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Olfactory and Sexual Attractiveness of Western Mosquitofish (*Gambusia affinis*) Exposed to the Commonly Used Insecticide Endosulfan

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To know whether a short-term exposure to a commonly used insecticide induces subtle negative toxic effects, female western mosquitofish, *Gambusia affinis*, were exposed to 0.1, 0.5, and 1 ppb endosulfan for one week and subsequently examined for their olfactory and sexual attractiveness to conspecific males. A short-term exposure to endosulfan did not impair the physical conditions investigated in this study nor did it disrupt olfactory attractiveness of female mosquitofish. However, 1 ppb endosulfan significantly reduced sexual attractiveness of exposed females. Test males showed significantly less copulation attempts with the exposed females. Our results suggest that in the field, a short term exposure of endosulfan may disrupt mating processes in non-targeted aquatic organisms.

Many pesticides and fungicides are used on agricultural crops. After administration, some chemicals persist as both parent compounds and their metabolites. Stream water near agricultural areas is often contaminated as a result of surface runoff following storm events, airborne spray drift, or direct accidental spills (Frank et al., 1990). More than 60% of rural ponds investigated in Ontario, Canada were contaminated with pesticides (Frank et al., 1990). In southern Sweden, Kreuger (1998) detected 38 different pesticides in stream water within an agricultural catchment. Such contamination of surface water has posed a risk of direct exposure to organisms in aquatic ecosystems.

Numerous studies have investigated impacts of chemical exposure on aquatic organisms. Since environmental chemicals can affect populations via disruption of reproductive systems of individuals, researchers have focused their investigations on disrupted reproductive systems. In the field, unlike detection of overt toxic effects of chemicals, conventional toxicological screening and testing methods are generally poor at exploring more subtle negative effects such as changes in behavioral or hormonal responses. For these reasons, there are relatively few studies that have investigated such subtle negative effects of exposure to environmental chemicals (Park et al., 2001a). Particularly, little is known of subtle negative effects of those toxic chemicals following short-term exposure.

Endosulfan is a chlorinated hydrocarbon insecticide of the cyclodiene subgroup, which acts as a poison to a variety of insects. It is primarily used on a wide variety of crops including tea, coffee, cotton, fruits, and vegetables, as well as on rice, cereals, maize, or other grains. Several studies reported the presence of endosulfan in cotton crop soils (0.1-0.4 mg/kg; Luchini et al., 2000) and estuaries (0.5-4.0 μg/L; Fox and Matthiessen, 1982), and relatively high residual levels have been found in some marketed fruits such as Brazilian strawberries and tomatoes (4-510 ppb endosulfan; Araujo et al., 1999). These reports indicate that endosulfan exposure can occur in both human and wildlife populations. Endosulfan not only acts as a hormone disruptor, but also affects neurotransmitter systems of many species such as rat, catfish, and bullfrog tadpoles. For example, endosulfan suppressed testosterone and 17β-estradiol concentrations in neonatal rats (Ahmad et al., 1993), increased thyroxine (T4) levels in catfish (Sinha et al., 1991), 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 fresh-water fishes, pigeons, and rats (Gopal et al., 1981; Gopal et al., 1985; Anand et al., 1986; Lakshmana and Raju, 1994)

To investigate whether a short-term exposure of endosulfan disrupts mating processes in non-targeted

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aquatic organisms, female western mosquitofish, *Gambusia affinis*, were exposed to 0.1, 0.5, and 1 ppb endosulfan for one week and subsequently their olfactory and sexual attractiveness to conspecific males were examined.

Materials and Methods

Collecting fish and husbandry

Male and female western mosquito fish were collected from the Bubbling Ponds Fish Hatchery, Yavapai County, Arizona, USA with a specific approval from the Game and Fish Commission of the State of Arizona (# SP844495). Information about general ecology and behavior of the mosquitofish is easily found in several papers (Hughes, 1985; Bisazza et al., 1989; McPeek, 1992; Koya et al., 1998). Upon arrival at the laboratory, the fish were placed in aquaria (80×40×50 cm) at a density of no more than 40 same-sex individuals per tank. The water temperature varied between 15-21℃. The photoperiod followed the local photoperiod. The fish were fed frozen brine shrimps (0.3 g per fish) daily. The holding tanks were cleaned weekly by siphoning and replacing 5 liters of water.

