swam in short bursts between long pauses of rest (Fig. 1). In contrast, immediately upon contacting adult-derived waterborne compounds, all 10 larvae swam significantly faster without pause (Table 1). Once a refuge was apparently detected visually, however, they moved rapidly and on a straight trajectory to the hiding place. From the point of stimulus contact, larvae swam directionally, upstream or downstream, depending on refuge location, and thus, the behavior was not simply an aversive reaction to a noxious chemical.

Adult skin is the source of a hiding cue

Clean stream water, when used to bathe adults, caused strong larval hiding reactions relative to controls (Fig. 2A). Blocking of the adult cloaca with inert gel did not diminish bathwater bioactivity, indicating that the signal molecule was not an excretory product (Fig. 2B). Similarly, adult feeding status did not affect the outcome (Fig. 2A). In bioassays using sterile cotton swabs of the backs, sides, and bellies of adults (rinsed with tap water), larvae ignored the swab controls and always sought refuge in response to swabs of adults, irrespective of body part (Fig. 2C). In contrast, swab rinses of tree frog (Hyla cadaverina) and newt larva skins failed to induce a behavioral reaction. Taken together, these behavioral studies strongly infer newt adult skin as the source of the cue.

Tetrodotoxin (TTX) is the hiding signal molecule

Newt larvae showed an unequivocal hiding response to tetrodotoxin. Reversed-phase HPLC analysis revealed the presence of TTX in adult skin swab rinses, and in bathwater at 1 × 10⁻⁷ mol/L (Fig. 3). Moreover, adults released TTX into bathwater at ~5 × 10⁻¹³ mol/s/cm² of skin surface. In contrast, this poison was not detected by HPLC in newt larva or tree frog swab rinses

![Diagram](https://via.placeholder.com/150)

**FIG. 1.** Swimming behavior of a four-week-old larva. In this trial, dilute fluorescein dye (0.1 g/L) was added to visualize the distribution of adult-conditioned stream water following introduction in a flume. The larva alternated movement with nearly motionless pauses ("p") before making contact with adult scent. Once contact was made ("hits," small black arrow), the larva swam quickly in a straight line to a refuge. Closed circles correspond to video images captured at 0.1 s, and the arrowhead shows the direction of travel. The starting position is denoted by an "X."

<table>
<thead>
<tr>
<th>Adult scent</th>
<th>Mean speed (cm/s)</th>
<th>Maximum speed (cm/s)</th>
<th>Maximum acceleration (cm/s²)</th>
<th>Pause frequency (to/s)</th>
<th>Pause duration (s)</th>
</tr>
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<tbody>
<tr>
<td>Present</td>
<td>13.9 ± 0.5</td>
<td>17.9 ± 0.7</td>
<td>80.4 ± 14.8</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Absent</td>
<td>4.2 ± 0.4</td>
<td>8.7 ± 0.5</td>
<td>58.1 ± 6.4</td>
<td>0.17 ± 0.03</td>
<td>5.9 ± 0.9</td>
</tr>
</tbody>
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**Note:** Values reported are means ± SE.

**P < 0.01; ***P < 0.001. Significance was determined using a Student's two-tailed t test with 18 degrees of freedom.
(data not shown). A TTX standard was bioassayed at concentrations from $1 \times 10^{-7}$ to $1 \times 10^{-9}$ mol/L, along with equivalent dilutions of bathwater. At each concentration, bathwater and the corresponding TTX standard triggered almost identical hiding responses (Fig. 4). The presence of dissolved TTX was therefore necessary and sufficient to promote observed bioactivity in larvae. **Larvae exhibit an age-dependent hiding response to TTX**

Striking ontogenetic changes in larval chemosensory-mediated behavior were associated with major developmental events. For the first 1–2 weeks after hatching, larvae lacked a fully grown tail or legs. While incapable of much propulsion, they thus remained hidden at most times under refuges and failed to behaviorally react when exposed to adult bathwater or TTX (Fig. 5). Beginning in weeks 2–3 (post-hatch), larvae have a functional tail and forelegs, become able swimmers, and venture away from refuges to search for food. At this stage, larvae first reacted to waterborne chemical stimuli. Hiding responses to adult bathwater and TTX were strongest during weeks 3–5, weaker during week 6, and disappeared thereafter (Fig. 5). No longer suscep-

