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Environmentally relevant atrazine exposures cause DNA damage in cells of the lateral antennules of crayfish (*Faxonius virilis*)

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Abstract

The herbicide atrazine is heavily applied in agricultural areas in the Midwestern United States and can run-off and seep into surrounding aquatic habitats where concentrations can reach over 300 ppb. It is known that acute exposures to 80 ppb atrazine cause lasting deficiencies in the chemoreception of food and mate odors. Since atrazine impairs chemosensory responses, the goal of this study was to determine the effect of atrazine on cells, including olfactory sensory neurons, located in the lateral antennules of crayfish. In this experiment, we treated crayfish for 10 days with ecologically relevant concentrations of 0, 10, 40, 80, 100 and 300 ppb ($\mu\text{g L}^{-1}$) of atrazine. Following treatments, the distal portion of the lateral antennules was cryosectioned. We used a TdT mediated dUTP nick-end labeling (TUNEL) assay to determine if any cells had DNA damage and may be thus undergoing apoptosis. We found that as atrazine concentrations increase above 10 ppb, the number of TUNEL-positive cells, visualized in the lateral antennules, significantly increases. Our data show that atrazine exposure causes DNA damage in cells of the lateral antennules, including olfactory sensory neurons, thus leading to impairments in chemosensory abilities. Because crayfish rely heavily on chemoreception for survival, changes in their ability to perceive odors following atrazine exposure may have detrimental effects on population size.

Keywords

Atrazine; Lateral Antennules; Crayfish; TUNEL; DNA Damage; Chemoreception

1.0 INTRODUCTION:

Atrazine (ATR; 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is one of the most commonly used herbicides in the United States (U.S.). ATR has been used since the 1950s and applied to crops such as corn, sorghum, and sugarcane to control broadleaf plants and grassy weeds. Although ATR use has been banned in the European Union, approximately 33–36 million kilograms are sold annually in the U.S. (Kiely et al., 2004; EPA, 2013). ATR

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can enter aquatic habitats around agricultural areas through run-off, ground water, and through evapotranspiration and be deposited by rainwater (LeBlanc et al., 1997; Tong and Chen, 2002). ATR is the most commonly detected herbicide in the U.S. Midwestern region at concentrations over 300 ppb ($\mu\text{g L}^{-1}$), and these high concentrations may last for several weeks (EPA, 2007, 2014; Belanger et al., 2016). Along with the high concentrations detected, ATR can be present for long periods of time, given that the half-life of ATR ranges from six days to several months or years (Comber, 1999). The chemical properties of ATR allow for high susceptibility to leaching and run-off, especially during heavy rainfalls. ATR has a moderate potential for soil sorption, high soil mobility, and has been detected in soils for up to 22 years following its application (Walther, 2003; Jablonowski et al., 2011). This is a cause for concern as ATR can leach into aquatic ecosystems and affect aquatic organisms.

