Endosulfan Exposure Disrupts Pheromonal Systems in the Red-Spotted Newt: A Mechanism for Subtle Effects of Environmental Chemicals

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Because chemicals introduced into the environment by humans can affect both long-term survivorship and reproduction of amphibians, discovering the specific mechanisms through which these chemicals act may facilitate the development of plans for amphibian conservation. We investigated the amphibian pheromonal system as a potential target of common environmental chemicals. By treating female red-spotted newts, *Notophthalmus viridescens*, to a commonly used insecticide, endosulfan, we found that the pheromonal system is highly susceptible to low-concentration exposure. The impairment of the pheromonal system directly led to disrupted mate choice and lowered mating success. There were no other notable physiologic or behavioral changes demonstrated by the animals at the insecticide concentrations administered. Our findings suggest that the amphibian pheromonal system is one of the systems subject to subtle negative effects of environmental chemicals. *Key words:* amphibian declines, electro-olfactogram, endosulfan, environmental chemicals, insecticides, pheromones, olfaction.

Chemicals released into the environment may have either overt toxic effects or more subtle effects that influence long-term survival and reproduction. In several amphibian populations, such toxic effects of environmental chemicals have been reported as one of the major pathways underlying population declines (*1–3*). Unlike the detection of overt toxic effects of chemicals, conventional toxicologic screening and testing methods are generally poor at exploring more subtle effects such as changes in behavioral or hormonal responses. For this reason, there are relatively few studies that have investigated such subtle effects of exposure to environmental chemicals (*4*). Discovering more subtle, but nevertheless damaging, mechanisms through which environmental chemicals act may help in the development of plans for amphibian conservation.

Endosulfan is a chlorinated hydrocarbon insecticide of the cyclodiene subgroup, which acts as a poison to a variety of insects. It is used on a wide variety of crops including tea, coffee, cotton, fruits, and vegetables, as well as on rice, cereals, maize, or other grains (*5*). Several studies reported the presence of endosulfan in cotton crop soils [0.1–0.4 mg/kg (*6*)] and estuaries [0.5–4.0 µg/L (*7*)], and relatively high residual levels have been found in some marketed fruits such as Brazilian strawberries and tomatoes [4–510 ppb (*8*)]. These reports indicate that endosulfan exposure can occur in both human and wildlife populations. Endosulfan not only acts as a hormone disruptor, but it also affects neurotransmitter systems of many species such as rats, catfish, and bullfrog tadpoles (*9–14*). For example, endosulfan suppressed testosterone and 17β-estradiol concentrations in neonatal rats (*9*),

increased thyroxine (T_4) levels in catfish (*10*), and induced neurotoxic effects including increased excitability, trembling, and deficits in operant learning performance via the disruption of cholinergic, dopaminergic, and serotonergic neurotransmitter systems in freshwater fishes, pigeons, and rats (*11–14*).

Pheromonal systems play important roles during many stages of amphibian life history including searching for migration routes, advertising individual territories, identifying conspecific individuals, presenting aggressive or alarm signals, and alluring potential mates (*15*). The pheromonal systems of the red-spotted newts, *Notophthalmus viridescens*, are well studied (*16,17*). Females have cloacal glands that produce attractants for males, and males have hedonic and cloacal glands. Male hedonic glands open into epidermal pits that are arranged in a row on either side of the head, and the pheromone produced increases female receptivity (*18*). Male cloacal pheromones may function by both increasing female receptivity (*18*) and repelling conspecific males (*19*). The growth of alveoli in the glands and the production of pheromones are under the control of prolactin, dopamine, and sex steroid hormones (*20*). Cholinergic neurotransmitter systems are responsible for the secretion of pheromones (*21*).

The purpose of our study was to determine whether exposure of female red-spotted newts to endosulfan limits individual interactions by disrupting pheromonal communication.

