Bull. Environ. Contam. Toxicol. (2002) 69:609–616

DOI: 10.1007/s00128-002-0104-8

Bulletin of Environmental Contamination and Toxicology

Endosulfan Affects Pheromonal Detection and Glands in the Male Red-Spotted Newt, *Notophthalmus viridescens*

D. Park, C. R. Propper

Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA

Received: 1 November 2001/Accepted: 26 April 2002

Chemicals released into the environment by human beings have affected many non-targeted wildlife species (Colborn and Clement 1992). Impacts range from subtle negative effects in an individual to the decline of wildlife populations. Many different organs are targeted by exposure to environmental chemicals. One possible, but under-studied, target system is the olfactory system (Park et al. 2001). Impairment of the olfactory system can occur via disrupting the developmental processes that organize the system or by impairing the adult olfactory neuroepithelium (Brittebo et al. 1991). Such disturbances in normal physiological function may result in failed olfactory perception and failed pheromonal communication (Saglio and Trijasse 1998).

Throughout an amphibian's life, olfactory input is used for such vital functions as searching for food, advertising territories, identifying and attracting conspecific individuals, and presenting alarm signals. Studies investigating the response of pheromonal systems to low dose exposure to environmental chemicals may provide a potentially novel mechanism that could impair amphibian reproduction. In this study, we investigate the effect of low-dose exposure to the insecticide, endosulfan, on the olfactory response to pheromones and pheromonal production in male red-spotted newts, *Notophthalmus viridescens*, and we determine if such disruptions affect mating outcomes and other endpoints.

MATERIALS AND METHODS

Experiments were conducted from April 20th to May 11th during the breeding season in 1999. 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 IACUC of the Northern Arizona University (#98-584). They were kept as described by Park et al. (2001). Endosulfan (ps-81, 99% mix of isomers, Chem. Service, West Chester, PA) dissolved in 10 μL of acetone was delivered into an aquarium containing four liters of dechlorinated tap water and four males. Final endosulfan concentrations were 0.5, 1, and 5 ppb. These concentrations were chosen because higher doses induced acute toxic responses in frog tadpoles (Gopal et al. 1981; Berrill et al.

Correspondence to: D. Park

1999). Furthermore, these levels are similar to those the animals may encounter in the field (Fox and Matthiessen 1982; Reyes et al. 1999). Animals were treated for 4 days. We neither changed the water containing endosulfan nor re-introduced endosulfan. During exposure, control animals received 10 μL of acetone alone.

After endosulfan treatment, males were placed in fresh water for 1 hr to remove potential endosulfan residues from the skin. Olfactory response tests were conducted using a standard Y-maze olfactometer (Park et al. 2001). Each side arm of the Y-maze contained one female in order to attract males and received 150 mL/min aged tap water from a reservoir. The amount of water was controlled by a flow meter (Gilmont Ins., Barrington, IL). At this flow rate, water flowing through the two arms remained laminar to a drain at the end of the Y-maze. Endosulfan-treated or control male newts were placed at the end of the maze behind a start gate for 3 min to allow them to adjust to the test situation. After 3 min, we slowly raised the gate. Each newt was allowed 10 min to crawl or swim up the main body of the maze. The latency time to determine an olfactory response for the test newts was defined as the time when a test male completely passed through the gate toward the odor sources. A completed female selection was considered made when the test newt moved more than 1/3 of the length of a given arm. After each trial, the Y-maze was rinsed with aged tap water. Data for latency time were analyzed by one-way ANOVA, followed by Tukey test when the ANOVA demonstrated significant differences among groups. The frequency of starting and finishing olfactory preference tests among groups was analyzed by Chi-square test.

To measure mating success, a randomly selected female was placed with either a control or an endosulfan-treated male in an experimental aquarium (32 × 15 × 9 cm) containing 1 L of aged tap water at 21:00hr. The next morning at 08:00hr, 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 test.

After the olfactory response and mating tests, each individual was anesthetized by immersion in 0.1 % MS 222 solution. After measuring snout-vent length (SVL) and weight, individuals were killed by exsanguination, and the trunk blood was used to measure testosterone concentrations (see below). We removed and weighed the testes. Ten hedonic glands, randomly selected for the anatomical study of pheromonal glands, were excised from each group, and then fixed in Histochoice (Amresco, OH) with 20 % ETOH. The glands 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. On one half of each hedonic gland for each fifth section, we captured and measured the alveolar and luminal size for alveoli following a previously described methodology (Park et al. 2001). We used the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) to

capture and measure the images. Final sample sizes for data analysis were; N=9, control; N=9, 0.5 ppb; N=10, 1 ppb; and N=9, 5 ppb. Data obtained were analyzed by one-way ANOVA, followed by Tukey test when the ANOVA demonstrated significant differences among groups.