Exposure to endosulfan

For investigation of the short-term exposure effects of endosulfan on olfactory and sexual attractiveness, 80 females (20 individuals in 4 groups) from February 3rd to 10th (Experiment I) and 84 females (42 for control and 14 individuals in 3 groups) from February 24th to March 3rd (Experiment II) were exposed to endosulfan in 1999. For treatment groups, endosulfan (ps-81, 99% Mix of isomers, Chem. Service, West Chester, PA) dissolved in 1 mL of acetone was delivered into the aquarium containing 10 L of dechlorinated tap water and fishes. Final endosulfan concentrations were 0.1, 0.5, and 1.0 ppb. These concentrations have been detected in the field (Fox and Matthiessen, 1982). The control group only received 1 mL acetone.

Olfactory attractiveness test

After one week the fishes were kept for 5 h in 10 L fresh water to remove endosulfan from the skin. To collect odor sources for the olfactory attractiveness test we put 10 individuals from each group into each different experimental aquarium (80×40×50 cm) containing 500 mL aged tap water for 3 h. The collected odor sources were filtered through double layers of gauze, kept at 4℃, and then used within 2 d.

For the olfactory test, two fresh males were placed in the experimental aquarium (80×40×50 cm) containing 10 L of tap water for 4 h before the start of each test. Each aquarium was divided into 2 halves by a centerline. Only when males frequently and regularly used both sides of the aquarium (determined by measuring the time spent on both sides of the aquarium) without any stimuli, did we introduce odors (at a flow rate of 70 mL/min into both sides of the aquarium). One side of the aquarium received either control or treated females odors. The other side of the aquarium coincidently received matched odors of either control or treated females. The flow rate was controlled by flow meters (GJ502, Gilmont Ins., Barrington, IL). During preliminary studies using a dye, two odors did not mix at this flow rate during a 5-min test period. The side of each odor was randomly decided by a coin toss. After introducing the odors, we recorded the amount of time that the two males spent at each side of the aquarium. Thirteen to fourteen trials for each test were made. During any given experiments, the observer was blind to which side received which odor. Data obtained were analyzed by one sample t-test (Sokal and Rohlf, 1981).

Sexual attractiveness test

After odor collection, to measure sexual attractiveness, each pair of control and treated females matched by body mass and gravid spot (if it bears) was allowed a courting stage for 5 min with two fresh males. We placed the two males in open field boxes (49×26×14 cm) containing 3 L of tap water 1 h before the test begun. We used two, rather than one, test males in the test box because sexual attempts from two different males are more easily measurable (McPeek, 1992). We recorded the amounts of male copulation trials during the 5 min as an indicator of female sexual attractiveness. Copulation attempts were separated into three steps: 1, a male approaches the female genital pore with gonopodium erected at an angle less than 90° with relation to his body; 2, a male approaches the female genital pore with gonopodium erected at an angle greater than 90°; and 3, a male thrusts his gonopodium into the female genital pore. Each level received a score for statistic analysis of 1, 2, and 3, respectively. The total sexual attractiveness was a sum of all scores during the 5 min test period. We used matched Willcoxon singed rank test to analyze data from each matched control and exposed female group (Sokal and Rohlf, 1981).

Following the behavioral experiments, each control and treated fish was anesthetized by immersion in 0.1% MS-222 solution. After measuring body length, body weight, and anal fin length, all animals were dissected. We determined if the fish were gravid and then removed and weighed the ovaries. The condition factor (body weight/body length), relative anal fin length (anal fin/body length), and gonadosomatic index (ovary weight/body weight; GSI) were determined from these measurements. By the end of experiments, individuals in some treatments were reduced; 1) 10 individuals from each experimental group in Experiment I were randomly selected and reserved for a future

Table 1. Physical conditions of control and exposed female mosquitofish to endosulfan for one week