**Fig. 2.** Percentage of larvae hiding under a refuge in response to adult-conditioned or control stream water. Ten larvae were bioassayed with each solution, and significance levels were determined (test vs. control) using a Fisher exact test ($***P < 0.001$). Each individual larva was tested only once. (A) Field-collected adults were handled with sterile polyethylene gloves and bathed in containers of clean stream water (0.45-μm filtered) for one hour at ambient stream temperature. Bathwaters were prepared with 15 g (wet mass) live adult per liter. Following adult removal, conditioned water was refiltered, placed on dry ice, and stored at −80°C until used in bioassays. In the laboratory, adults were fed an ad libitum diet of California black worms (*Lumbriculus variegatus*) and water fleas (*Daphnia pulena*) or else starved for three weeks before solution preparation. (B) To test if behavior-altering chemicals were derived from excretory products, bathwater was conditioned as above using adults with cloaca sealed shut by inert silicon gel. Bathwater controls were prepared using (1) adults with gel placed on their tails instead of cloacae, (2) microscope slides with gel, or (3) adults without gel additions. The gel seal was watertight (see Methods). (C) To determine if skin was the source of behavior-altering chemicals, sterile cotton (0.5 g), dipped into dechlorinated tap water, was used to gently swab either the back, side, or belly of an adult for one minute. Swabs of frog (*Hyla cadaverina*) or newt larvae backs were prepared as controls. Ultimately, each swab was immersed in 1.5-mL dechlorinated tap water and diluted (1000×), before bioassay. Tap water rinses of sterile cotton swabs were also tested as additional controls.
tible to adult cannibalism, complete cessation of larval response to TTX coincided with hind-leg development and the capacity to climb out of water onto land.

**Larvae hide in response to TTX, but not to other neurotoxins**

Larval hiding behavior is not a generalized reaction to all neurotoxins. Neither of two additional sodium channel blockers, saxitoxin (STX) and μ-conotoxin GIIIB (CTX), each bioassayed at $1 \times 10^{-7}$ mol/L, evoked a hiding response (Table 2). Larvae exhibited a minor sublethal toxic reaction (muscle tremors) to CTX, distinct from all other solutions. After being tested, however, each larva survived to the end of this study (through week 7) with no significant difference in growth rate between treatment groups (Table 3, Fig. 6).

Although chemical structures were similar for all three neurotoxins tested, TTX was the only effective stimulant of larval behavior.

**TTX evokes an excitatory response from larval olfactory epithelium**

Newt larvae possess an olfactory epithelium that lines the ventromedial walls of a nasal cavity. The cavity has external and internal openings, or nares, and extends from the outer skin surface to the inner roof of the mouth (Fig. 7A, B). In behavioral experiments, larvae with nares blocked by inert silicon gel did not react to $1 \times 10^{-7}$ mol/L TTX, whereas control animals immediately took refuge in response to the same stimulus (Fig. 8). When recording electrodes were placed near the external nares of larvae, electro-olfactograms showed no
responses by the epithelium to TTX (data not shown; \( N = 6 \) animals). In contrast, identical electrodes, positioned near the internal naris, recorded excitatory (depolarization) responses to forskolin and TTX (Fig. 7C, \( N = 2 \) animals). Relative to TTX, forskolin responses exhibited longer lags in onset and offset times. Because forskolin does not bind to receptor proteins, it takes longer for this compound to diffuse across olfactory cell membranes and activate a cAMP transduction pathway (via adenylyl cyclase). Alternatively, application of artificial freshwater control (AFW) did not induce a change in electrical activity.

**DISCUSSION**

**TTX has opposing physiological effects with contrasting ecological consequences**

California newt larvae (*Taricha torosa*) escape cannibalism by detecting a poison well known as a chemical defense for conspecific adults (Buchwald et al. 1964, Mosher et al. 1964, Hanifin et al. 1999, Brodie et al. 2005). Following release from adult skin, TTX acts as a reliable olfactory cue, warning larvae of imminent danger. For individuals at different life-history stages of the same newt species, TTX thus operates in a dual role, both alerting (conspecific larval) prey and defending (adults) against predators. Stimulating a behavioral response in one case and inhibiting neural activity in the other, this compound has opposing physiological effects with strong, but contrasting, ecological consequences.