To measure plasma testosterone concentration, trunk blood was collected in heparinized tubes and stored on ice until centrifugation at 1,200 × g for 15 min from 19 individuals, control; 21, 0.5 ppb; 21, 1 ppb; and 23, 5 ppb. After centrifugation, the plasma was removed and stored at -80 °C until the steroids were extracted and assayed by radioimmunoassay (RIA; Park et al. 2001). In order to measure recovery efficiency during steroid extractions, plasma was incubated with 800-1000 cpm of the tritiated analog of the steroid for 1hr. Plasma was then extracted with diethyl ether, dried, and re-suspended in RIA buffer (0.81g Na_2HPO_4, 1.0 g sodium azide, 0.39 g NaH_2PO_4, 8.17 g NaCl, 1.08 g Gelatin in 1000mL double-distilled water, pH 7.0). Testosterone was measured using T3-125 testosterone antisera diluted 1:200 (Lot 33 A, Batch 1343, Endocrine Sciences Ria Agents, CA). Standards ranged from between 0.98 and 1000 pg / tube and all tubes were incubated with 5,000 cpm [1, 2, 6, 7-^{3}H] testosterone (Amersham, Life Science). Serial dilutions of extracted plasma showed parallelism with a standard curve. The intra-assay coefficient of variation was 5.97 %, sensitivity was 1.18 pg / tube, and specific binding was 29.51 %. Data obtained were analyzed by one-way ANOVA.

RESULTS AND DISCUSSION

During the olfactory response test, endosulfan-exposed males showed significantly longer latency times in response to female odors (Figure 1, $F_{3, 85}$=3.14, p=0.03). The response was not dose dependent, however, as the lowest treatment group was the only group that differed significantly from the control. The number of individuals that completed the olfactory selection task was not different among groups (Control, 15 / 22 inds; 0.5 ppb, 9 / 22 inds; 1 ppb, 11 / 22 inds; 5 ppb, 8 / 23 inds; Chi-square, p=0.59). Also, the ratio of individuals that started the olfactory selection task within the first 10 min of the test was not different among groups (Chi-square, p=0.96). Our results demonstrate that the newt olfactory system was affected by exposure to a low dose of endosulfan. Several studies have previously documented impairment of the olfactory system following exposure to pesticides and herbicides. Dichlobenil (2,6-dichlorobenzonitrile) is an herbicide that morphologically and pathologically affects olfactory systems in many different species ranging from fish to mice (Brittebo et al. 1991; Andreini et al. 1997). Impairments by dichlobenil include degeneration of neurosensory and sustentacular epithelium, necrosis of olfactory mucosa, and changes in concentrations of olfactory G-proteins like $G_{\alpha q}/G_{\alpha 11}$ in the olfactory neuroepithelium. Such impairments are primarily caused by damage to Bowman's glands (Andreini et al. 1997). Other environmental chemicals such as atrazine, diuron, ethylsulfony 1-2,5-and 2,6-dichlorobenzene, and deltamethrin have been shown to interfere with olfactory perception, generation of olfactory

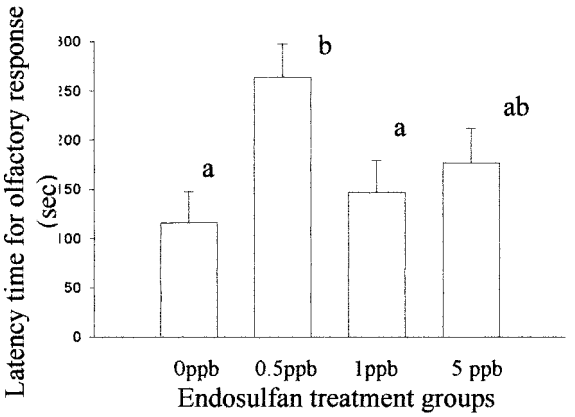

Figure 1. Males exposed to endosulfan demonstrated significantly delayed olfactory responses following the exposure of female odor cues. Data are represented as mean ± s.e.m throughout all figures. Groups that do not share the same letters differed significantly from one another ($P < 0.05$).

