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Diminished Conspecific Odor Recognition in the Rusty Crayfish (*Orconectes rusticus*) Following a 96-h Exposure to Atrazine

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Abstract The presence of agricultural contaminants has been shown to disrupt olfactory-mediated behaviors in aquatic animals. We assessed the effects of atrazine on the ability of reproductively active (form I), male crayfish (*Orconectes rusticus*) to identify and respond to conspecific chemical signals involved in mating. Male crayfish were exposed to atrazine (80 ppb) and water (control) for 96 h. We analyzed odor localization and locomotor behaviors of herbicide-treated and control male crayfish to two different odor sources: female odor or water (control) delivered from the proximal end of a test arena. Control crayfish spent more time in the proximal region of the test arena and at the odor source. Atrazine-exposed crayfish showed no preference for the proximal region of the test arena and odor source when female odor was delivered. Atrazine exposure did not affect locomotor behaviors. Overall, atrazine-mediated chemosensory deficits have the potential to disrupt mating and affect population size.

Keywords Crayfish · Atrazine · Chemosensory deficits · Conspecific odors

Land-use practices have the ability to impact the aquatic habitats surrounding them. Specifically, run-off from the agricultural industry and urban areas can carry with it residues and contaminants that may enter local streams and rivers. In addition to run-off, contaminants can enter the aquatic environment through evapotranspiration and groundwater percolation (LeBlanc et al. 1997). Transport of agricultural chemicals, such as pesticides, can therefore have a significant impact on water quality in aquatic environments and can subsequently impact native flora and fauna (Tong and Chen 2002). Pesticides are frequently used in agricultural areas in order to expand crop yield and to remove unwanted vegetation. Roughly 234 million kilograms of pesticides are applied to cropland in the U.S. yearly (Fernandez-Cornejo et al. 2014). Atrazine (ATR; 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is used to stop the growing of broadleaf plants and grassy weeds. It is listed among the top four herbicides used in the U.S. in 2008 and is the most frequently used herbicide in the U.S. Midwest with 33–36 million kilograms being sold annually to the U.S. agricultural industry (EPA 2013; Fernandez-Cornejo et al. 2014; Kiely et al. 2004). The half-life of ATR in surface waters and in ground water can vary from 8 days to several years so its persistence and pollution in surface waters and streams is a concern (Comber 1999, EPA 2007). Because of storm events and ground water percolation, large amounts of ATR can reach local streams and rivers. ATR concentrations greater than the maximum safe concentrations of 10 µg/L have been found repeatedly in the environment (see Table 1, Belanger et al. 2016). Exposure levels above the maximums can last for over 21 days during the Spring and may subsequently affect non-target species (EPA 2014).

Previous research has shown that exposure to sublethal, ecologically relevant concentrations of ATR can have negative effects on the physiological and behavioral performance in many aquatic organisms (Solomon et al. 2008). When frogs (*Xenopus laevis*) were exposed to sublethal concentrations of ATR, it had damaging effects on their sexual development; this exposure produced the demasculinization of the

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Table 1 Verification of stock and treatment ATR solutions using RPLC-MS/MS

Intended [ATR] (ppb)	ATR solvent/ATR diluent	Measured [ATR] (ppb)
17,000 (stock)	H_2O/H_2O	16,900
80 (treatments)	H_2O/H_2O	77.9
80 (treatments)	H_2O/H_2O	76.6

male larynx and hermaphroditism (Hayes et al. 2002). After fathead minnows (*Pimephales promelas*) were treated with ATR, they showed a decrease in egg production and gonadal abnormalities (Tillitt et al. 2010). Tierney et al. (2007) showed that ATR exposure can impair olfactory responses rainbow trout (*Oncorhynchus mykiss*). They showed that electroolfactogram (EOG) responses to amino acids were significantly reduced following a subacute (30 min) exposure of 10 and 100 µg/L ATR. Normal olfactory responses returned after at 20 min recovery period. Further, goldfish (*Carassius auratus*) displayed a reduction in olfactory-mediated actions in response to social odors and alarm cues when exposed to sublethal concentrations of carbofuran, ATR, and diuron, a phenylurea herbicide (Saglio and Trijasse 1998). Crayfish also demonstrate changes in olfaction and locomotory behavior following ATR exposure. Signal crayfish (*Pacifastacus leniusculus*) exposed to ATR moved with difficulty and often stayed in the corners of the test arena following exposure to a high dose (>10 mg/L) of ATR (Velisek et al. 2013). Further, exposure to environmentally relevant concentrations of ATR led to lasting decreases in attraction to food odors in crayfish *Orconectes rusticus* and *Orconectes virilis* (Belanger et al. 2016, 2015). Overall, ATR exposure has been shown to have negative impacts on many aquatic organisms, including crayfish.

