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The scent of danger: arginine as an olfactory cue of reduced predation risk

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Summary

Animal perception of chemosensory cues is a function of ecological context. Larvae of the California newt (*Taricha torosa*), for example, exhibit predator-avoidance behavior in response to a chemical from cannibalistic adults. The poison tetrodotoxin (TTX), well known as an adult chemical defense, stimulates larval escape to refuges. Although they are cannibals, adult newts feed preferentially on worms (*Eisenia rosea*) over conspecific young. Hence, larval avoidance reactions to TTX are suppressed in the presence of odor from these alternative prey. The free amino acid, arginine, is abundant in fluids emitted by injured worms. Here, we demonstrate that arginine is a natural suppressant of TTX-stimulated larval escape behavior. Compared to a tapwater control, larvae initiated vigorous swimming in response to 10^{-7} mol l^{-1} TTX. This excitatory response was eliminated when larval nasal cavities were blocked with an inert gel, but not when gel was placed on the forehead (control). In additional trials, a binary mixture of arginine and 10^{-7} mol l^{-1} TTX failed to induce larval swimming. The inhibitory effect of arginine was, however, dose dependent. An arginine concentration as low as 0.3-times that of TTX was significantly suppressant. Further analysis showed that suppression by arginine of TTX-stimulated behavior was eliminated by altering the positively-charged guanidinium moiety, but not by modifying the carbon chain, carboxyl group, or amine group. These results are best explained by a mechanism of competitive inhibition between arginine and TTX for common, olfactory receptor binding sites. Although arginine alone has no impact on larval behavior, it nevertheless signals active adult predation on alternative prey, and hence, reduced cannibalism risk.

Key words: arginine, amino acid, tetrodotoxin, TTX, newt, salamander, *Taricha torosa*, predator, prey, cannibalism, chemical signal, olfaction, adult–larval interaction, predator avoidance.

Introduction

A number of poisons have dual or multiple functions, including acting as chemosensory stimuli for resistant consumer species (Weller et al., 1999; Macel and Vrieling, 2003). Foraging caterpillars, for example, respond to host plant pyrrolizidine alkaloids as contact chemical cues and feeding stimulants (Bernays et al., 2000; Bernays et al., 2002). Once consumed, however, the alkaloids are sequestered by caterpillars in their own defense (Hartmann and Witte, 1995; Schulz, 1998; Nishida, 2002). Later in life, they are used by post-metamorphic adult female moths as precursors for the synthesis of mate attractants (Bell and Meinwald, 1986; Schulz et al., 1993). Poisonous substances can thus play critical roles in mediating a wide range of ecologically relevant behaviors.

Chemosensory environments in most terrestrial and aquatic habitats are characterized by mixtures of odors, rather than by the isolated scent of any single compound (Knudsen et al., 1993; Ache and Young, 2005). Behavioral responses of animals to chemical mixtures often are greater than (synergism) or less than (suppression) the predicted sum of responses to individual mixture components (Zimmer-Faust et al., 1984; Carr and Derby, 1986; Daniel and Derby, 1987; Smallegange et al., 2003). Chemical attraction of prey animals to food, for example, is suppressed in the presence of predator odor (Lima and Dill, 1990; Gillette et al., 2000). By contrast, adult male attraction is amplified significantly by release of a pheromone mixture (as compared to single compounds) from a sexually receptive female (Cardé et al., 1975; Murlis et al., 1992; Cummins et al., 2006). Consequently, animal perception of chemosensory cues is a function of ecological context (Zimmer-Faust, 1987; Zimmer-Faust, 1993; Ziesmann, 1996).

The California newt (*Taricha torosa*) has a rich history of ecological interactions mediated by neurotoxins (Buchwald et al., 1964; Mobley and Stidham, 2000; Brodie et al., 2005). Larvae escape cannibalism by detecting the poison tetrodotoxin (TTX) in adult conspecifics, where it functions as a chemical defense (Zimmer et al., 2006). By contrast, the toxin is not found in the larval stage. Following release from adult skin, TTX acts as a reliable behavioral cue, warning young of

imminent danger. The presence of TTX-sensitive cells within larval olfactory epithelium has been confirmed by electrophysiological recordings. Concentrations of 10^{-7} to 10^{-9} mol l^{-1} TTX in natural, adult-scented stream water stimulate effective predator-avoidance and refuge-hiding behaviors in larvae (Zimmer et al., 2006). Once a refuge is detected visually, larvae move rapidly and on a linear trajectory to the hiding place. From the point of TTX contact, they swim directionally, upstream or downstream, depending on refuge location, and thus, the behavior is not simply an aversive reaction to a noxious chemical.