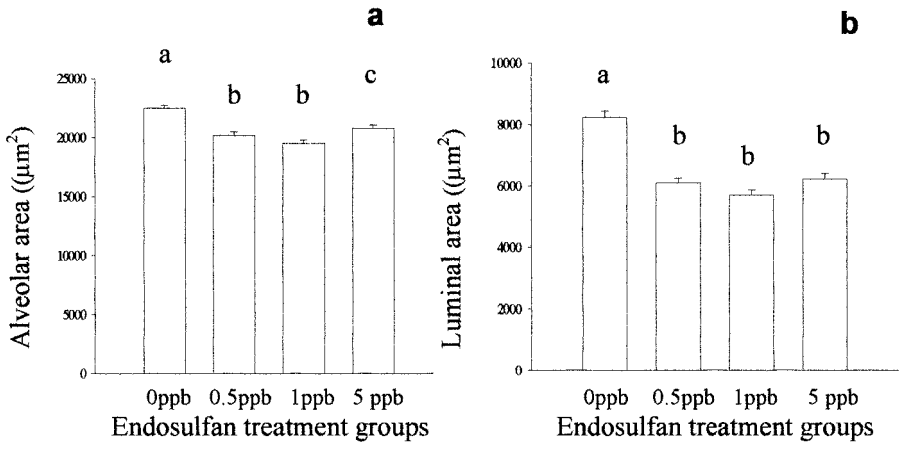

Figure 2. Morphological measurements of pheromone glands; **a**, alveolar size and **b**, luminal size. Groups that do not share the same letters differed significantly from one another ($P < 0.05$).

neuroepithelium, and olfactory signal processing in several species including moths, goldfish, and mice (Saglio and Trijasse 1998; Bahrami et al. 1999; Delpuech et al. 1999). Our current results imply that a variety of environmental chemicals including endosulfan may also affect amphibian olfactory and sensitivity to pheromones.

Histomorphometric investigation of hedonic glands revealed that treated male newts had significantly smaller alveoli in all treatment groups (Figure 2a, $F_{3, 8379}$

=22.95, $p<0.001$), suggesting that endosulfan exposure disrupts the growth of alveoli. In male newts, prolactin and steroid hormones (Pool and Dent 1977) control the growth of alveoli. Dopamine is a neurotransmitter that regulates prolactin production and secretion from the anterior pituitary (Pool and Dent 1977). Dopaminergic pathways are vulnerable to endosulfan and the disruption depends on the brain regions investigated and the time period of exposure (Gopal et al. 1985; Lakshmana and Raju 1994). Thus, 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.

The area of lumen within the alveoli was also significantly reduced by endosulfan treatment in all groups (Figure 2b, $F_{3, 8379}=41.88$, $p<0.001$). This result indicates that pheromonal secretion is also vulnerable to low-dose endosulfan exposure. The cholinergic system controls secretion of pheromones from these glands in male newts (Pool and Dent 1977). Cholinergic substances bind to the muscarinic receptor and induce the myoepithelium to contract, thus stimulating secretion of pheromones from the glands. The contraction results in a decrease in the luminal area of the alveoli. The effects of endosulfan on cholinergic systems have been also reported in several species. For example, when the fish *Channa punctatus* is exposed to this insecticide, there is a decrease in concentration of acetylcholine in the brain (Gopal et al. 1985). In pigeons (*Columba livia*), endosulfan exposure alters the cholinergic system and mediates seizure activity in the brain (Anand et al. 1986). These results suggest that during our study, endosulfan may affect the secretion of pheromones via disruption of the cholinergic system.

Endosulfan-treated male newts showed significantly reduced mating success in all treatment groups (Figure 3, Chi-square=12.42, df=3, $p=0.006$). Endosulfan exposure may affect the mating success of red-spotted newts possibly via the above documented disruption of the pheromonal detection and production system and possibly through neurological damage in regions of the brain affecting sexual behavior. Considering that hedonic pheromones play a critical role in increasing female receptivity during courtship, deficits in the production or release of these pheromones may prevent males from completing courtship because the females may never be becoming fully receptive. Furthermore, if a male's olfactory system was disrupted, he may be less likely to be sexually stimulated by a female's pheromones. Such deficits then could lead to lower mating success.