The olfactory capabilities of an organism provide vital links between chemical signal production/transmission and behavior, and between individual behavior and larger scale dynamics that structure populations and communities (Zimmer and Butman 2000). The nervous system is thus an essential filter for translating environmental features into sensory stimuli, and then into a behavioral task. Studies on the molecular identities of signal molecules, particularly in conjunction with behavioral and population studies, can fill a critical need in establishing linkages between stimulus space, behavior, and demographic consequences of decisions made by individual organisms.

Ultimately, field and laboratory experiments must be dynamically scaled to reproduce natural patterns of contact between signal molecules and biological sensors.
The type and magnitude of behavioral response are dependent on rates of signal production and release (concentration, flux), and hence, on stimulus encounter frequencies (Elkinton et al. 1984, Zimmer-Faust et al. 1988, Moore and Atema 1991, Weissburg 2000). Chemical identification is therefore required as a first step toward achieving dynamic scaling. Surprisingly, given their seminal roles in mediating kin recognition and cannibalism, especially among amphibian species (Petranka et al. 1987, Mathis et al. 1995, Kiesecker et al. 1996), few signal molecules have been determined. For Taricha torosa, knowledge of TTX as an adult-cannibal avoidance cue in the larvae leads to insights about physiological mechanisms, ecological consequences, and the evolution of trophic interactions, as discussed next.

**Opposing TTX effects may arise from differences in protein binding affinities**

TTX is a remarkably versatile molecule. As a poison, it targets voltage-gated, sodium channel proteins on membranes of electrically excitable cells. Binding tightly and competitively to these proteins, TTX blocks sodium ion conductances, while inhibiting action potential generation in nerve and muscle tissues (Narahashi 1994, Cestèle and Catterall 2000). Like other alkaloids, however, TTX effects are ultimately dose dependent. A number of poisons have dual or multiple functions, causing lethality at high concentration and acting as feeding attractants or pheromones at low concentrations in resistant consumer species (Weller et al. 1999, Bernays...
Figure 8. Effect of eliminating olfactory input on behavioral responses to TTX. Data are percentages of larvae hiding under refuges in response to 1 × 10−2 mol/L TTX or control tap water. The larvae had either gel applied to block the nares, gel put on the forehead (control), or no gel addition (control). For each test or control treatment, 15 larvae were bioassayed with each solution. Significance levels were determined as in Fig. 2 (***P < 0.01). Even though larvae were younger (2–3 weeks post-hatch) and less responsive, as compared to other experiments, TTX evoked highly significant hiding reactions in the gel-on-forehead and no-gel treatments.

et al. 2002). Similarly, we found that unnaturally high concentrations of TTX (1 × 10−5 mol/L TTX) in stream water induce muscle tremors and morbidity in California newt (Taricha torosa) larvae (R. P. Ferrer and R. K. Zimmer, unpublished data). As a signal molecule, however, 100- to 10 000-times more dilute TTX concentrations (1 × 10−7 to 1 × 10−9 mol/L) invoke predator-avoidance behavior in T. torosa larvae. These opposing physiological effects are dose dependent, inhibiting/stimulating larval neuromotor activity at high/low concentrations, respectively. Such disparate effects may arise because of differences in TTX binding affinities of the olfactory receptors (high) and voltage-gated sodium channel (low) proteins. In fact, protein sequences of sodium channels varying by even one amino acid residue express notably different TTX binding affinities (Kaneko et al. 1997, Yotsu-Yamashita et al. 2000).

Not all receptors are created equal. Voltage-gated sodium channels and chemosensory receptor proteins apparently diverged in their TTX-binding specificities. The heterocyclic guanidines, tetrodotoxin (TTX) and saxitoxin (STX), and peptide μ-contraxin GIIB (CTX) are all structurally mimetic. Each of these compounds binds to the same extracellular receptor site 1 of sodium channel protein surfaces (Satin et al. 1992, Cestéle and Catterall 2000, Olivera 2002). Until recently, TTX and STX were thought to bind with sodium channels in exactly the same manner (Penzotti et al. 1998). Thus, TTX-binding requirements of sodium channels are relatively tolerant of differences in ligand structure. In contrast, predator-avoidance responses of newt larvae, presumably mediated by olfactory receptor proteins, are intolerant and more narrowly tuned. Larvae hide in the presence of dilute TTX, but exhibit no behavioral response to CTX or STX. This high degree of specificity may reflect very stringent requirements for ligand binding by TTX-sensitive olfactory receptors. Electrophysiological findings for rainbow trout (Salmo gairdneri) and Arctic char (Salvelinus alpinus) support the hypothesis of narrow tuning at the cellular, or receptor, level (Yamamori et al. 1988). Each fish species has two different populations of gustatory neurons, harboring receptors for either TTX or STX, respectively. Whereas one cell population responds only to TTX, the other detects exclusively STX.

