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Pairing behavior and reproduction in *Hyalella azteca* as sensitive endpoints for detecting long-term consequences of pesticide pulses

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ABSTRACT

The aim of the present study was to examine acute and delayed effects of pulse exposure of the pyrethroid pesticide, permethrin, on precopulatory pairs of *Hyalella azteca*. Pairs of *H. azteca* were exposed to a single 1 h pulse of different nominal concentrations of permethrin: 0, 0.3, 0.9 or 2.7 μg/L. During exposure, pairing behavior was observed, and during a 56 day post-exposure period the treatments were monitored for pairing behavior, survival and reproductive output. All permethrin-exposed pairs separated within minutes during exposure and shortly thereafter became immobile; however they regained mobility after transfer to clean water. The time to re-form pairs was significantly longer in all tested concentrations compared to the control, although all surviving pairs re-formed within the 56 day test period. Nevertheless not all pairs exposed to 0.9 and 2.7 μg/L reproduced. Furthermore the numbers of juveniles produced by pairs exposed to 0.9 and 2.7 μg/L, but not 0.3 μg/L, were lower throughout the entire post-exposure period compared to the control groups, and the total numbers of juveniles produced during 56 days were significantly lower in organisms exposed to 0.9 and 2.7 μg/L, but not 0.3 μg/L, compared to the control groups. The long-term effects of short-term exposure on reproductive behavior of pairs could potentially have consequences for the population dynamics of *H. azteca*. However, since individual-level responses can both overestimate and underestimate effects at the population level, appropriate population models are needed to reduce the uncertainty in extrapolating between these levels of biological organization.

1. Introduction

Non-target aquatic organisms are likely to be exposed to pulses of pesticides following spray drift, accidental spills, drain flow and surface run-off after rainfall. These pulses may extend anywhere from a few minutes to several hours (Kreutzweiser and Wood, 1991; Liess et al., 1999; Rawn et al., 1982; Reinert et al., 2002). Standard acute ecotoxicity tests to assess the risks of pesticides to non-target organisms typically use juvenile organisms. In addition, there is limited chronic toxicity testing looking at sublethal effects such as reproduction. Both standard acute and chronic test methods use constant exposure concentrations and such tests are not designed to assess the effects of intermittent exposure or the potential for delayed effects. Here we explore the extent to which a brief pulse of pesticide exposure has long-term effects on pairing behavior and reproductive output.

We have chosen permethrin as a representative pyrethroid insecticide since it is widely used and is found to be highly toxic to aquatic organisms (US EPA, 2000). Permethrin has been detected in the aquatic environment (CCME, 2006; Starner et al., 2008), and approximately 0.5–1.5 h after aerial applications surface water concentrations ranged from 0.070 to 7.2 μg permethrin/L (Helson et al., 1993; Kreutzweiser and Wood, 1991; Lizotte et al., 2012a, b). US EPA estimated a worst case environmental concentration ranging from 0.2 to 5.32 μg permethrin/L depending on the crop (US EPA, 2009).

We have chosen the freshwater amphipod, *Hyalella azteca*, as a test species since it is widely distributed and provides an important part of the food source for many fish, birds and large invertebrates throughout North America (De March, 1981). Also, *H. azteca* has been extensively used in aquatic toxicity tests, and its life cycle is well described.

H. azteca is iteroparous and reproduces sexually. Reproduction starts with amplexus in which the larger adult male grasps the female from the dorsal side with its gnathopods. The precopulatory pair is able to swim and feed together. After 1 to 7 days in amplexus the pair separates briefly while the female sheds her old

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exoskeleton. Thereafter the two organisms reunite for a short period, and copulation occurs. After copulation the female releases eggs into the marsupium where they are fertilized and the offspring develop and hatch. The offspring are kept in the marsupium until the next molt (Cooper, 1965; Environment Canada, 1997; Strong, 1973; US EPA, 2000).

Swimming in amplexus plays a key role in the reproductive cycle and is therefore essential for successful reproduction in *H. azteca*. However, very few studies have investigated effects of pesticides on pairing behavior of *H. azteca*. Previous studies have found that precopulatory pairing in *H. azteca* was impaired by pesticide exposure (Blockwell et al., 1998; Pandey et al., 2011), and disruption of pairs of other amphipod species due to environmental changes including the presence of toxicants has been described in the literature (Davis, 1978; Linden, 1976; Malbouisson et al., 1994; Pascoe et al., 1994). Pairs of *Gammarus pulex* exposed for 1 h to the pyrethroid esfenvalerate separated during exposure, and subsequent reproductive output was significantly reduced during a 14 day monitoring period in clean water (Cold and Forbes, 2004).

The specific aims of the present study were to examine sublethal effects of a 1 h pulse exposure of different concentrations of permethrin on precopulatory pairs of *H. azteca* and to determine whether there were any acute or delayed effects on pairing behavior or subsequent reproductive output over a 56 day period following the pulse exposure.