Materials and Methods

Animal husbandry and endosulfan exposure.
Experiments were conducted from 26 March to 11 June during the breeding season in 1998. A total of 99 red-spotted female and male newts were purchased from a commercial supplier (Charles D. Sullivan Co. Inc., Nashville, TN). The use of animals in this study was approved by the institutional animal care and use committee of Northern Arizona University (#98-584). Newts were kept as described by Verrell (*22*). Endosulfan (ps-81, 99% mixture of isomers, Chemical Service, West Chester, PA) dissolved in 10 µL acetone was delivered into individual containers containing one female and 1 L of water, and then immediately and gently mixed throughout the water using a glass rod. Final exposure concentrations were 0, 5, and 10 ppb endosulfan. These concentrations were based on the results of a study that exposed frog tadpoles to endosulfan (*12*) and concentrations found in the field (*7*). Animals were treated for 4 days. We neither changed the water containing endosulfan nor reintroduced endosulfan. During exposure, control animals only received 10 µL acetone.

General behavioral tests.
We measured behavioral activity and food uptake just before (0 h) and at 24, 48, and 96 hr after the onset of endosulfan exposure. The activity was measured by calculating the number of squares crossed in an open field box (30 × 15 × 9 cm) for 10 min. We also recorded the number of air gulps each animal took during a 10-min observation period as an indication of breathing rate. To measure the amount of food consumed, five young crickets (less than 1 week old) were randomly selected and supplied to each individual tank at 2200 hr. The next morning (0800–0900 hr), we counted the remaining crickets. Data for the behavioral activity were analyzed by two-way analysis of variance (ANOVA) (*23*). For the amount of food consumed and breathing rates, we used Kruskal-Wallis tests (*23*).

Visual mate selection. To determine if endosulfan impairs visual aspects of mate choice, visual selection tests were conducted following Verrell's method (*16*). An aquarium measuring 80 × 40 × 50 cm was divided lengthwise into two halves by drawing a vertical line. Two transparent plastic boxes measuring 12 × 10 × 15 cm were submerged, one in the center of each half of the aquarium, and each box had either a control or an endosulfan-exposed female. The two females were paired by weight (< 0.5 g). A test male was then released into the center of the aquarium. We recorded which half of the aquarium the male was in at the end of each minute for 30 min. During selection tests, the boxes were watertight to prevent female pheromones from reaching the test males. Therefore, the males could show mate preferences based only on female visual cues. After each experiment, the aquarium and test boxes were rinsed with aged tap water. Data obtained were analyzed by Wilcoxon signed-rank tests (*23*).

Olfactory mate selection. After the endosulfan treatment, we placed females in fresh water for 12 hr to remove potential endosulfan residues from the skin. Olfactory selection tests were conducted using a standard Y-maze olfactometer (*16*). Two arms of the Y-maze, containing either experimental or control individuals, who were paired by weight (< 0.5 g), each received 150 mL/min aged tap water from a reservoir. Each chamber of the arm could reserve 10 mL water until overflowing. The amount of water was controlled by a flow meter (Gilmont Instruments, Barrington, IL). At this flow rate, water flowing through the two arms remained laminar to a drain at the end of the Y-maze. Test newts were placed at the end of the maze behind a start gate for 5 min to allow them to adjust to the test situation. After 5 min, we slowly raised the gate when the newt was facing away from the gate. Each newt was allowed 20 min to crawl or swim up the main body of the maze. A choice was considered to be made when the test newt moved more than one-third of the length of a given arm. After each trial, the Y-maze was rinsed with aged tap water. Data obtained were analyzed by binomial tests (*23*).

Mating test. To measure mating success, we placed a randomly selected male with either a control or an endosulfan-treated female in an experimental aquarium (32 × 15 × 9 cm) containing 1 L aged tap water at 2100 hr. The next morning at 0800 hr, the presence of a spermatophore substrate on the aquarium bottom or of a spermatophore in the female's cloaca was used to determine mating success. Data obtained were analyzed by chi-square tests followed by *Z*-tests when differences between groups were statistically significant (*23*).

Histologic experiments. After the behavioral and mating tests, each individual was anesthetized by immersion in 0.1% MS222 solution (Sigma Chemical Company, St. Louis, MO). After measuring snout–vent length and weight, individuals were killed by exsanguination, and the trunk blood was used to measure estradiol concentrations. We removed and weighed the ovaries and oviducts. Ten cloacae, randomly selected for the anatomical study of pheromonal glands, were also excised from each group, and then fixed in Histochoice (Amresco, Solon, OH) with 20% ethanol. Cloacae were embedded in paraffin, sectioned at 10 μm, stained by the Mallory method, and observed under a Zeiss Axioskop microscope (Atto Instruments, Thornwood, NY), interfaced with a Macintosh computer (Apple Computer, Inc., Cupertino, CA). On one-half of each cloaca for each fifth section, we captured and measured alveolar and luminal size for alveoli following previously described methodology (*20*). We used the public-domain NIH Image program [developed at the National Institutes of Health (*24*)] to capture and measure the images. Data obtained were analyzed by one-way ANOVA, followed by Tukey test when the ANOVA demonstrated significant differences among groups (*23*).