Crayfish rely heavily on chemoreception to find food, mates, and to avoid predators, changes in their ability to perceive odors (Dunham et al. 1997; Dunham and Oh 1992; Hazlett 1990). Because they are a keystone species in aquatic systems, changes in their ability to perceive odors can have detrimental effects on aquatic ecosystems (Lodge et al. 1994). Moreover, crayfish have measureable responses (e.g. changes in behavior, chemoreception, mating success, morphology, tissue structure and toxicant accumulation, etc.) to sublethal concentrations of various contaminants (Belanger et al. 2017). Because of this, they can be used as a sentinel species to assess the broad effects of ATR on vital olfactory-mediated behaviors, including responses to conspecific mate odors. As male crayfish rely heavily on chemosensory information to locate female odors in order to mate and reproduce, any reduction in this ability could have detrimental effects on their population size. We exposed reproductive (form I) male crayfish (*O. rusticus*) to environmentally-relevant concentrations of ATR and assessed their abilities to localize a conspecific female odor source. We hypothesized that ATR-exposed crayfish would have a difficult time locating mate odors following exposure while not affecting overall locomotion. Given that Belanger et al. (2015) showed that chemosensory responses to food odors are reduced following ATR exposure, we expect to see changes in time spent near the odor source following ATR exposure. Because *O. rusticus* are known to mate in the Spring, chemosensory impairments caused by increased ATR exposure could interfere with mating activities (Berrill and Arsenault 1982). We hope that clear evidence demonstrating the detrimental effects of ATR on the chemosensory responses of non-target species may lead to tighter regulations and a search for alternatives.

Materials and Methods

Form I (reproductive) male and reproductive female crayfish (*O. rusticus*) were collected by seine near Bowling Green, Ohio, USA. Crayfish were acclimated to laboratory at Bowling Green State University for 2 weeks prior to experimentation. Reproductive (form I) male crayfish were identified and isolated from each other both visually and mechanically in a flow-through holding system (each container was 11 cm depth × 17 cm width × 27 cm length). The flow-through tanks were housed in an environmental chamber [Mean water chemistry parameters: pH 7.5, dissolved oxygen = 8.29 mg/L, Dissolved oxygen percent saturation = 96.45%, Temperature = 22.3°C, Hardness = 250 ± 25 mg/L, and TOC = 2.54 mg/L; 14:10 h light dark cycle to maintain reproductive status]. Crayfish mass, carapace and chelae lengths (±S.D.) were measured. Intact form I males (11.5 ± 4.3 g; 3.1 ± 0.4 cm carapace length; 2.8 ± 0.6 cm chelae length, N = 40) were used in this experiment. There was no difference in the size of crayfish used in each of the test groups ($p > 0.05$, 1-way ANOVA). Reproductive female crayfish (8.67 ± 3.40 g and 3.01 ± 0.43 cm carapace length, N = 35) were housed in a similar manner prior to collection of female odor. Crayfish were fed a diet of rabbit pellets three times per week. All experimental trials were conducted between May and August 2011 between 0900 and 1800 h.

Stock solutions of ATR (17 mg/L) were prepared using methods of Belanger et al. (2015) and were kept in the dark at 4°C (ATR; Sigma-Aldrich, 99% purity). For treatments, crayfish were placed in marked 1500 mL plastic containers with 500 mL of dechlorinated water (water chemistry similar to that of the holding system), a lid and an air stone for aeration. To ensure constant concentration during the 96-h treatment period, the water was changed every morning and 2.24 mL of stock solution was added to the pot using a

pipette (final concentration −80 ppb (µg/L) ATR). Crayfish were exposed to 80 ppb ATR as previous studies have shown that this concentration is environmentally-relevant and may cause changes in olfaction in fish and crayfish (Belanger et al. 2016, 2015; Tierney et al. 2007). Control crayfish also received daily water changes.