When ATR enters local streams and rivers, it may cause sublethal effects on non-target aquatic organisms. Several studies have shown that acute and subacute exposure to ATR leads to changes in olfactory-mediated behaviors and chemoreception in aquatic organisms. When tadpoles (*Osteopilus septentrionalis*) were exposed to $178 \mu\text{g L}^{-1}$ ATR for 7 d, they displayed hyperactive behaviors and did not avoid predator odors (Ehrsam et al., 2016). Steele et al. (2018) found alterations in agonistic interactions in crayfish (*Faxonius virilis* (formally *Orconectes virilis*, Crandall and De Grave (2017))) following a 23 h exposure to $40 \mu\text{g L}^{-1}$ ATR. Exposure to ATR is also known to affect foraging and feeding behaviors, as well as chemosensory responses. Nieves-Puigdoller et al. (2007) showed that a 21 d exposure to $100 \mu\text{g L}^{-1}$ ATR decreased feeding behavior in Atlantic salmon (*Salmo salar*). A short-term (24 h) ATR exposure ($5 \mu\text{g L}^{-1}$) led to decreased grouping behavior in the presence of an alarm cue in goldfish (*Carassius auratus*) and ATR concentrations above $0.04 \mu\text{g L}^{-1}$ (5 d exposure) impaired the priming effect of ovulated female urine on male Atlantic salmon (Moore and Waring, 1998; Saglio and Trijasse, 1998). Further, following acute 96 h ATR ($80 \mu\text{g L}^{-1}$) exposures in crayfish (*Faxonius rusticus* and *F. virilis* (formally *Orconectes rusticus*)), chemosensory deficits were found and crayfish could not localize a food or mate odor source (Belanger et al., 2015; Belanger et al., 2017). These ATR exposures were shown to have lasting effects on chemosensory-mediated behaviors (Belanger et al., 2016). Moreover, when ATR is applied to the olfactory epithelium of Atlantic salmon parr and rainbow trout, a noticeable decrease in electro-olfactogram (EOG) response was noted (Moore and Waring, 1998; Moore and Lower, 2001; Tierney et al., 2007; Tierney et al., 2008). ATR exposure ($2.0\text{--}20 \mu\text{g L}^{-1}$) led to decreases in EOG responses by male Atlantic salmon parr to prostaglandin $\text{F}_{2\alpha}$, a priming pheromone, released by females (Moore and Waring, 1998; Moore and Lower, 2001). Further, decreased EOG responses were recorded to a known food odor (L-histidine) in rainbow trout after exposure of their olfactory epithelium to concentrations of ATR above $10 \mu\text{g L}^{-1}$ (Tierney et al., 2007; Tierney et al., 2008). Overall, ATR exposure is known to impair olfactory physiology and olfactory-mediated behaviors in aquatic animals.

Crayfish depend heavily on chemoreception in order to gain information from their environment, such as determining social status, accessing food, mates, and shelters, as well as avoiding predation (Bergman and Moore, 2005). Chemoreceptors are found lining the surface of the body of the crayfish, specifically on their chelae, antennae, antennules, and other mobile appendages. Crayfish possess chemoreceptors located on the ventral side of the

distal portion of the lateral antennule called aesthetasc sensilla (or hairs) (Tierney et al., 1986). Aesthetasc sensilla play an important role in chemoreception, such as identifying food odors and chemical cues from conspecifics (Tierney et al., 1984; Tierney et al., 1986). These sensilla are characterized by their thin cuticle, allowing odorants to pass through, with clusters of 25–500 olfactory sensory neurons (OSNs) at the base of the sensilla with supporting cells surrounding it, and a dendrite that projects into the lumen of the aesthetasc sensilla. Axons run beneath the exoskeleton of the lateral antennules and project from the OSNs and extend to the olfactory lobe (Laverack, 1965; Tierney et al., 1986; Sandeman and Sandeman, 2003). Aesthetasc sensilla, OSN dendrites, cell bodies, and axons play a key role in the olfactory system of the crayfish and chemoreception of odorants. Impairment of OSNs would have profound effects on the ability of crayfish to perceive odors, ultimately affecting their survival.

Environmentally relevant ATR exposures have been shown to negatively affect olfactory-mediated behaviors in crayfish (Belanger et al., 2015; Belanger et al., 2016; Belanger et al., 2017; Steele et al., 2018). This suggests that chemoreceptors, including OSNs, could be damaged following exposure to ATR. Several studies have shown that ATR treatment causes DNA damage in the cells of animals that are exposed to ATR (Liu et al., 2006; de Campos-Ventura et al., 2008; Cavas, 2011; Zhu et al., 2011; Santos and Martinez, 2012). The purpose of this study was to determine if exposure to environmentally relevant concentrations of ATR cause DNA damage in cells of the lateral antennules, including OSNs. We exposed crayfish to ATR (0, 10, 40, 80, 100 and 300 ppb) for 10 d and used a TdT mediated dUTP nick-end labeling (TUNEL) assay to determine if ATR exposure caused DNA damage in cells of the distal portion of the lateral antennule associated with aesthetasc sensilla. The TUNEL assay has been performed on crustaceans prior to this study, making it an appropriate marker for DNA-fragmentation (Sahtout et al., 2001; Sandeman and Sandeman, 2003; Wongprasert et al., 2003). We hypothesized following ATR exposure, the number of TUNEL-positive cells in the lateral antennules would increase in a dose-dependent manner. Results of this study would link changes in olfactory-mediated behaviors post-ATR exposure with damage to cells of the lateral antennules, including OSNs. If DNA damage increases following realistic ATR exposures, chemosensory abilities may also be impaired. Because crayfish rely heavily on chemoreception to detect food, mates, and predator odor and for locating shelters and determining social status, any change in chemosensory abilities may impair survival and affect crayfish population size. Alterations in crayfish populations may have cascading effects on other organisms, as crayfish are a keystone species in the aquatic food web (Dorn and Wojdak, 2004).