Electro-olfactogram study. The electro-olfactogram (EOG) experiment was conducted 10–22 June 1999. Females were exposed as previously described to three different concentrations of endosulfan; 0 ppb ($n = 7$), 5 ppb ($n = 6$), and 10 ppb ($n = 7$) for 96 hr. After the treatment, females were placed in 5 L fresh water for 5 hr with two changes of water to facilitate the removal of any endosulfan residues on the skin. Females were then individually placed in a small, glass box (17 × 13 × 11 cm) containing 50 mL distilled water for 3 hr to collect odor sources. Collected odor sources were stored at 4°C and randomly used within 4 days.

To measure olfactory potential, subject males ($n = 4$) were anesthetized by subcutaneous injection of ketamine hydrochloride (100 mg/kg body weight) (*25*) and then were double-pithed. EOG recordings were made from the surface of the main olfactory epithelium. Odor sources (0.5 mL) were injected with a disposable syringe into the constant flow of distilled water, 3 mL/min, which flowed into the olfactory chamber.

To record signals, a glass capillary electrode (50–100 μm tip diameter) was filled with Ringer's solution in 1% agar and bridged to a chloride-coated silver wire. An Ag-AgCl reference electrode was placed under the head skin (*26*). Electrodes were coupled to a Grass P-18 DC/AC preamplifier (Grass, West Warwitk, RI), and the signals were displayed on a V-552 Hitachi oscilloscope (Tokyo, Japan) and simultaneously recorded on a Vetter 420-M instrumentation tape recorder (Reversburg, PA). To assure the use of the same sensory cells during a recording, we did not change the position of the electrode until finishing a replicate of stimuli consisting of one control and two treated samples. Before and after each replicate recording, we confirmed that the response of the olfactory epithelium was constant using 10^{-2} M L-cysteine, which is a potent olfactory stimulus. Response magnitudes were defined as a relative peak phasic displacement measured from the baseline in millivolts. The magnitude and time course of the EOGs were analyzed using pCLAMP 5.5 software (Axon Instruments, Foster City, CA), and the means were compared using one-way ANOVA, and followed by Tukey test (*23*).

Plasma collection and measurement of plasma 17β-estradiol. To collect plasma, trunk blood was collected in heparinized tubes and stored on ice until centrifugation at 1,200 × *g* for 15 min. After centrifugation, the plasma was removed and stored at –80°C until the steroids were extracted and assayed by radioimmunoassay (RIA) (*27*).

To measure recovery efficiency during steroid extractions, we incubated plasma with 800–1,000 cpm of the tritiated analog of the steroid for 1 hr. Plasma was then extracted with diethyl ether, dried, and resuspended in RIA buffer. We measured 17β-estradiol using rabbit estradiol antiserum. The antiserum showed cross-reactive binding to 5α-dihydrotestosterone (3%), estrone (6%), and estriol (16%). Six other steroids showed no cross-reactivity. Standards ranged from between 0.98 and 1,000 pg/tube, and all tubes were incubated in 5,000 cpm [2,4,6,7,16,17-³H]estradiol (Amersham Life Science, Buckinghamshire, UK). Serial dilutions of extracted plasma showed parallelism with a standard curve. The intra-assay coefficient of variation was 7.7%, sensitivity was 1.2 pg/tube, and specific binding was 24.6%. Data obtained were analyzed by one-way ANOVA (*23*).