Endosulfan exposed groups	Physical parameters (mean ± SE)						
	Body mass (g)	Body length (mm)	Ovary weight (g)	Anal fin length (mm)	Condition factor	Anal fin/ Body mass	GSI*
Control (N=10)	0.90 ± 0.28	35.98 ± 4.14	0.094 ± 0.07	5.77 ± 0.89	0.025 ± 0.01	0.16 ± 0.01	0.10 ± 0.07
0.1 ppb (N=10)	0.93 ± 0.27	36.53 ± 3.36	0.085 ± 0.04	6.03 ± 0.78	0.025 ± 0.01	0.17 ± 0.01	0.12 ± 0.05
0.5 ppb (N=10)	0.81 ± 0.31	36.13 ± 3.96	0.076 ± 0.04	5.78 ± 0.70	0.022 ± 0.01	0.16 ± 0.01	0.10 ± 0.05
1.0 ppb (N=10)	0.96 ± 0.28	37.37 ± 3.62	0.104 ± 0.05	5.99 ± 0.61	0.020 ± 0.01	0.16 ± 0.01	0.13 ± 0.05

* Gonadosomatic index

hormonal study and 2) some individuals were infected with the tapeworm, *Bothriocephalus acheilognathi* and removed from the analysis. Differences in physical conditions among treatment groups were analyzed by one-way ANOVA (Sokal and Rohlf, 1981). To compare the ratio of gravid females between Experiments I and II, we used Chi-square test (Sokal and Rohlf, 1981).

Results

In Experiment I, there were more gravid females (28 gravid and 11 non-gravid females) than in Experiment II (33 gravid and 38 non-gravid females) and the difference was significant (Chi-square = 5.546, $p = 0.01$).

Physical conditions of the female fish exposed to endosulfan in February were not different among the control and treated groups (Table 1, one-way ANOVA; $p > 0.05$ for all parameters). The female fish exposed to 1.0 ppb of endosulfan received significantly less number of courting attempts from test males than the control females who matched by body mass and gravity spot (Matched Wilcoxon singed ranks test; $z = 2.38$, df = 17, $p = 0.017$, Fig. 1A). On the other hand, courting attempts foward the 0.1 and 0.5 ppb exposed female fish were not different from the matched control females (Fig. 1A). In the olfactory attractiveness test, attractiveness of the exposed females odors was not different from that of the control female odors (data not shown, one sample t-test, $p > 0.05$ for all paired groups).

In Experiment II, one-week exposure of endosulfan did not change physical conditions (data not shown, one-way ANOVA; $p > 0.05$ for all parameters). The test males did not show different preferences among odors from the exposed and matched control females, indicating that olfactory attractiveness of control and treated females was not different (data not shown, one sample t-test, $p > 0.05$ for all paired groups). Finally, no differences in sexual attractiveness were found between the control and treated females (Fig. 1B).

Discussion

Female mosquitofish exposed to 1.0 ppb endosulfan in February were significantly less attractive sexually to conspecific males. This result suggests that a short-term exposure to low doses of endosulfan may disrupt mating processes in mosquitofish and that in the field, such impact may result in lowered mating success of exposed fish.

One-week treatment of endosulfan did not change any physical conditions of female mosquitofish, including condition factor, GSI, and relative anal fin length. These results suggest that although endosulfan exposure affects sexual attractiveness of females, the concentrations of endosulfan and the period of exposure we used may not be enough to induce overt toxicosis. Other studies, using higher concentrations or longer exposure of endosulfan, however, have reported the conventional toxic effects. For example, female red-spotted newts, *Notophthalmus viridescens*, exposed