2.0 MATERIALS AND METHODS:

2.1 Animals:

Male and female crayfish (weight: 11.1 ± 6.0 g, carapace length: 3.1 ± 0.9 cm, chelae length: 2.2 ± 0.8 cm; mean $\pm$ standard deviation, N=24) were collected using traps from Belle Isle Park and William G. Milliken State Park (Detroit, MI). They were housed at the University of Detroit Mercy in large tanks [mean water chemistry parameters: pH 7.5, dissolved oxygen = 8.29 mg L^{-1} , Dissolved oxygen percent saturation = 96.45%,

Temperature = 22.3°C, Hardness = $250 \pm 25 \mu\text{g L}^{-1}$, and TOC = $2.54 \mu\text{g L}^{-1}$; 14:10 h light dark cycle] for at least one week prior to treatments. During this time and throughout the treatments, crayfish were fed rabbit pellets three times per week.

2.2 Treatments:

During the 10-day treatment period, three crayfish were placed in 3000 mL translucent plastic containers (31 cm length $\times$ 18.5 cm width $\times$ 11 cm height; N = 3 crayfish per container) with secured lids and three polyvinyl tubes as shelters. Each container was filled with control or ATR-treatment solution and aerated using an air stone. Stock solutions (17 mg L^{-1}) were of ATR were prepared by dissolving 17 mg of ATR (Sigma-Aldrich, 99.1% purity) in 2.5 mL of ethanol using a vortexer and subsequently diluted with 997.5 mL distilled water (Awali et al., 2019). Crayfish were treated with environmentally relevant ATR concentrations (10, 40, 80, 100, and 300 ppb) and control solution of 0 ppb (final ethanol concentration of 0.004%). These concentrations and exposure time was selected, as they are known to be ecologically relevant (EPA, 2014; Belanger et al., 2016). Reversed-phase liquid chromatography-tandem mass spectrometry (LC/MS) analysis was performed to verify and standardize all ATR solutions (see Table S1 in Awali et al. 2019).

2.3 Tissue Collection:

Following ATR treatment, crayfish were placed in a -20°C freezer for 10–15 min before the lateral antennules were removed. The lateral antennules were fixed in 4% paraformaldehyde (PFA) and stored in labeled microcentrifuge tubes at 4°C for at least 48 h prior to preparing the antennules for cryosectioning. Following fixation, the lateral antennules of crayfish were rinsed with 0.1 M phosphate-buffered saline (PBS), decalcified in Immunocal Formic Acid (StatLab, McKinney, TX) for 24 hours before being cryoprotected using a sucrose gradient (10% and 20% for 1 hour each and 30% sucrose overnight).

2.4 Sectioning of Tissues:

Following cryoprotection, the distal portion of the lateral antennules from control and treated crayfish were embedded in cryomatrix (FSC 22 Clear, Leica Biosystems) in plastic cryomolds (Standard, $25 \times 20 \times 5 \text{ mm}$) and frozen on a cryotome (Leica CM1860). Frozen tissue was wrapped in parafilm and stored in a -20°C freezer until they were sectioned. Gelatin (1%) coated slides were prepared and $10 \mu\text{m}$ sagittal sections of the distal portion of the lateral antennules of crayfish were sectioned on a cryotome. Microscope slides were initially examined with a light microscope to determine if aesthetasc sensilla and olfactory sensory neurons were present. An aesthetasc sensillum is a long, thin hair located on the lateral antennules of crayfish. Aesthetascs occur ventrally on the distal segments of the lateral antennules. They are identified as sensilla that contain a single annulation, which divides them into a basal and distal region. Each aesthetasc sensillum is innervated by hundreds of olfactory sensory neurons in a neuron bundle (Tierney et al., 1986). An ideal section would show the structure of an aesthetasc sensillum along with neuron bundle attached to it.