On the other hand, 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 following endosulfan exposure. Rats treated with endosulfan showed increased aggressive behavior and deficits in operant learning performance (Lakshmana and Raju 1994). One study in the fish, *Tilapia rendalli*, reported that brains from animals (Matthiessen and Roberts 1982). Thus, in our study, it is possible that endosulfan not only disrupted the male olfactory and pheromonal systems, but also interfered

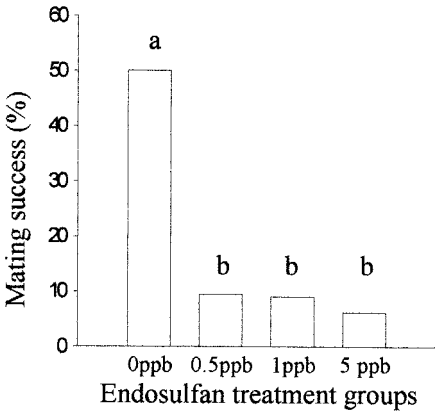

Figure 3. Mating success. Groups that do not share the same letters differed significantly from one another ($P < 0.05$)

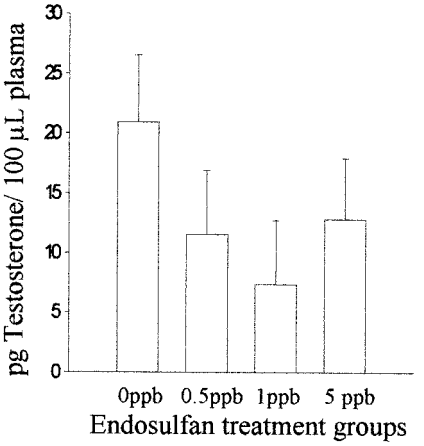

Figure 4. Testosterone concentration in plasma samples.

with male neural pathways involved in the performance of appropriate reproductive behavior.

Treatment with endosulfan did not change testis mass or the ratio of testis/body mass (one-way ANOVA, p=0.86, p=0.63 respectively). Control and treated newts did not have different snout-vent lengths (SVLs), body masses, or condition factors (body mass/ SVL; one-way ANOVA, p=0.12, p=0.08, p=0.09 respectively). These results suggest that although endosulfan exposure affects olfactory, pheromonal, and mating systems, our administrated concentrations of endosulfan do not induce overt toxicosis. Other studies, where higher concentrations of endosulfan were administered, however, have reported traditional toxicological effects. For example, neonatal rats exposed to 4.5 mg endosulfan /kg body weight by subcutaneous injection had decreased weights of testis, ovary, and oviduct and also had lowered levels of testosterone and estradiol (Ahmad et al., 1993). Fresh water fish, *C. punctatus*, exposed to only slightly higher levels of endosulfan as compared to our study (6 -10 ppb) showed tremors, convulsions, increased surfacing activity and increased general activity, and altered concentrations acetylcholine, serotonin and dopamine in the brain (Gopal et al. 1985). Considering these previous results, our findings cannot rule out the possibility that the sensitivity in pheromonal function and mating processes in newts may be similar to that of more overt toxicological effects that indicate marked neurologic dysfunction in *C. punctatus* (Gopal et al.1985).

Finally, plasma testosterone concentrations were measured. The difference in testosterone concentrations among groups was not significant (Figure 4., one-way ANOVA, p=0.37). Endosulfan has been demonstrated to have both antiestrogenic and estrogenic effects depending on the target system investigated and the species. Several studies have documented decreased concentrations of plasma

sexual hormones following treatment with endosulfan (e.g. Singh and Pandey 1990; Ahmad et al. 1993). For example, neonatal rats exposed to 4.5 mg/kg body weight endosulfan by subcutaneous injection had decreased plasma testosterone concentrations (Ahmad et al. 1993). On the contrary, in male fresh water fish, *C. punctatus*, exposed to 6 ppb and 10 ppb endosulfan, the plasma concentration of 11-ketotestosterone did not change (Matthiessen and Logan 1984). These previous and our current results suggest that 5 ppb concentration of endosulfan in male newts is not enough to induce detectable changes in testosterone levels.

Park and Propper (2001) found similar results in when female red-spotted newts were treated with endosulfan. Female cloacal pheromonal glands were reduced in size, males showed lowered olfactory responses to females, and the mating success of courting pairs was reduced when females were exposed to low dose of endosulfan. These results demonstrate that the effects of endosulfan on newts may not be sex-specific.