These behavioral responses of larvae to TTX are, however, context dependent. 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 feed preferentially on worms over conspecific young, and there is no evidence for adult adaptations specifically for cannibalism. Moreover, larval avoidance and hiding responses to TTX are suppressed in the presence of odor from alternative worm prey (Kerby and Kats, 1998). Suppression frees the larvae to perform other fitness-enhancing activities (such as foraging), rather than wasting time and energy seeking refuge. In this study we identified the free-amino acid, arginine, as a natural suppressant of TTX-stimulated behavior in California newt larvae. Arginine was chosen for study because: (1) it is abundant in fluids emitted by injured worm prey (*Eisenia rosea*) of adult newts (Ferrer and Zimmer, 2007), and (2) the structural moiety (guanidinium group) also present in TTX plays a seminal role in receptor binding (Hille, 1975; Kao, 1986; Lipkind and Fozzard, 1994). This is the first of two companion papers describing the behavioral mechanisms and ecological consequences of an ontogenetic shift in chemosensory reception in the California newt. The present paper is written from the perspective of the larval prey. By contrast, the accompanying paper is written from the perspective of an adult cannibal, with comparisons to the larval stage (Ferrer and Zimmer, 2007).

Materials and methods

Collection of egg masses and larval culture

Egg masses of *Taricha torosa* Rathke 1883 were collected from Arroyo Sequit Creek and Tuna Canyon Creek (Malibu, California, USA) during April and May, 2005, and transported in fresh stream water to the laboratory. For 2 weeks prior to hatching, they were incubated in 5 μm-filtered, continuously flowing (single-pass), dechlorinated tapwater and maintained on a 12 h:12 h dark:light cycle (light on: 07.00 h) at 17–20°C. After hatching, groups of 20 larvae were placed in free-floating plastic trays (2-l capacity) containing refuges. Larvae were fed *ad libitum* on water fleas (*Daphnia pulex*) and black worms (*Lumbriculus variegates*) prior to use in experiments. At 21–28 days post-hatch, at a mean length of 1.7 cm (±0.2 cm; s.d.) their responses were examined to various chemical stimuli.

Bioassays

Given the vagaries of simulating the hydrodynamic environments of natural stream habitats, bioassay chambers were constructed that reduced turbulent flow, and thus, minimized inputs of test chemicals (including TTX). The tanks (7 cm×3 cm×1 cm; length×width×depth) fabricated from clear acrylic, were flow-through systems, using dechlorinated, 5 μm-filtered, tap water at stream (17–20°C) temperature. Water was introduced through a porous foam diffuser (100 μm pore diameter) at the chamber entrance, and an adjustable vertical weir was positioned at the downstream end of each tank. These controls on water flow greatly reduced the scales of water motion and evenly distributed momentum across the chamber width. Both dye visualization and velocity measurements within test tanks confirmed laminar flow. Reynolds numbers of 280 and 560 were calculated at a mean flow speed of 0.8 cm s^{-1} at 3.5 cm (chamber center) and 7.0 cm (chamber tail), respectively, downstream of the foam diffuser. There was essentially no cross-stream variation in mean velocity (coefficient of variation <0.1). Trials were run simultaneously in six tanks, positioned side by side.

Opaque blinds around bioassay chambers allowed observation without disturbing test animals. Incandescent bulbs (General Electric Corp. Daylight Ultra, Cleveland, Ohio, USA) were placed within baffled housings to provide diffuse overhead lighting. The mean photon flux (20 μmoles m^{-2} s^{-1}), spectral composition (intensity peaks between 435 and 543 nm), and angular light distribution (sharp decline in intensity between 40° and 50° relative to the zenith) in each bioassay chamber were similar to morning sunlight within natural stream habitats (Zimmer et al., 2006). Lighting was completely confined by the blinds, and the surrounding laboratory was maintained in darkness to reduce further disturbances to test animals.