2.5 TdT-mediated dUTP nick-end labeling (TUNEL) Assay:

Apoptosis in neuronal cells was detected by performing TdT-mediated dUTP nick-end labeling (TUNEL) assay (DeadEnd™ Fluorometric TUNEL System) based on the methods described by (Sahtout et al., 2001). Slides were dipped in 4% PFA for 1 min in order for the tissue sections to adhere to gelatin-coated slides. Cover wells (300 µL) were used and applied to the slides so tissue would not be lost during the staining procedure. The tissue was washed twice in PBS for 5 min each time by pipetting it into the wells. After the slides were washed, they were treated with 0.2% Triton X-100 in PBS for 5 min and washed two times in PBS for 5 min each time. DNase I (100 µL) was directly added to positive control sections for 5 min at room temperature. The coverwells were removed and all tissue sections were equilibrated by adding 100 µL equilibration buffer directly to the tissue sections (at room temperature for 7 min). After equilibration, 50 µL of rTdT reaction mix was added to the tissue and covered with plastic coverslips to ensure the distribution of the mix. rTdT reaction mix was added to all concentrations except for negative control, to which distilled water was substituted for the rTdT enzyme in the rTdT reaction mix. Slides were incubated for 60 min at 37 °C in a humidified chamber in the dark to avoid exposure to light. Plastic coverslips were removed from the slides and the coverwells were reapplied. Slides were then treated with 2X SSC for 15 min. Slides were washed with 0.1 M PBS (three times for 5 min each). Mounting medium (VECTASHIELD with 4',6-diamidino-2-phenylindole (DAPI), Vector Labs) was added to the slides, coverslips were applied and sealed with clear nail polish. Slides were visualized using fluorescence microscopy (Zeiss Axio Scope.A1). Nuclei of the cells fluoresce blue after being exposed to UV light due to DAPI which labels nuclei and TUNEL-positive cells fluoresce green after excitation with the fluorescein isothiocyanate (FITC) filter. TUNEL and DAPI micrographs were collected using ZEN Blue (Zeiss), merged and analyzed using Fiji (National Institutes of Health), an imaging processing system, which provides plugins for image analysis.

2.6 TUNEL-Positive Cell Counting:

For each concentration, three animals were used for counting. Two to three independent slide images were counted per animal. Images with visible aesthetasc sensilla and olfactory sensory neuron clusters were used for cell counting. The total number of cells and the number of TUNEL-positive cells were counted by individuals in the lab using Cell-Counter, a Fiji plugin. DAPI images were used to count the total number of cells. Prior to counting, images were inverted in order to better identify individual cells. DAPI stained cells were counted if they were in focus and stained deeply. Merged images (TUNEL and DAPI) were used to count TUNEL-positive cells. As described in (Sahtout et al., 2001), (Sandeman et al., 1998), and (Wongprasert et al., 2003), an intense, green fluorescent cell was scored as a TUNEL-positive cell. A percentage of TUNEL-positive cells was calculated for each image.

2.7 Statistical Analysis:

The percent TUNEL-positive cells data was arcsine transformed. A linear mixed models method followed by analysis of deviance tables using Type III Wald v2 tests with Satterthwaite's method (Zuur et al., 2009) were used to determine the effect of concentration on the presence of TUNEL-positive cells in the lme4 package in R statistical software (Bates

et al., 2015; R Development Core Team, 2016). Differences of least squares means ('diffsmeans') from the lmerTest package in R was used as a post hoc test to discern which concentrations were significantly different from each other (Kuznetsova et al., 2017). Fixed factors include concentration and length of exposure and random factors include TUNEL-positive cell counts from individual crayfish.