Results

Effects of endosulfan on nonreproductive female behaviors, visual selection of females by males, and ovary and oviduct weight. Endosulfan-exposed females did not exhibit any nonreproductive behavioral deficits. General activity (two-way ANOVA, $p = 0.53$), amount of food consumed (Kruskal-Wallis test, $p = 0.42$), and surfacing to breathe (Kruskal-Wallis test, $p = 0.51$) did not differ among control and treated females. Also, there were no significant differences in ovary/body mass and oviduct/body mass among groups (one-way ANOVA, $p = 0.37$ and 0.55, respectively). Furthermore, the

visual selection of females by males was not different between control and exposed females (Table 1).

Effects of endosulfan on olfactory selection of females by males. Males less frequently selected water containing odor sources from low-concentration–treated females than water from control females (Table 2). Males did not show any preferences between control and high-concentration–treated females or between low- and high-concentration–treated females (Table 2).

Effect of endosulfan exposure on mating success. Female newts exposed to endosulfan had lower mating success than that of controls (chi-square, $p = 0.035$; Figure 1). The decrease in mating success was more distinctive in the high-concentration group (z-test, $p = 0.002$) than in the low-concentration group (z-test, $p = 0.009$).

Effects of endosulfan on gland morphology. Histomorphometric examination of pheromonal glands of control and exposed females revealed anatomical changes. The alveolar size and luminal area of alveoli differed significantly as a function of treatment (Figure 2A; one-way ANOVA, $F = 15.32$, $p < 0.001$ for alveoli; Figure 2B; $F = 15.59$, $p < 0.001$ for lumen). Females exposed to the high concentration of endosulfan had smaller alveoli than control females (Figure 2A; Tukey test, $p < 0.001$), and the alveoli of the low-concentration exposure group exhibited reduced luminal area (Figure 2B; Tukey test, $p < 0.001$).

Effects of endosulfan on pheromone production. Compared to odor sources from control females, odor sources from endosulfan-exposed females at both concentrations induced a significantly lower magnitude in EOG responses from male olfactory sensory cells (Figure 3; one-way ANOVA, $F = 9.15$, $p < 0.001$). There was no significant difference in response between the two different concentrations of endosulfan (Tukey test, $p = 0.40$).

Effects of endosulfan on plasma estradiol concentrations. The plasma concentration of 17β-estradiol was not different between control and treated groups (mean ± SD: control, 11.90 ± 1.40 pg/100 μL plasma, $n = 20$; low concentration, 14.80 ± 2.60, $n = 22$; high concentration, 13.80 ± 1.60, $n = 20$; one-way ANOVA, $p = 0.77$).

Discussion

Our morphologic, electrophysiologic, and behavioral results suggest that low-concentration exposure to a commonly used insecticide disrupts the mate selection process in red-spotted newts specifically by interfering with olfactory communication and that this disruption consequently leads to lowered mating success.

Previously reported toxicologic observations were not induced by the concentration of endosulfan we administered. During our study, endosulfan did not affect general activity, food consumption, surfacing to breathe oxygen, or weight of the ovaries and oviducts. Nor did exposure affect visual attractivity of females. Other studies where higher concentrations of endosulfan were administered, however, have reported traditional toxicologic effects. For example, neonatal rats exposed to 4.5 mg/kg body weight endosulfan by subcutaneous injection had decreased testis, seminal vesicle, ovary, and oviduct weight and also had low levels of testosterone and estradiol (9). The freshwater fish *Channa punctatus* exposed to similar levels of endosulfan as in our study (6 ppb and 10 ppb) showed tremors, convulsions, increased surfacing activity, and increased general activity, as well as altered concentrations of neurotransmitters in the brain including acetylcholine, serotonin, and dopamine (11). Harris et al. (28) reported deformities in American toads (*Bufo americanus*) and northern leopard frogs (*Rana pipiens*) exposed to 0.1 ppm endosulfan. Considering these previous results, our findings suggest that the sensitivity to changes in pheromonal function in newts may be similar to that of more overt toxicologic effects that indicate marked neurologic dysfunction in *C. punctatus*.