Our results have documented that the disruption of amphibian pheromonal communication systems can be induced by low concentration exposure to environmental chemicals without there being overt toxicosis, suggesting that disruption of amphibian pheromonal systems could mediate subtle negative 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 (Stebbins and Cohen 1995; Klesecker et al. 2001). Therefore, because pheromonal systems play a critical role in the daily life of amphibians, environmental chemical disruption of this sensory mechanism may be another mechanism leading to population declines

Acknowledgments. We thank Drs. S Hempleman, L Drickamer, K Nishikawa for their intellectual input with this manuscript and Dr. L Mayer, and R Lania, S Overstreet, and M Minor for their technical help. International Rotary Foundation Scholarship to DP and The Council for Tobacco Research, USA, Grant # 4661R1 to CRP supported this study.

REFERENCES

Ahmad MM, Ahmad MMaqsood, Sarvat S (1993) Effects of endosulfan and chlorpyrifos on the reproductive organs and sex hormones of neonatal rats. Pakistan J Zool 25:11-14

Anand M, Agrawal AK, Gopal K, Sur RN, Seth PK (1996) Endosulfan and cholinergic (muscarinic) transmission: effect on electroencephalograms and [^{3}H]quinuclidinyl benzilate in pigeon brain. Environ Res 40:421- 426

Andreini I, DellaCorte C, Johnson LC, Hughes S, Kalinoski DL (1997) G-protein (s), Gαq/Gα11, in the olfactory neuroepithelium of the channel catfish (*Ictalurus punctatus*) is altered by the herbicide, dichlobenil. Toxicology 117:111-122

Bahrami F, Brittebo EB, Bergman A, Larsson C, Brandt I (1999) Localization and comparative toxicity of methylsulfonyl 1-2,5- and 2,6-dichlorobenzene in the olfactory mucosa of mice. Toxicol Sci 49:116-123

Brille M, Coulson D, McGillivray L, Pauli B (1998) Toxicity of endosulfan to aquatic stages of anuran amphibians. Environ Toxicol Chem 17:1738-1744.

Brittebo EB, Erikson C, Fiel V, Bakke J, Brandt I (1991) Toxicity of 2,6 dichlorothiobenzamide (Chlorthiamid) and 2,6-dichlorobenzamide in the olfactory nasal mucosa of mice. Fundam Appl Toxicol 17:92-102

Colborn T, Clement C (1992) Chemically-induced alterations in sexual and functional development: the wildlife/ human connection. Princeton Scientific Publishing Co., INC, New Jersey

Delpuech J, Legallet B, Terrier O, Fouillet P (1999) Modifications of the sex pheromonal communication of trichogramma brassicae by a sublethal dose of deltamethrin. Chemosphere 38:729-739

Fox PJ, Matthiessen P (1982) Acute toxicity to fish of low-dose aerosol applications of endosulfan to control tsetse fly in the Okavango Delta, Botswana. Environ Pollut 27:129-142

Gopal K, Anand M, Mehrotra S, Ray PK (1985) Neurobehavioral changes in fresh water fish *Channa punctatus* exposed to endosulfan. J Adv Zool 5:74-80

Klesecker JM, Blaustein AR, Belden LK (2001) Complex causes of amphibian population declines. Nature 410:681-684

Lakshmana MK, Raju TR (1994) Endosulfan induces small but significant changes in the levels of noradrenaline, dopamine and serotonin in the developing rat brain and deficits in the operant learning performance. Toxicology 91:139-150

Matthiessen P, Roberts RJ (1982) Histopathological changes in the liver and brain of fish exposed to endosulfan insecticide during tsetse fly control operations in Botswana. J Fish Dis 5:153-159

Matthiessen P, Logan JWM (1984) Low concentration effects of endosulfan insecticide on reproductive behavior in the tropical cichlid fish *Sarotherodon mossambicus*. Bull Environ Contam Toxicol 33:575-583

Park D, Hempleman SC, Propper CR (2001) Endosulfan exposure disrupts pheromonal systems in the red-spotted newt: A mechanism for subtle effects of environmental chemicals. Environ Health Perspect 109:669-674

Pool TB, Dent JN (1977) Neural regulation of product discharge from the hedonic glands of the red-spotted newt. J Exp Zool 201:203-220

Reyes GG, Villagrana C, Alvarez GL (1999) Environmental Conditions and Pesticide Pollution of Two Coastal Ecosystems in the Gulf of California, Mexico. Ecotoxicol Environ Saf 44:280-286

Saglio P, Trijasse S (1998) Behavioral response to atrazine and diuron in goldfish. Arch Environ Contam Toxicol 35:484-491

Singh SK, Pandey RS (1990) Effect of sub-chronic endosulfan exposures on plasma gonadotrophins, testosterone, testicular testosterone, and enzymes of androgen biosynthesis in rat. Indian J Exp Biol 28:953-956