3.0 RESULTS:

TUNEL-positive cells were identified in micrographs from lateral antennules of crayfish. Both negative and positive procedural controls were used in this experiment to ensure that the validity of the results obtained (Figure 1 A, B). TUNEL-positive cells were identified as a discrete green fluorescent cell. Sections from crayfish treated with 0 ppb ATR contained very few TUNEL-positive cells (Figure 2A). When crayfish were treated with ATR, the appearance of TUNEL-positive cells increased. TUNEL-positive cells were found throughout the lateral antennules and associated with aesthetasc sensillum of crayfish following ATR treatment at concentrations ranging from 10 to 300 ppb (Figure 2 B–F). The occurrence frequency of TUNEL-positive cells increased as ATR concentrations increased.

There was a significant difference in the percentage of TUNEL-positive cells following 10-day exposures to ATR ($F_{5,43,0.05} = 80.92$, $P < 0.0001$; linear mixed-model with Satterthwaite's method; Figure 3). Lateral antennules of crayfish treated with 0 ppb ATR had $0.2 \pm 0.2\%$ TUNEL-positive cells. When crayfish were exposed to ATR concentrations at 10 ppb and above, the percentage of TUNEL-positive cells was significantly different from those treated with 0 ppb ATR. Crayfish, treated with 10 ppb ATR, had $8.4 \pm 1.5\%$ TUNEL-positive cells in the lateral antennules ($P < 0.001$ compared to the 0 ppb ATR treatment). Following a 10-day 40 ppb ATR exposure, there were $11.5 \pm 1.5\%$ TUNEL-positive cells present in the lateral antennules ($P < 0.001$ compared to the 0 ppb ATR treatment). When crayfish were exposed to 80 and 100 ppb ATR, they had $22.8 \pm 3.4\%$ and $22.8 \pm 2.4\%$ TUNEL-positive cells in the lateral antennules respectively ($P < 0.001$ compared to 0 ppb treatment). Following a 10-day 300 ppb ATR exposure, the lateral antennules contained $38.7 \pm 1.5\%$ TUNEL-positive cells. ($P < 0.001$ compared to 0 ppb treatment).

4.0 DISCUSSION:

ATR is persistent in the aquatic environment as it has a half-life that varies from multiple days to many years, and aquatic organisms, like, crayfish can therefore be exposed to ATR for long periods (Comber, 1999; EPA, 2014). Previous research by Belanger et al. (2015, 2017), demonstrated that crayfish, treated with 80 ppb ATR, could no longer detect and respond to food and mate odors. Further, ATR exposure was found to cause lasting chemosensory deficits (Belanger et al. 2016). Taken together, these studies suggest that environmentally relevant ATR exposures impair chemosensory cells and OSNs which are located in the lateral antennules. We found that treatment with 10, 40, 80, 100 and 300 ppb ATR for 10 days caused an increase in the number of cells with DNA fragmentation/damage in the lateral antennules of crayfish in a dose-dependent manner (Figure 2). As the lateral antennules of crayfish contain aesthetasc sensilla and 25–500 OSNs in addition to other chemosensory and mechanosensory sensilla (Tierney et al. 1984, 1986), damage to the DNA

of these cells, may lead to loss of function and subsequent apoptosis. ATR exposure has been shown to cause morphological changes and formation of apoptotic bodies in epithelial cells (cell line ZC7901) of the grass carp (*Ctenopharyngodon idellus*) in a dose and time-dependent manner (Liu et al. 2016). This study, therefore, suggests that changes in olfactory-mediated behaviors following ATR exposures are caused by damage to chemosensory cells.