2. Materials and methods

2.1. Culturing conditions

H. azteca were obtained from Ghent University and maintained in our laboratory at Roskilde University since February 2009. *H. azteca* were cultured in a temperature-controlled room at 23 °C with a photoperiod of 16 h of light and 8 h of dark and a light intensity of 500 ± 50 lux. Cultures were maintained in 63 L glass aquaria in which water quality was controlled by means of a biological filter containing mussel shells, stones and sand. The water in the aquaria was a mixture of tap water from Roskilde University diluted with deionized water (2:1) which was further mixed with artificial freshwater (Borgmann, 1996) in the ratio of 2:1 (Pedersen, unpublished data). A screening study of nine different mixtures of water showed that this combination was the most favorable for survival and reproduction of *H. azteca* (Pedersen, unpublished data). pH was 7.8 and hardness was 221 measured as mg/L $CaCO_3$. Three times a week approximately 1.4 g rabbit chow (Chrisco, Koege, Denmark) was added to each aquarium, and every second week cotton gauze was added as a substrate.

2.2. Experimental procedures

The experiment was conducted under the same light and temperature conditions as the cultures (described above). The experiment lasted for 56 days, and measured endpoints were mobility, survival, pair separation, pair re-formation, time to first reproduction and reproductive output.

Each treatment consisted of 6 replicates (1 pair of *H. azteca* in each) per concentration. Each of the replicate pairs was exposed in a 400 mL glass beaker containing 150 mL of test water and 5 × 5 cm gauze as a substrate. The test water used in the experiments was the same as used for the cultures.

Pairs used in the experiment were randomly selected from a culture of 10 week old *H. azteca*. The pairs were pulse-exposed to different nominal concentrations of permethrin: 0, 0.3, 0.9 and 2.7 μg/L. The pulse lasted for 1 h to simulate a realistic run-off event. There were two control groups. Control group 1 (C1) was pulse exposed in control water and treated identically to exposed organisms to ensure that any differences among treatments were not due to differences in handling and that any possible separation was not caused by transferring the pairs. Control group 2 (C2) was pulse exposed in control water, and after the pulse the pair was gently separated with a pipette. This control group was included to enable comparison of the time to pair re-formation with pairs exposed to permethrin.

After exposure, the pairs were transferred to clean water to be rinsed of residual pesticide and subsequently transferred to another beaker with clean water and cotton gauze as a substrate. Three times a week, 50 mL of the water was removed and gently replaced with fresh aerated water, after which each pair was fed with 3 mg of homogenized rabbit chow diluted in water.

Each beaker was monitored for separation of pairs, pair re-formation and mobility with an interval of 1 min for the first 10 min and thereafter every 15 min until the end of the pulse exposure, once immediately after transfer to clean water and then at 2, 3, 6, 20, and 24 h from the start of the experiment. From day 1 to 4 the beakers were monitored twice a day and then daily until day 9, after which they were monitored approximately three times a week. The beakers were also monitored for survival and reproduction. Every second week the juveniles produced by a pair were counted and removed from the beakers. For each monitoring day, only those replicates with a surviving pair were included in the calculation of reproductive output.

H. azteca were defined as immobile when lying at the bottom of the beakers, with no observed swimming behavior after gentle disturbance. Mortality was recorded when animals were partly or completely decomposed.

2.3. Preparation of test solutions

Permethrin was provided by Syngenta Ltd., as a mixture of cis and trans isomers (chemical purity of 98%) (Sigma–Aldrich, Fluka: 45614). A stock solution of permethrin was made in acetone to prepare the test solutions. The same amount of acetone, 0.01% of the total volume, was added to each treatment including the two controls. A screening experiment was made with two control groups, one with and one without acetone. We found no significant difference in behavior or survival between the two control groups and therefore used only acetone control groups in the experiment.

2.4. Water quality

Temperature, pH, dissolved oxygen level, hardness, alkalinity and ammonium were measured weekly. The pH was measured with a pH electrode (HANNA Instruments), dissolved oxygen level with a handheld dissolved oxygen meter (YSI 550 DO), water hardness with a Total Hardness Test (Aquamerck, No. 1.08020.0001), and ammonium with a JBL test kit. Water quality met the US EPA acceptability criteria (US EPA, 2000) such that ammonium concentrations never exceeded 0.05 mg/L, and the oxygen concentrations were above 6.18 mg/L in all treatments.

2.5. Permethrin extraction and analysis

Methods were based on Hladik et al. (2009) with modification. Strata-X 33μ Solid Phase Extraction (SPE) cartridges (30 μg/3 mL) (Phenomenex) were conditioned with 2 × 1 mL of dichloromethane and 2 × 1 mL of deionized water. Phenanthrene D10 and chrysene D12 were used as internal and recovery standards, respectively. The 200 mL water samples were passed through a SPE cartridge with a maximum flow rate of 10 mL min^{-1}. The cartridges were eluted with 2 × 1.5 mL of dichloromethane, collected in a 12 mL