For each trial, a single larva was transferred to an experimental chamber and acclimated for 1 h. The larvae usually swam continuously during the first 15–30 min, and then rested on the bottom for long (5–10 min) periods of time. Thereafter, they occasionally swam for brief (5–10 s) intervals before resting again. Field observations (using mask, fins and snorkel) confirmed that such behavior was characteristic of animals within natural stream habitats (R.P.F. and R.K.Z., unpublished data). Larvae were tested only when inactive. Following acclimation, either a test or control solution was introduced continuously (at 1.7 μl s^{-1}) over 1 min. A positive response was defined as initiation of swimming behavior during the trial interval. Within a given experiment, the order of test, or control, presentation and chamber use were selected at random (*via* a random numbers table). Each larva was tested once only and then discarded.

A test or control stimulus was presented to each chamber by a delivery system consisting of a syringe, polyethylene tubing (0.85 mm i.d.), and a glass microcapillary (10-μl capacity), with the tip positioned 2 cm upstream of the larva. To minimize flow disturbance, the capillary was mounted vertically and perpendicular to the direction of tap water flow. A constant

volume flow rate through the capillary was maintained by a microprocessor-controlled syringe pump (KD Scientific Inc., Holliston, Massachusetts, USA). The entire capillary and tubing assembly was mounted on a three-dimensional micromanipulator, and its position controlled by an investigator from outside the opaque blinds. This procedure enabled precise solution delivery to the head of a larva from almost anywhere in a chamber. In 12 trials, fluorescent dye (Rhodamine WT, at 1 g l^{-1}) was substituted for a test or control solution. Following capillary release, dye moved in a thin ribbon (~1 mm diameter) directly downstream for several centimeters. Tubing, glassware and capillary were either rinsed clean with ddH_2O (Nanopure, HPLC grade), or replaced, between each successive test or control trial. All chemical solutions were prepared with 0.45-μm filtered, dechlorinated tapwater and the highest quality analytical-grade reagents (Sigma-Aldrich Chemical Co., St Louis, Missouri, USA).

The role of nasal chemoreception in mediating behavioral response

Newt larvae swim in response to trace concentrations of TTX (Zimmer et al., 2006). The presence of TTX-sensitive cells within larval olfactory epithelium was revealed by electrophysiological recordings. We conducted an experiment that unequivocally confirmed the role of the nasal cavity in chemoreception. The cavities of 30 larvae were occluded by applying inert silicon gel (0.05 ml) to the external openings (nares) with a sterile cotton swab. Otherwise, larvae behaved without apparent ill effect. Each individual was ultimately assessed with either 10^{-7} mol l^{-1} TTX or dechlorinated tapwater (control). Fifteen individuals were tested with each solution. To control for animal handling, a second group of 30 larvae was tested in the same manner, but gel was applied to their foreheads, rather than nares. Strong responses to TTX of control larvae, but not of those with blocked nares, implicate the nasal cavity as a critical conduit for chemical-stimulus laden water to the chemoreceptor organ(s).

Test solutions for bioassays

The following experiments were performed to (1) test for arginine suppression of TTX-mediated behavior, (2) generate a dose–response curve for arginine suppressant effects, and (3) determine the relationship between suppression effects and arginine structure. Each test or control solution was bioassayed on 20 different larvae. In the first experiment, larvae were presented with solutions of 10^{-7} mol l^{-1} TTX, 10^{-7} mol l^{-1} TTX plus 10^{-7} mol l^{-1} arginine, 10^{-7} mol l^{-1} arginine, and dechlorinated tapwater (control). These conditions were chosen to simulate natural arginine and TTX fluxes from tissues of injured worm prey and from skin surfaces of adult cannibals, respectively (Zimmer et al., 2006; Ferrer and Zimmer, 2007). Actual experimental input rates were 100- to 1000-times lower than those occurring naturally, compensating for dilution in turbulent stream flows. Suppression would be indicated by a significantly lower larval response to the TTX–arginine mixture, than to TTX alone. In the second experiment, solutions of 10^{-7} mol l^{-1} TTX were bioassayed with, or without, the addition of 10^{-6} mol l^{-1}, 10^{-7} mol l^{-1}, 3.31×10^{-8} mol l^{-1}, 10^{-8} mol l^{-1} or 10^{-9} mol l^{-1} arginine. Dechlorinated tapwater, as well as solutions of 10^{-6} mol l^{-1}, 10^{-7} mol l^{-1}, 3.31×10^{-8} mol l^{-1}, 10^{-8} mol l^{-1} and 10^{-9} mol l^{-1} arginine alone, served as controls. Arginine concentration effects on TTX-stimulated larval responses were determined by comparing results for tests and controls. The third experiment was almost identical in design to the first, but with six mixtures combining TTX with a different arginine structural analog, modifying either the carbon chain, or the guanidinium, amine or carboxyl group (Fig. 1). Each chemical was prepared at a concentration of 10^{-7} mol l^{-1}. Arginine (at 10^{-7} mol l^{-1}), tested alone, and dechlorinated tapwater again served as controls.