ATR exposures ranging between 5 and 178 ppb ($\mu\text{g L}^{-1}$) and between 23 h to 21 d are known to affect chemosensory abilities and alter olfactory-mediated behaviors in several aquatic organisms (Moore and Waring, 1998; Saglio and Trijasse, 1998; Nieves-Puigdollér et al., 2007; Tierney et al., 2007; Belanger et al., 2015; Belanger et al., 2016; Ehrsam et al., 2016; Belanger et al., 2017; Steele et al., 2018). We found that ATR treatment caused DNA damage in cells of the lateral antennules, which include OSNs, in crayfish. Significant increases in TUNEL-positive cells were noted following exposure to concentrations of 10 ppb ATR and above (Figure 3). Other assays performed in blood, liver (or hepatopancreas), and gills have shown damage to cells, including the presence of micronuclei, occurred following exposures to concentrations as low as $5 \mu\text{g L}^{-1}$ pure ATR or in an ATR-based herbicide such as Gesaprim® (de Campos Ventura et al., 2008; Cavas, 2011; Zhu et al., 2011; Santos and Martinez, 2012). When ATR is combined with other pesticides, synergistic effects can occur and subsequently lead to mixture toxicities which may intensify the damage experienced post-exposure (Schmidt et al., 2017). (Liu et al., 2006) showed that following exposure to ATR, cultured cells of the grass carp display cytotoxic effects including condensation of the nucleus, margination of chromatin to form dense granular caps, and formation of apoptotic bodies. They also found that ATR exposure causes mitochondrial membrane potential disruption, elevation in intracellular calcium, generation of reactive oxygen species, and intracellular ATP depletion. Other herbicides (e.g. dichlobenil) have been shown to cause olfactory neurotoxicity by altering G-protein expression (Andreini et al., 1997). Further, (Genter et al., 1998) suggest that bioactivation of herbicides by cytochrome P450 and glutathione-S-transferase may lead to differential and targeted damage in tissues of the body, including OSNs.

We have previously shown that a 96 h exposure to 80 ppb ATR causes lasting chemosensory deficits (Belanger et al. 2016). Even when crayfish were placed in ATR-free water for 72 h, they did not recover their ability to detect food odors, and subsequently consumed less food when compared to control. When Tierney et al. (2007) exposed the olfactory epithelium of rainbow trout to 10 and $100 \mu\text{g L}^{-1}$ for 30 min, there was a significant decrease in EOG response to L-histidine. EOG responses to histidine returned to control levels once the ATR was washed off the olfactory epithelium. This suggests that aquatic animals receiving subacute ATR exposures (e.g. 30 min) can quickly recover, whereas longer exposures (> 96 h) cause lasting effects as they lead to DNA damage, the formation of micronuclei, and apoptosis. We found that 8.4% of the cells in the lateral antennules had DNA damage following a 10 day, 10 ppb ATR exposure. The amount of DNA damage increased in a dose-dependent manner. Like this study, other studies have found that ATR exposure causes DNA damage in cells of aquatic organisms in a dose and time-dependent manner (e.g. Santos and Martinez 2012, Cavas 2011, de Campos Ventura et al. 2008, Zhu et al., 2011, Liu et al. 2016), although this is the first study to link changes in olfactory-mediated behaviors following ATR exposure to DNA damage in chemosensory cells.

Crayfish are heavily reliant on chemoreception due to crayfish being both nocturnal and bottom-dwellers. Because of this, the studies showing that their ability to perform olfactory – mediated behaviors being compromised following ATR exposure demonstrates that ATR can negatively impact their survival. Following dynamic and static exposures to ATR and ATR-based “weed killers,” crayfish could not localize food or mate odors and did not respond appropriately to conspecific odors. We have shown that acute ATR exposure results in the DNA-fragmentation in cells of the lateral antennules, which include OSNs. ATR has been found to induce DNA damage in zebrafish (*Danio rerio*) liver cells exposed to 10 µg L⁻¹ ATR on all days tested (5, 10, 15, 20, and 25 d). Similar exposures (5, 12.5, and 25 µg L⁻¹, for 72 h) in the cichlid (*Oreochromis niloticus*) also resulted in genotoxic endpoints that include increases in the frequencies of both micronuclei and DNA damage (deCampos-Ventura et al., 2008). One of the key biochemical changes that occur while a cell undergoing apoptosis is DNA fragmentation (Green and Reed, 1998). Because of this, apoptotic activity could be the underlying reason why chemosensory cells are experiencing DNA fragmentation, but this would need to be further investigated. We are unaware of any studies that examine apoptotic activity in OSNs after exposure to ATR; however, apoptotic activity was found in cells of ATR-treated grass carp cells. Future studies will examine apoptotic markers (e.g. cleave caspase-3 activity) to determine if apoptosis is occurring (Wu et al., 2012). Further, because chemosensory cells of the lateral antennules are capable of regeneration in crustaceans (Harrison et al., 2003), we seek to determine if crayfish can recover their chemosensory abilities and regenerate chemosensory cells following an acute ATR exposure.