All ATR treatment solutions were verified and standardized via liquid chromatography-mass spectrometry (LC-MS) analysis. All reagents were purchased from Sigma-Aldrich (St. Louis, MO). Water was Sigma-Aldrich HPLC grade, and acetonitrile was Fluka Analytical LC-MS grade. ATR and ATR-d5 stock solutions of 0.500 and 1.00 mg mL^{-1}, respectively, were prepared in methanol. Standard solutions of 0, 5, 10, 50, 100, 500, 1000, and 5000 ppb ATR were prepared by dilution in 5% acetonitrile and 0.1% formic acid, and 100 ppb ATR-d5 was included in all solutions for use as an internal standard. The ATR standards and exposure solutions were analyzed by reversed phase liquid chromatography-mass spectrometry/mass spectrometry (RPLC-MS/MS) using an Agilent 1200 LC coupled to an Agilent 6410 tandem quadrupole mass spectrometer (Santa Clara, CA). The column used was a Phenomenex Kinetex 2.6 µm C18 column (2.1 × 75 mm) (Torrance, CA). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The LC flow rate was 0.2 mL/min, and the gradient consisted of a 5-min linear ramp from 0 to 90% B, a 2-min wash at 90% B, and a 5-min re-equilibration period at 0% B (total run time 12 min). The injection volume was 5 µL, and the column temperature was 30°C. Detection was performed using multiple reaction monitoring (MRM) in positive ion mode using the following precursor/product ion transitions: ATR, *m/z* 216 → 96 (quantifier) and *m/z* 216 → 174 (qualifier); ATR-d5, *m/z* 221 → 101 (quantifier) and *m/z* 221 → 179 (qualifier). The collision energy voltage for all transitions was 20 V. Other mass spectrometer parameters were as follows: fragmentor voltage 130 V, cell accelerator voltage 7 V, capillary voltage 4000 V, gas temperature 325°C, gas flow 10 L min^{-1}, and nebulizer pressure 40 psi. LC-MS validation of ATR treatment solutions can be found in Table 1.

To examine the effects of acute ATR exposures on chemoreception of female odor in *O. rusticus*, reproductive female conditioned-water (n = 7) was obtained using a procedure similar to Belanger and Moore (2009). This water was filtered and used in the behavioral trials. Water for control trials was prepared similarly in the absence of crayfish. The experiments consisted of 2 × 2 design with two treatments (80 ppb ATR and control) with two odor response treatments (female conditioned-water and control (water)). Ten crayfish were tested for each treatment/odor combination and each crayfish was used only once. Trials were conducted in a flow-through test arena (120 cm long × 21 cm wide × 19 cm high) described by Belanger et al. (2015). Before each trial, the test arena was filled with 10.5 L of dechlorinated water (water chemistry similar to that of the holding system). The female odor or water (control) was contained in a 4 L vat and connected to the test arena at the proximal end via plastic tubing and a flow meter (Manostat Riteflow #3, Manostat, Peaquannock, New Jersey). Following every trial, the tank was thoroughly rinsed to remove any residual odors.

ATR-treated and control male crayfish were individually placed at the distal end of the test arena, furthest away from the inflow valve. Crayfish were acclimated to the test arena by initially covering them with a polyvinylchloride pipe (10-cm diameter) with holes drilled into it and mesh covering the top for 5 min. Following this, the pipe was removed and the crayfish were free to move about the test area for 15 min (range 15–17 min) before the introduction of test odor. When the crayfish returned to the distal area of the arena, the behavioral trial began. Test odor (female odor or water) was introduced at the proximal part of the test arena at a flow rate of 50 mL/min (Belanger and Moore 2009). Crayfish movements within the tank were subsequently recorded for 15 min using a Panasonic HDC-HS250 digital video camera mounted above the test arena. The tank was marked with tape to delineate different areas of the test arena for behavioral data collection.