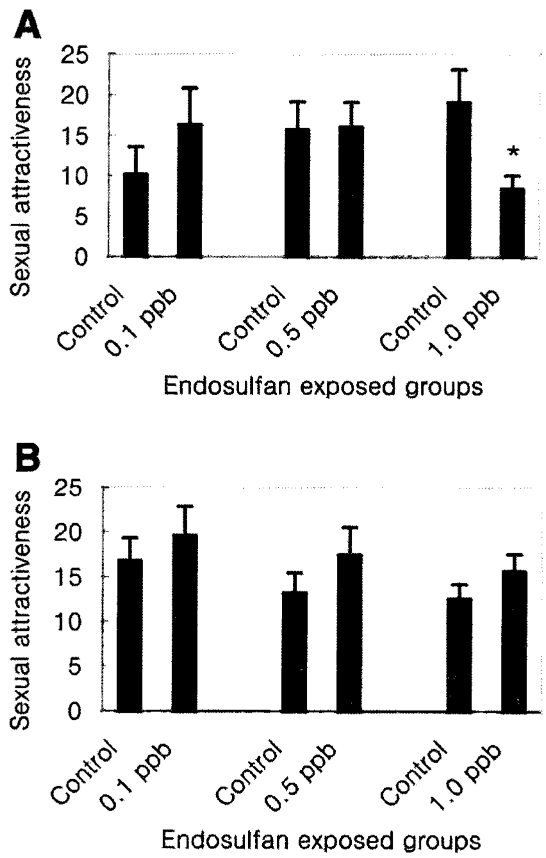

Fig. 1. Females exposed to 1.0 ppb endosulfan in Experiment I were sexually less attractive to conspecific males (A), but the disruption was not observed in Experiment II (B). * $p < 0.05$.

to 5 and 10 ppb for 96 h had anatomically and functionally impaired pheromonal systems (Park et al., 2001a). Female mosquitofish exposed to the same doses of endosulfan for 5 weeks had significantly elongated anal fin in female mosquitofish (Park et al., 2001b).

Appropriate secretion of specific pheromones in a specific step of mating is critical for successful mating in aquatic vertebrates such as mosquitofish, Atlantic salmon (*Salmo salar* L.), and red-spotted newts (Moore and Waring, 2001; Park et al., 2001a; Park et al., 2001b). Several pesticides have been documented to affect the pheromonal systems and subsequently result in disrupted mating. For example, red-spotted newts exposed to 5 ppb of endosulfan for 96 h had impaired pheromonal systems, with lowered mating success (Park et al., 2001a). Male Atlantic salmon exposed to 0.004 ppb of cypermethrin, synthetic pyrethroid pesticide, for 5 d had lowered olfactory responses to female pheromones (Moore and Waring, 2001). In our olfactory attractiveness tests, however, test males did not show any different preferences to control and exposed female odors. Our current results suggest that one week exposure to 0.1, 0.5, and 1.0 ppb endosulfan may not disrupt pheromonal systems in this species.

How does endosulfan impair sexual attractiveness of female mosquitofish? It is possible that endosulfan causes changes in sexual attractiveness through mechanisms that were not detected in this study. Several investigations have documented neurological changes after endosulfan exposure. Fresh water fish, *Channa punctatus*, exposed to 6 ppb and 10 ppb of endosulfan showed tremor, convulsion, increased surfacing activity and increased general activity, as well as altered concentrations of neurotransmitters in the brain including acetylcholine, serotonin and dopamine (Gopal et al., 1985). One study in the fish *Tilapia rendalli* reported that brains from animals exposed to endosulfan exhibit abnormalities such as encephalitis, meningitis, and edema in several different areas of the brain (Matthiessen and Roberts, 1982). Thus, it is possible that endosulfan may interfere with female neural pathways involved in the performance of appropriate reproductive behavior. Males may have ceased copulation attempts with females who were non-responsive.

Our results about different gravid ratio between Experiment I and II females suggest that different reproductive conditions may be in part responsible for the disrupted sexual attractiveness in the 1.0 ppb females in Experiment I. Females in Experiment I had significantly heavier ovary than those in Experiment II, suggesting that more females in Experiment I might be in breeding conditions. Several studies have documented different toxic responses in different breeding conditions. Chambers and Yarbrough (1979) found that enzymes involved in metabolizing pesticides vary both seasonally and between *G. affinis* populations. The highest levels of organophosphate pesticides were found in *G. affinis* during non-breeding season (Porte et al., 1992). These results suggest that reproductive conditions could affect toxic response patterns in the fish. Interestingly, our result that Experiment I females, possibly in higher breeding conditions, only showed impaired sexual attractiveness is contrary to the results from the previous studies. In our previous study, only non-breeding female mosquitofish exposed to the same concentration of endosulfan for 5 weeks had impaired anal fin (Park et al., 2001b). At present it is difficult to precisely explain the mechanism of this insecticide in acting on sexual attractiveness. Further studies about mechanisms underlying disrupted mating processes and seasonal different responses are necessary.

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