This study has shown that ATR causes DNA-damage in cells of the lateral antennules, which includes OSNs. Crayfish are dependent on their chemosensory abilities for survival and reproduction, and if chemosensory abilities are compromised, it can negatively impact crayfish population size. Crayfish are considered a keystone species, playing a major role in linking and transferring the energy within the aquatic environment and between the aquatic and terrestrial ecosystems (Dorn and Wojdak, 2004; Sullivan and Rodewald, 2012). As a keystone species, crayfish play a pivotal role in aquatic food webs. The decline in the crayfish populations could, therefore, have cascading ecosystem effects.

5.0 CONCLUSIONS:

This study showed that environmentally relevant ATR exposures of 0, 10, 40, 80, 100 and 300 ppb lead to a dose-dependent increase in DNA-damage in lateral antennules of crayfish. Our results show that there is a significant increase in the percentage of TUNEL-positive cells when crayfish were exposed to ATR concentrations of 10 ppb and above. These results support previous research that shows that after exposure to environmentally relevant concentrations of ATR leads to chemosensory deficits.

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7.0 REFERENCES:

- Andreini I, DellaCorte C, Johnson L, Hughes S, Kalinoski D, 1997 G-protein (s), $G_{\alpha}qG_{\alpha}11$, in the olfactory neuroepithelium of the channel catfish (*Ictalurus punctatus*) is altered by the herbicide, dichlobenil. *Toxicology* 117, 111–122. [PubMed: 9057890]
- Awali S, Abdulelah S, Crile K, Yacoo K, Almouseli A, Torres V, Dayfield D, Evans K, Belanger R, 2019 Cytochrome P450 and Glutathione-S-transferase activity are altered following environmentally relevant atrazine exposures in crayfish (*Faxonius virilis*). *B. Environ. Contam. Tox* 10.1007/s00128-019-02674-2
- Bates D, Mächler M, Bolker B, Walker S, 2015 Fitting linear mixed-effects models using lme4. *J. Stat. Softw* 67, 1–48.
- Belanger R, Evans K, Abraham N, Barawi K, 2017 Diminished conspecific odor recognition in the rusty crayfish (*Orconectes rusticus*) following a 96-h exposure to atrazine. *B. Environ. Contam. Tox* 99, 555–560.
- Belanger RM, Mooney LN, Nguyen HM, Abraham NK, Peters TJ, Kana MA, May LA, 2016 Acute atrazine exposure has lasting effects on chemosensory responses to food odors in crayfish (*Orconectes virilis*). *Arch. Environ. Con. Tox* 70, 289–300.
- Belanger RM, Peters TJ, Sabhapathy GS, Khan S, Katta J, Abraham NK, 2015 Atrazine exposure affects the ability of crayfish (*Orconectes rusticus*) to localize a food odor source. *Arch. Environ. Con. Tox* 68, 636–645.
- Bergman DA, Moore PA, 2005 The role of chemical signals in the social behavior of crayfish. *Chem. Senses* 30, I305–I306. [PubMed: 15738171]
- Cavas T, 2011 In vivo genotoxicity evaluation of atrazine and atrazine-based herbicide on fish *Carassius auratus* using the micronucleus test and the comet assay. *Food Chem. Toxicol* 49, 1431–1435. [PubMed: 21443921]
- Comber SDW, 1999 Abiotic persistence of atrazine and simazine in water. *Pestic. Sci* 55, 696–702.
- Crandall K, De Grave S, 2017 An updated classification of the freshwater crayfishes (Decapoda: Astacidea) of the world, with a complete species list. *J. Crustacean Biol* 37, 615–653.