Videos obtained were analyzed using Noldus Ethovision XT software (Leesburg, Virginia, USA). Using methods originally developed by Belanger and Moore (2009), the test arena was divided into proximal (0–60 cm from the odor source) and distal regions (60–120 cm from the odor source), and the time spent in each region was evaluated. Time spent within 10 cm of the odor source, time spent moving, total distance travelled, and walking speed (cm/s) were also analyzed. To determine if ATR exposure affected male chemosensory responses to female odors, a three-way multiple analysis of variance (MANOVA) [factors = treatment group (ATR-treated, control), odorant type (female odor, water) and test arena location (proximal, distal, odor source)]. A Fisher least significant difference (LSD) post-hoc test was performed to examine the dependent variable [time differences (s)] in each area of the test arena between groups. A three-way MANOVA was also used to compare time spent moving, total distance moved, and walking speed in the test arena [factors = treatment group (ATR-treated, control)], odorant type (female odor, water) and activity type [time spent moving, total distance travelled, walking speed]. Dependent variables analyzed were time spent moving (s), total distance travelled (cm) and walking speed (cm/s).

Results and Discussion

ATR is used in the agricultural industry to manage the growth of broad leaf weeds and to increase yields of crops

like corn, sorghum, and sugar cane; however, ATR concentrations in waterways surrounding agricultural fields can reach concentrations greater than 100 ppb for as long as 21 days (EPA 2014). We exposed reproductive male crayfish to 80 ppb ATR for 96 h and found that this acute treatment causes significant changes in their ability to localize a reproductive female odor source (Fig. 1, $F_{(1,108,0.05)} = 565.4$, Fisher LSD, $p < 0.0001$). In the control experiment in which water was delivered into the proximal end, both control and ATR-treated crayfish spent more time in the distal portion of the test arena. When female odor was introduced into the proximal end, ATR-treated males also spent more time in the distal end of the test arena. When control male crayfish were exposed to female odor, they spent significantly more time in the proximal region of the tank (Fig. 1). Not only did control male crayfish spend more time in the proximal region of the tank, they spent more time at the odor source when female odor was delivered (Fig. 2). Exposure to ATR and other herbicides has been shown to affect olfaction and olfactory-mediated behavior in other aquatic species. Saglio and Trijasse (1998) found that after a 24 h exposure to 0.5 µg/L, responses to conspecific social odors were affected. Goldfish decreased grouping behavior and sheltering and increased surfacing when conspecific skin extract or alarm cues were present. ATR thus altered the chemical perception of natural conspecific social odors in goldfish, which is similar to what we found in crayfish. Moreover, perception of social odors by crayfish has also been altered after herbicide exposure. Metolachlor exposure (80 ppb) diminished

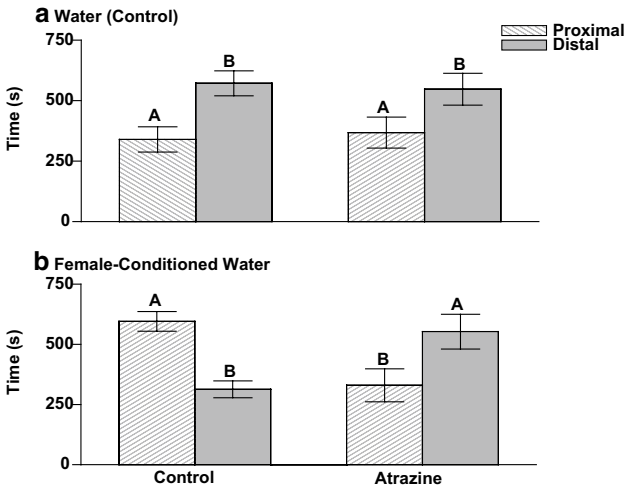

Fig. 1 Control and ATR-treated crayfish spent significantly more time (s ± S.E.) in the distal portion of the test arena when water was delivered from the odor source (**a**). When female odor was delivered in the proximal end of the test arena, control crayfish spent significantly more time in the proximal region of the tank while ATR-treated crayfish spent more time in the distal portion of the arena (**b**). Significant differences ($p < 0.05$) between experimental groups within the same graph are indicated by different capital letters