Asymmetrical selection drives the evolution of TTX-mediated trophic relationships

TTX-mediated interactions between adult newt prey (Taricha granulosa and T. torosa) and garter snake predators (Thamnophis sirtalis and T. couchii) are valuable systems for investigating the evolution of physiological adaptations. Although garter snakes are adapted for consuming dangerous newt prey (Brodie 1968, Geffeney et al. 2002, 2005), their relationships with Taricha species may not be reciprocal and coevolutionary, as proposed previously (Brodie and Brodie 1999, Brodie et al. 2005). Coevolution within a predator/prey system requires a tight association between species. Typically, a given prey has one (or, at most, a few) predator(s), and vice versa (Janzen 1980, Futuyma and Slatkin 1983, Berenbaum and Zangerl 1992). To date, no quantitative field data link garter snake predation and adult newt mortality rates. Gut content analyses also reveal that garter snakes do not forage preferentially on adult newts (Kephart 1982, Arnold 1992). In fact, there is no predictive relationship between garter snake predation pressure and frequencies of genotypes within a newt population. A compelling alternative to the predator/prey arms race hypothesis is asymmetrical selection. Over its lifetime, a newt is potentially vulnerable to a wide range
of invertebrate (insect larvae, crayfish) and vertebrate (fish, snakes, bullfrogs, owls, waterfowl, adult cannibals) predatory species (Kaplan and Sherman 1980, Marshall et al. 1990, Gamradt and Kats 1996, McAllister et al. 1997, Jennings and Cook 1998, Kats et al. 1998, Mobley and Stidham 2000). To be effective, TTX must therefore act as a general chemical defense against a diffuse predator network. Adult survivorship might not be a principal demographic bottleneck. As with other animal taxa, embryos and larvae could be especially vulnerable to predation and suffer the highest mortality rates (Orians and Janzen 1974, Fox 1975, Morgan 1995). These early life-history stages may be the most critical bottlenecks to population growth, and thus would be prime targets for intense selective pressures that drive the evolution of behavioral, morphological, and/or chemical defenses.

The California newt (Taricha torosa) is defended by TTX as an embryo, juvenile, and adult (Twitty 1937, Buchwald et al. 1964, Mosher et al. 1964, Kats et al. 1992, Brodie et al. 2003), but not in the larval stage. Our HPLC analyses and bioassays of swab rinses demonstrate an absence of TTX on larval skin, thereby suggesting a window of vulnerability. This absence of poison is a trait shared by larvae of other, chemically defended newt species (Mathis and Vincent 2000). Evidently, toxin is provisioned by females in eggs (Hanifin et al. 2003), but retained only until embryos hatch from protective gelatinous masses. Poison glands develop after larvae undergo rapid growth and limb generation in an initial post-hatch period (of several weeks; Mathis and Vincent 2000).

The apparent window of larval vulnerability to predation might be explained by the growth–differentiation balance (GDB) hypothesis. As conceptualized originally for plants, this theory is premised upon a physiological trade-off between growth (cell division and enlargement) and differentiation (chemical and morphological changes leading to cell maturation and specialization) processes (Herm and Mattson 1992, Cronin and Hay 1996). Shared biosynthetic pathways give rise to amino acid precursors of protein and chemical defense compounds (including alkaloids, such as TTX). Alkaloid synthesis, with its amino acid...
precursors and huge energetic expense, can compete directly with protein synthesis, and consequently with growth (Lindsey and Yeoman 1983, Jensen 1985, Hegnauer 1988). Hence, all physiological requirements may not be met simultaneously, and a strong inverse relationship can occur between the allocation of resources to growth and chemical defense.