The California newt (*Taricha torosa*) is registered as a US Fish and Wildlife Service 'species of concern' and native southern California populations are at historical lows. To

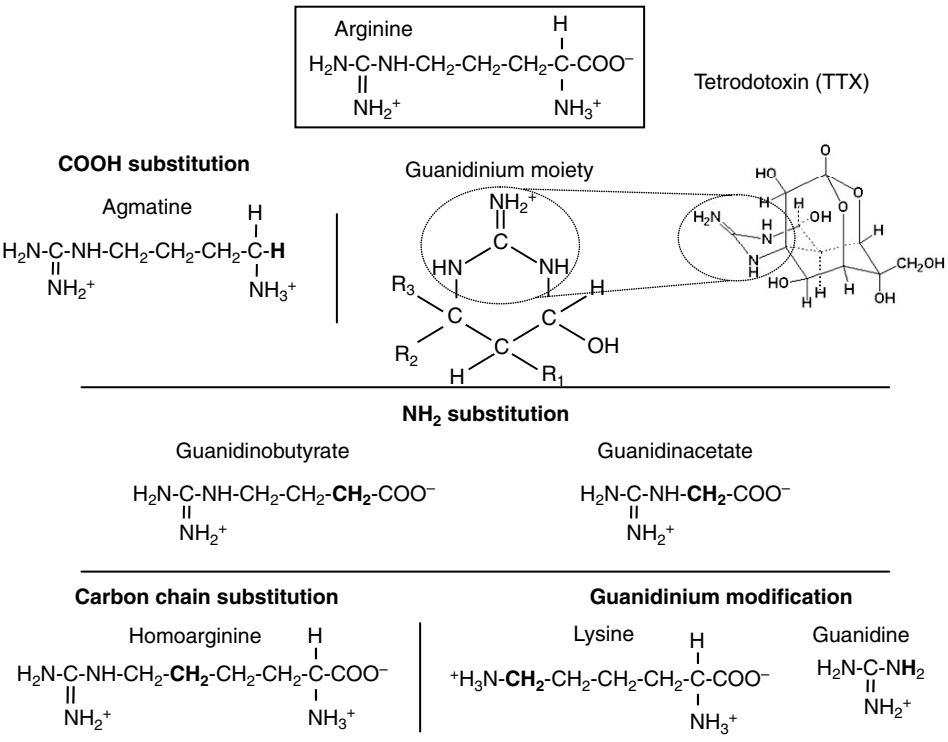

Fig. 1. Structural formulae of tetrodotoxin (TTX) and arginine analogues, as tested in this study.

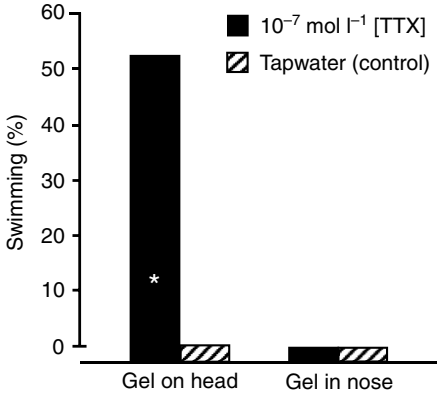

Fig. 2. Percentage of larvae swimming in response to 10^{-7} mol l^{-1} tetrodotoxin (TTX) or tapwater (control). Inert silicon gel was applied either to the forehead (control) or to the external nares of larvae to block nasal cavities. Asterisk denotes a significant difference between larval responses to TTX and tapwater, using a Fisher exact test (P<0.001).