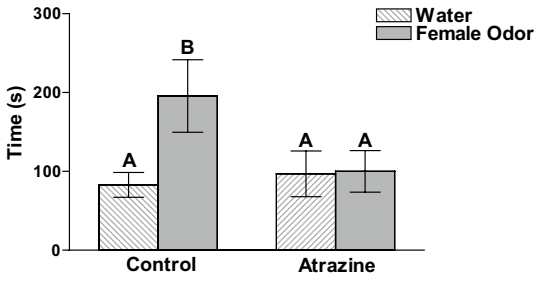

Fig. 2 ATR-treated crayfish spend significantly less time (s ± S.E.) within 10 cm of a female odor source when compared to control crayfish. Significant differences ($p < 0.05$) between experimental groups are indicated by different capital letters

responses to conspecific alarm cues and social odors in crayfish (Cook and Moore 2008; Wolf and Moore 2002). Browne and Moore (2014) also found that exposure to 2,4-dichlorophenoxyacetic acid (2,4-D) reduced the ability of crayfish to locate a food odor source. Similar reductions in food odor detection were observed in crayfish following an environmentally-relevant ATR-exposure (Belanger et al. 2015). Taken together, ATR exposure at environmentally-relevant concentrations affects olfactory-mediated responses by crayfish to both food and reproductive odors. Because herbicides are often found in mixtures, additive effects may be observed when herbicides like ATR, metolachlor, and 2,4-D are combined and applied to agricultural fields as a mixture.

This study showed that after an 80 ppb ATR exposure that there were no differences between the groups with respect to time spent moving and not moving, as well as total distance moved and walking speed of the crayfish ($F_{(1,144,0.05)} = 0.52$, $p = 0.5$). This is similar to what Belanger et al. (2015) found in crayfish after a similar ATR exposure; there was no change in locomotion after ATR exposure. Previous studies have shown that ATR and other herbicides affect locomotor behaviors in fish and crayfish. Browne and Moore (2014) found that after exposure to 2,4-D, crayfish altered their searching behaviors and displayed increased walking speeds. Additionally, changes in bursting or swimming behavior was noted in goldfish when they were being exposed to 0.5 µg/L ATR (Saglio and Trijasse 1998). Bursting behavior could be an olfactory-mediated ATR avoidance behavior where goldfish may be trying to avoid being exposed to the chemical after detection. Velisek et al. (2013) found that after exposure with high concentrations of ATR (>10 mg/L), crayfish moved with difficulty and often stayed in corners. They did however note when they were exposed to lower concentrations (1 mg/L), crayfish displayed normal locomotor behaviors during the treatments. Since we found that there were no changes in locomotor behaviors in crayfish post-ATR

exposure, we believe that an acute exposure to ATR acts on chemoreceptors, leading to olfactory impairments in crayfish. Prolonged exposures, commonly found in nature, may lead to larger chemosensory deficits or acclimation.

Crayfish are nocturnally active and often live in turbid waters; therefore, they rely heavily on chemoreception for perception of conspecific social odors, detection of alarm cues and predators, as well as for locating food sources. We have shown that ATR exposure affects the ability of male crayfish to detect and respond to female odors. ATR-treated crayfish spend more time in the distal area of the test arena when female odors are delivered in the proximal region. This is similar to the behavior control and ATR-treated crayfish display when water is being delivered from odor source (Fig. 1). Additionally, we know that acute ATR exposures can have a lasting effects on olfaction, taking over 72 h before crayfish regain their abilities to detect odors post-exposure (Belanger et al. 2016). An inability to detect essential social and food odors can have a huge impact on crayfish populations where herbicides are applied heavily. Moreover, exposure to mixtures of herbicides including metolachlor and 2,4-D may compound chemosensory deficits in crayfish and other aquatic organisms. Thus, herbicide exposure may alter populations of crayfish and other organisms. Of interest is the mode of action of ATR on chemoreceptors. Tierney et al. (2007) found that during a 30 min ATR-exposure, rainbow trout were unable to detect amino acids. However, once the ATR was removed, rainbow trout regained their ability to detect *L-histidine* within 20 min. This suggests that during subacute exposures, ATR may cause changes to olfactory receptors or act as a competitive inhibitor or antagonist of the odorant receptors. Belanger et al. (2016) showed that after a 96 h ATR exposure, crayfish did not regain their ability to detect and respond to food odors when examined 72 h post-exposure. It is therefore clear that exposure to ATR for environmentally-relevant lengths of time can impair chemoreception long-term. Overall, impairments to chemoreception may affect crayfish population size, especially on more sensitive/displaced crayfish species. Changes in crayfish population sizes may have cascading negative effects on the aquatic ecosystem. ATR exposure has the possibility of affecting the chemosensory abilities and thus population sizes of many different species of crayfish, especially those that are already imperiled by invasive species. Further research studies examining ATR accumulation and long-term cellular effects should be performed to help gain a better understanding of the cellular and subcellular mode of action of ATR.

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