Females exposed to endosulfan had smaller pheromonal gland alveoli than did control animals, suggesting that endosulfan exposure disrupts alveolar growth. In male newts, prolactin and steroid hormones (20,29) control the growth of alveoli.
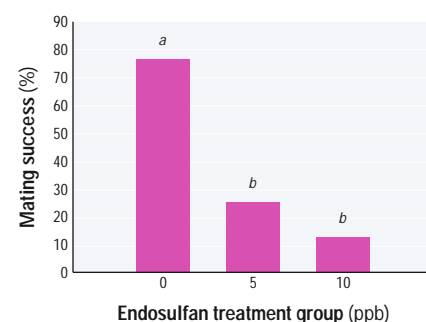

Figure 1. Females exposed to endosulfan demonstrated lower mating success. Groups that do not share the same letters differed significantly from one another ($p < 0.05$).
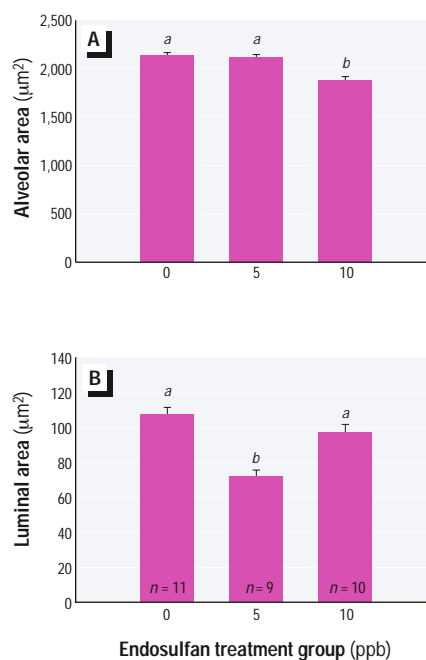

Figure 2. Morphologic measurements of pheromone glands from endosulfan-treated females demonstrated significant differences among groups. (*A*) Alveolar size. (*B*) Luminal size. Data are represented as mean ± SEM. Groups that do not share the same letters differed significantly from one another ($p < 0.05$).

Table 1. Visual mate selection tests demonstrated that endosulfan exposure did not affect visual female attractivity.

Test set (*n*)	Endosulfan treatment (ppb)	Median number stayed with male	*p*-Value[a]
1 (30)	0.0	14	0.094
	0.5	16	
2 (28)	0.0	15	0.701
	1.0	15	

[a]Wilcoxon signed-rank test.

Table 2. Olfactory mate selection tests demonstrated that males select control females over those exposed to 5 ppb endosulfan.

Test set (*n*)	Endosulfan treatment (ppb)	No. chosen by male	*p*-Value[a]
1 (34)	0	25	< 0.01
	5	9	
2 (31)	0	17	0.360
	10	14	
3 (33)	5	15	0.364
	10	18	

[a]One-tailed binomial test.

Dopamine affects prolactin production (*30*). The dopaminergic system is vulnerable to endosulfan exposure, and the disruption of the system is dependent on the brain regions investigated and the duration of exposure (*14,31*). The level of dopamine in the hippocampus of Wistar rat pups exposed daily to 6 mg/kg body weight endosulfan by gastric intubation was decreased (*14*), whereas adult rats exposed daily to 3 mg/kg endosulfan by intraperitoneal injection showed an increase in whole-brain dopamine concentration (*30*). Although we did not measure changes in brain dopamine concentrations, previous results imply that the impact on the dopaminergic system by endosulfan may be responsible for the changes in pheromonal gland morphology.

In our study, endosulfan did not directly block the production of 17β-estradiol. However, endosulfan exposure may still affect estradiol-related responses including the growth of alveoli in the pheromonal glands, and consequently the production of pheromones. Endosulfan competes with 17 β-estradiol for binding to estrogen receptor (*32*). In the oviduct of the American alligator, *Alligator mississippiensis*, 50 μM endosulfan inhibits the binding of 17β-estradiol to the estrogen receptor (*32*). Endosulfan also interferes with progesterone activity. A 30-μM dose of endosulfan inhibits the binding of the synthetic progestin, R5020, to the progesterone receptor in the oviduct of the American alligator (*32*). In a transformed yeast assay system, endosulfan also decreased the activation of a reporter gene that is induced by progesterone (*33*). These studies suggest that endosulfan administered in our study may also affect pheromonal gland function via a steroid-mediated pathway without directly changing plasma concentrations of 17β-estradiol.