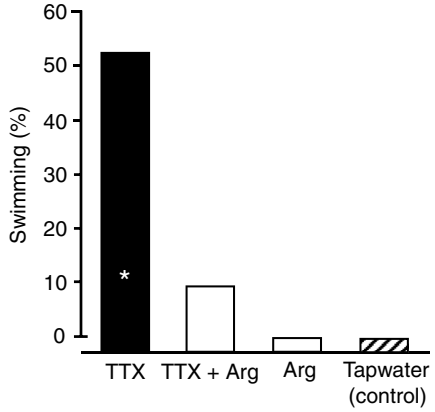

Fig. 3. Percentage of larvae swimming in response to 10^{-7} mol l^{-1} tetrodotoxin (TTX), a binary mixture of 10^{-7} mol l^{-1} TTX + 10^{-7} mol l^{-1} arginine, 10^{-7} mol l^{-1} arginine alone, or tapwater (control). Asterisk denotes a significant difference between larval responses to test and tapwater (control) solutions, using a Fisher exact test (P<0.001).

minimize impacts on wild populations, this study was performed using only six egg masses collected from each host stream. Our goal was to minimize the number of experimental treatments and replicate trials, yet generate a biologically meaningful and statistically rigorous investigation. Once removed, it is a violation of California State law to return newts to their native habitats. Thus, each research animal was sacrificed following use in experiments, according to approved UCLA animal care protocol.

Results

The role of nasal chemoreception in mediating behavioral response

Newt larvae (21–28 days post-hatch) possess a nasal cavity, extending from the outer skin surface to the inner roof of the mouth. In behavioral experiments, larvae with external nares blocked by inert silicon gel did not react to 10^{-7} mol l^{-1} TTX, whereas control animals immediately responded to the same stimulus (Fig. 2; Fisher exact test: P<0.001). Application of tapwater had no effect on animals of either group. In newt larvae, the nasal cavity is the necessary conduit for TTX-laden water in mediating chemosensory responses.

Arginine suppression of TTX-mediated behavior

The percentage of larvae swimming in response to a mixture of 10^{-7} mol l^{-1} TTX plus 10^{-7} mol l^{-1} arginine was not significantly different from the percentage responding to tap water (control; Fig. 3; Fisher exact test: P=0.89). By contrast, a solution of 10^{-7} mol l^{-1} TTX caused significant swimming activity relative to the control, demonstrating that effects of the TTX–arginine mixtures could be ascribed to chemical composition alone. Behavioral reaction to 10^{-7} mol l^{-1} TTX was significantly different from that to the mixture (P=0.012). Thus, results clearly show that arginine acts as a suppressant of TTX-stimulated behavior.

Some compounds are stimulants while also, through mixture interactions, suppressing the activity of alternative molecules. This outcome does not, however, appear to be the case for arginine. Newt larvae were behaviorally unreactive to arginine over a wide range of concentrations (10^{-6} to 10^{-9} mol l^{-1}) (Fig. 4; least squares regression, $F_{1,4}$=0.75, P=0.91). Still, the effect of arginine on TTX-stimulated behavior was dose dependent and described best as a logistic function ($F_{1,4}$=76.78, P=0.007). Arginine, at 0.3-times the dose, significantly reduced the percentage of larvae that swam in response to TTX. Although ineffective as a stimulant, arginine was a very potent suppressant.

Additional bioassays of arginine analogs were limited in scope, but meaningful in outcome. Results of these trials showed significant suppression only for modification to the guanidinium moiety and not for other small changes in parent structure. Deletion of, or alteration to, the carbon chain (homoarginine), the carboxyl group (agmatine) and the amine group (guanidinobutyrate, guanidinacetate) all significantly failed to reduce the suppressant effects relative to arginine (Fig. 5). Each of four equimolar mixtures, combining TTX with one structural analog described immediately above, was no more stimulatory than tap water (control; Fisher exact test: $P \geqslant 0.62$, all comparisons). By contrast, changes to the guanidinium group (lysine) caused the mixture to lose its inhibitory effect (P=0.02), even though guanidinium alone (as guanidine) did not revoke suppression (Fig. 5; P=0.75).