The benefit of morphological, or physiological, adaptation often comes at a cost in performance trade-offs (Futuyma and Moreno 1988, Roff 1996). Yet, a period of rapid growth and limb development may be more effective than TTX biosynthesis in reducing overall predation risk. Our results show elsewhere that six-week-old (post-hatch) newt larvae effectively escape capture by invertebrate predators, and achieve size refuge from gape-limited newt adults (R. P. Ferrer and R. K. Zimmer, unpublished data). Because newt adults consume TTX-laden conspecific eggs, they apparently have some resistance to the poison. For this reason, morphology (larger size), and concomitant neuromuscular performance (faster locomotory speed), as opposed to chemistry, should provide newt larvae with the best defense against cannibalism. Such traits are particularly valuable if intraspecific, not interspecific, predation is a principal source of larval mortality and a factor limiting prey fitness. Additional research clearly is needed to establish the relative contributions of morphological and chemical defenses in shaping survivorship, as integrated over an entire individual lifetime.

Because cannibalism is often a critical determinant of larval survivorship (Fox 1975, Polis 1981, Elgar and Crespi 1992), strong selective pressures can shape the evolution of prey behavior (Crowley and Hopper 1994). Newt larvae hide in response to a waterborne cue (TTX) emanating from their cannibalistic elders, but do not react to chemical stimuli of other predatory species (Elliott et al. 1993). Thus, these larvae are sensitive uniquely to the danger posed by cannibalism. In contrast, adult newts are, themselves, prey for a suite of vertebrate predators (Brodie 1968, McAllister et al. 1997, Jennings and Cook 1998, Kats et al. 1998, Mobley and Stidham 2000). Evidently, selection favors adult acquisition of TTX as a chemical defense, more than elimination of this signal molecule as a warning to conspecific larvae. As feeding generalists, adult Taricha torosa dine on a taxonomically diverse prey assemblage including, primarily, insects, worms, snails, and other small invertebrates (Stebbins 1972, Hanson et al. 1994). In fact, adults preferentially prey on worms over conspecific young (Kerby and Kats 1998), and there is no evidence for adult adaptations targeting, specifically, cannibalism. The trophic relationship between newt larvae and adults apparently has resulted from asymmetrical selection. Both predator and prey have evolved in response to their own enemies, rather than through a reciprocal, and coevolutionary, interaction with one another (Vermeij 1994).

TTX is a molecule of global ecological significance

The criticism of chemical ecology as a reductionist subdiscipline (sensu Brown 1995) is largely unsubstantiated. For the last 30 years, or longer, chemical ecologists have worked hard to advance empirical and general theoretical frameworks. As examples, optimal defense theory, the growth–differentiation balance hypothesis, and Jensen’s inequality (applied to plant–herbivore interactions) have provided benchmark intellectual achievements in weaving chemical ecological principles into the fabric of basic ecological theory (McKee 1974, Rhodes and Cates 1976, Herms and Mattson 1992, Ruel and Ayers 1999). Through empirical studies, chemical ecology also has demonstrated profound consequences for populations and communities (Steinberg et al. 1995, Hay and Fenical 1996, Peckarsky et al. 2002). Direct effects of TTX mediate multitrrophic interactions in California mountain streams. Moreover, dimethylsulfiniopropionate (DMSP), and its metabolites (dimethylsulfide [DMS] and acrylic acid), convey information that accelerates material exchange rates between bacteria (decomposers), phytoplankton (primary producers), zooplankton (secondary consumers), and seabirds (apex predators) in open ocean habitats (Nevitt et al. 1995, Zimmer-Faust et al. 1996, Wolfe 2000). To our knowledge, however, TTX is unique among all signal/defense molecules in two important aspects: (1) it is harbored by metazoans of diverse phylogenetic origins (e.g., phyla Nematoda, Platyhelminthes, Mollusca, Arthropoda, Chaetognatha, Echinodermata, and Chordata; Kim et al. 1975, Sheumack et al. 1978, Miyazawa et al. 1986, Thuesen et al. 1988, Matsumura 1995, Kogure et al. 1996) and (2) it has a near-cosmopolitan biogeographical distribution within marine, freshwater, and terrestrial habitats (Kim et al. 1975, Sheumack et al. 1978, Miyazawa et al. 1986, Thuesen et al. 1988, Matsumura 1995, Kogure et al. 1996). Globally, TTX functions as a chemical defense for prey, a predator venom to subdue prey, a predator-avoidance cue, and even a sex pheromone. Thus, it is the chemical equivalent of an ecological keystone species (sensu Paine 1966). Such remarkable flexibility arises from the contrasting impacts of different concentrations, and makes TTX a bioactive molecule of considerable ecological significance.

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