Secretion of pheromones is vulnerable to low-concentration endosulfan exposure. During our electrophysiologic experiments, odor sources from exposed females induced less intensive EOG responses, suggesting that exposed females released a lower concentration of pheromones during odor collection. Also, in olfactory selection tests, low-concentration–exposed individuals were significantly less attractive than control females to test males. These results suggest that endosulfan exposure disrupts pheromonal secretion. In male newts, the cholinergic system controls secretion of pheromones from their pheromonal glands (*21*). Acetylcholine binds to muscarinic receptors and stimulates the myoepithelium-induced secretion of pheromones. The effects of endosulfan on the cholinergic system have been reported in several species. For example, in the fish, *C. punctatus*, endosulfan exposure decreased the concentration of acetylcholine in the brain (*12*). In pigeons (*Columba livia*), endosulfan exposure changed cholinergic-mediated seizure activity in the brain (*13*). These results imply that during our study, endosulfan may have affected the secretion of pheromones via disruption of cholinergic systems.

Endosulfan exposure affected mating success of red-spotted newts possibly via the disruption of pheromonal systems and through potential neurologic system damage involved in sexual behavior as well. During our study, endosulfan exposure induced a significant decline in mating success, suggesting that decreased individual interactions via the disrupted pheromonal systems may result in decreased mating success. On the other hand, because we provided a relatively small courtship arena, which may allow male and female newts to easily come in contact, it is also possible that endosulfan may cause changes in reproductive interactions through mechanisms that were not detected in this study. Several investigations have documented neurobehavioral changes after endosulfan exposure. Rats treated with endosulfan showed increased aggressive behavior and deficits in operant learning performance (*14*). Such changes were correlated with changes in levels of neurohormones and neurotransmitters such as noradrenaline, dopamine, acetylcholine, and serotonin in the brain (*14*). One study in the fish *Tilapia rendalli* reported that brains from animals exposed to endosulfan exhibit abnormal encephalitis, meningitis, and edema, with an associated inflammatory infiltrate of eosinophilic granule cells (*34*). Thus, in our study, it is possible that endosulfan not only disrupted the female pheromonal systems, but that it also interfered with female neural pathways involved in the performance of appropriate reproductive behavior even though it did not disrupt behaviors associated with general activity. Males may not have continued courtship or insemination attempts with females who were nonresponsive.

Our results are the first to document that the disruption of pheromonal communication systems can be induced by exposure to low concentrations of environmental chemicals without overt signs of toxicosis. This study suggests that amphibian pheromonal systems could be one of the systems mediating subtle effects of environmental chemicals. In addition to local climate change, habitat fragmentation, infectious disease, and increased UV-B irradiation, increased use of environmental chemicals has been suggested to be one of the major factors underlying recent amphibian declines (*1–3,35–39*). Therefore, disruption of amphibian pheromonal systems by such chemicals are possibly one other mechanism facilitating population declines because the system plays a critical role in the daily life of amphibians such as conspecific recognition, migration, social behavior, and reproduction.

Figure 3. Males exposed to odor sources from endosulfan-treated females exhibited a significantly lower magnitude in olfactory response. (*A*) Representative EOGs from the treatment groups. Arrowheads on each EOG indicate the start of the responses; odor sources from endosulfan-exposed females induced smaller responses in EOG magnitude. (*B*) Comparison of magnitude in EOG (mean ± SEM) among groups was significantly different. Groups that do not share the same letters differed significantly from one another ($p < 0.05$).

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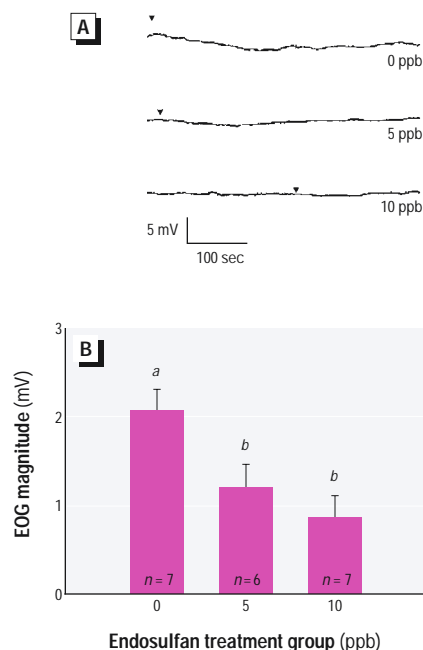
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