Discussion

The free amino acid, arginine, is a natural suppressant of TTX-stimulated cannibal avoidance behavior in California newt (*Taricha torosa*) larvae. Whereas arginine, by itself, has no effect on larval behavior, it nevertheless signals active adult predation on alternative worm (*Eisenia rosea*) prey. Following

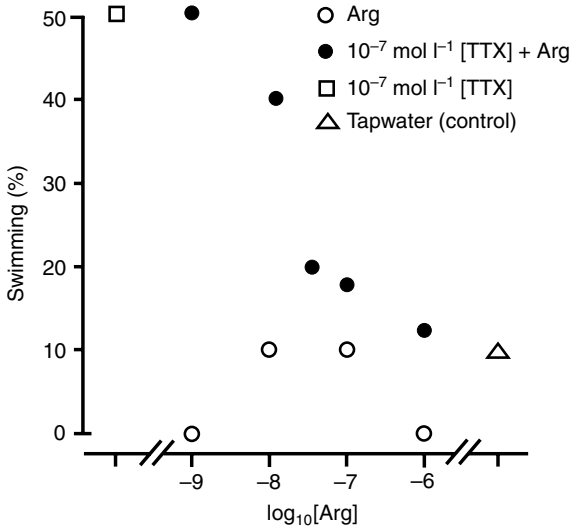

Fig. 4. Effects of concentration on percentage of larvae swimming in response to arginine alone (open circles), or a binary mixture of arginine + 10^{-7} mol l^{-1} tetrodotoxin (TTX; closed circles). Tapwater (open triangle) and 10^{-7} mol l^{-1} TTX (alone, open square) served as controls. Results of statistical comparisons are provided in the text.

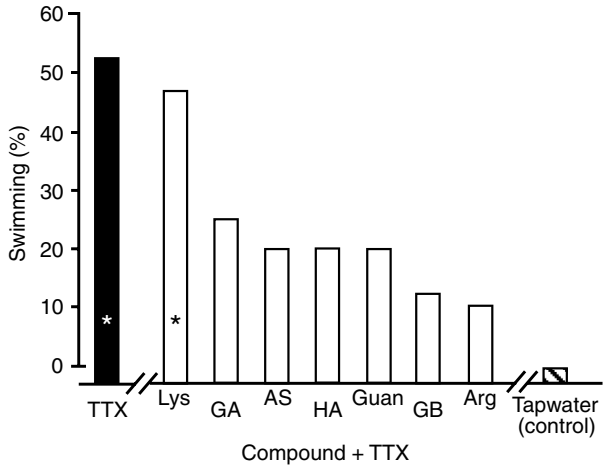

Fig. 5. Percentage of larvae swimming in response to 10^{-7} mol l^{-1} tetrodotoxin (TTX; alone), or binary mixtures of 10^{-7} mol l^{-1} TTX + 10^{-7} mol l^{-1} arginine (or an arginine analog) and tapwater (control). Asterisks denote a significant difference between larval responses to test and tapwater (control) solutions, using a Fisher exact test (P<0.01). Lys, lysine; GA, guanidinacetate; AS, agmatine; HA, homoarginine; Guan, guanidine; GB, guanidinobutyrate; Arg, arginine.

release in body fluids of injured prey, this molecule inhibits larval responses to TTX in a dose-dependent manner. The relative ratio of dissolved TTX:arginine is thus a meaningful indicator of ecological context – degree of cannibalism threat – dictating the appropriate larval behavioral response.

Combined results in the present study point to olfaction as mediating suppressant interactions between TTX and arginine. The nasal cavity is an essential conduit for chemical-stimulus-laden water and contains TTX-sensitive cells within the olfactory epithelium (Zimmer et al., 2006) (current study). Moreover, blocking external nares eliminates any behavioral reaction to TTX. When nares are obstructed, waterborne chemical stimuli can travel an alternative path through the mouth to taste buds and the vomeronasal organs, but not to the olfactory receptor neurons (Døving et al., 1993; Døving and Trotier, 1998). Notably, vomeronasal cells of some reptile species detect arginine (Hatanaka, 1990). Sensitivity to this compound in amphibians is, however, attributed to olfaction (Vogler and Schild, 1999; Manzini and Schild, 2004).

Behavioral and physiological mechanisms of mixture suppression

Mixture suppression is a basic property of quality coding in chemosensory systems (Rhein and Cagan, 1983; Derby et al., 1985; Danaceau and Lucero, 2000). Competitive inhibition at the peripheral level is one suppression mechanism (Gleeson and Ache, 1985; Cagan, 1986; Bruch and Rulli, 1988; Ache et al., 1988). Under this scenario, a binary mixture of TTX and arginine would compete for a single receptor subtype resulting in a loss of, or reduction in, neuronal stimulation relative to TTX alone. The guanidinium group on TTX is vital for effective interaction with voltage-gated sodium channel receptors (Kao, 1986; Hille, 2001). Presumably, this same moiety plays a seminal role in binding to TTX-sensitive chemosensory receptors in newt larvae. Likewise, guanidinium also is essential for arginine binding to G-protein coupled receptors on dendritic membranes of gustatory and olfactory receptor cells (Bryant et al., 1989; Kalinoski et al., 1989; Lipsitch and Michel, 1999). Whereas modifications to the carbon chain, carboxyl group, or amine of the parent molecule were without significant effect, arginine suppression of TTX-stimulated newt larval behavior was eliminated by altering the positively-charged guanidinium moiety. Combined results of current and past investigations favor a mechanism of suppression that arises from competitive interactions at a guanidinium sensitive receptor site.

Alternatively, suppression of TTX-stimulated predator avoidance responses in newt larvae might arise from non-competitive interactions within the peripheral (Johnson et al., 1984; Johnson et al., 1985) or central nervous system (Kang and Caprio, 1995; Giraudet et al., 2002; Wilson, 2003), or both (Derby and Ache, 1984). A feasible peripheral non-competitive mechanism is TTX and arginine binding at different receptor sites on the same chemosensory receptor cell. Subsequent activation of opposing second messenger pathways would either dampen depolarization or induce hyperpolarization (McClintock and Ache, 1989; Boekhoff et al., 1990; Restrepo et al., 1996) resulting in a reduced or blocked response. This type of mechanism has been described for several invertebrate species, but not for a vertebrate (including amphibians). If TTX and arginine stimulate receptors on different chemosensory neurons, inhibition also could take place through integration in the olfactory bulb (Shepherd and Greer, 1990; Wellis and

Kauer, 1993) or in other, higher-order brain centers (Shepherd, 1996).

Ecological consequences of mixture suppression and context-sensitive behavior

Prey behavioral responses to chemicals emitted from damaged conspecifics, or active predators, include rapid evasion, hiding or seeking refuges (for reviews, see Kats and Dill, 1998; Chivers and Smith, 1998). Although reducing predation risk, these behaviors also conflict with an individual's ability to maximize energy gain. Refuge environments are often nutrient poor (Holomuzki, 1986; Kohler and McPeek, 1989; Persson et al., 2000), and prey cease to forage for and consume food during evasion or avoidance (for a review, see Brown and Kotler, 2004). A reduction in energy consumption can negatively impact growth (Sih, 1987; Van Buskirk and Yurewicz, 1998; Nakaoka, 2000), health [e.g. resistance to parasites (Baker and Smith, 1997) and bacterial pathogens (Rigby and Jokela, 2000)] and reproductive success (Peckarsky et al., 1993; Loose and Dawidowicz, 1994; Peckarsky, 1996). It is therefore advantageous for prey to weigh all sensory information before committing to costly predator avoidance behaviors.

Context-sensitive behavior in larval newts is dependent on TTX and arginine playing uncharacteristic roles. For a biodiverse assemblage of animal and microbial species, TTX serves as a chemical defense or venom (Kim et al., 1975; Sheumack et al., 1978; Miyazawa et al., 1986; Thuesen et al., 1988; Ritson-Williams et al., 2006). It acts as a potent neurotoxin by binding to and blocking voltage-gated sodium channels on nerve and muscle cell membranes (Hille, 2001). Alternatively, for California newt larvae, this same compound evokes action potentials from olfactory receptor cells and stimulates antipredator behavior (Zimmer et al., 2006). Arginine, on the other hand, functions as a feeding stimulant/attractant for many aquatic and terrestrial animal species (Caprio and Byrd, 1984; Zielinski and Hara, 1988; Kang and Caprio, 1997; Carr et al., 1996), yet suppresses cannibal-avoidance in larval newts. The habitat, and associated fauna, of adult California newts are seasonally variable, dictating the need for a diverse diet of aquatic and terrestrial invertebrates (Kerby and Kats, 1998) (R.P.F. and R.K.Z., unpublished data). When food is limited, cannibalism on their young may forestall starvation, therefore enhancing the probability of adult survival (Fox, 1975; Polis, 1981; Elgar and Crespi, 1992). Using a mixture of waterborne chemical cues (TTX and arginine), however, larval newts appear to sufficiently abate adult predation pressure while minimizing the time and energy lost to cannibalism avoidance.

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