- de Campos-Ventura B, de Franceschi de Angelis D, Marin-Morales M, 2008 Mutagenic and genotoxic effects of the Atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. *Pestic. Biochem. Phys* 90, 42–51.
- Dorn NJ, Wojdak JM, 2004 The role of omnivorous crayfish in littoral communities. *Oecologia* 140, 150–159. [PubMed: 15064944]
- Ehram M, Knutie S, Rohr J, 2016 The herbicide atrazine induces hyperactivity and compromises tadpole detection of predator chemical cues. *Environ. Toxicol. Chem* 35, 2239–2244.
- EPA, 2007 Atrazine: Chemical Summary. United States Environmental Protection Agency Available at: http://www.epa.gov/teach/chem_summ/Atrazine_summary.pdf
- EPA, 2013 Atrazine updates. US Environmental Protection Agency Available at: www.epa.gov/pesticides/reregistration/atrazine/atrazine_update.htm
- EPA, 2014 Atrazine updates: 2014 atrazine ecological exposure monitoring program data. United States Environmental Protection Agency Available at: <http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2003-0367-0303>
- Genter M, Deamer-Melia N, Wetmore B, Morgan K, Meyer S, 1998 Herbicides and olfactory/neurotoxicity responses. *Rev. Toxicol* 2, 93–112.
- Green D, Reed J, 1998 Mitochondria and apoptosis. *Science* 281, 1309–1312. [PubMed: 9721092]
- Harrison PJH, Cate HS, Steullet P, Derby CD, 2003 Amputation-induced activity of progenitor cells leads to rapid regeneration of olfactory tissue in lobsters. *J. Neurobiol* 55, 97–114. [PubMed: 12605462]
- Jablonowski ND, Schaffer A, Burauel P, 2011 Still present after all these years: persistence plus potential toxicity raise questions about the use of atrazine. *Environ. Sci. Pollut. R* 18, 328–331.

- Kiely T, Donaldson D, Grube A, 2004 Pesticide industry sales and usage: 2000 and 2001 market estimates United States Environmental Protection Agency, Washington, DC.
- Kuznetsova A, Brockhoff P, Christensen R, 2017 lmerTest package: tests in linear mixed effects models. *J. Stat. Softw* 82, 1–26.
- Laverack MS, 1965 The innervation of the aesthetasc hairs of *Panulirus argus*. *Q. J. Microsc. Sci* 106, 45–60.
- LeBlanc R, Brown R, FitzGibbon J, 1997 Modeling the effects of land use change on the water temperature in unregulated urban streams. *J. Environmen. Manage* 49, 445–469.
- Liu XM, Shao JZ, Xiang LX, Chen XY, 2006 Cytotoxic effects and apoptosis induction of atrazine in a grass carp (*Ctenopharyngodon idellus*) cell line. *Environ. Toxicol* 21, 80–89. [PubMed: 16463256]
- Moore A, Lower N, 2001 The impact of two pesticides on olfactory-mediated endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. *Comp. Biochem. Phys. B* 129, 269–276.
- Moore A, Waring CP, 1998 Mechanistic effects of a triazine pesticide on reproductive endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. *Pestic. Biochem. Phys* 62, 41–50.
- Nieves-Puigdollers K, Björnsson BT, McCormick SD, 2007 Effects of hexazinone and atrazine on the physiology and endocrinology of smolt development in Atlantic salmon. *Aquat. Toxicol* 84, 27–37. [PubMed: 17610964]
- R Development Core Team, 2019 R: A language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria <https://www.R-project.org/>.
- Saglio P, Trijasse S, 1998 Behavioral responses to atrazine and diuron in goldfish. *Arch. Environ. Con. Tox* 35, 484–491.
- Sahtout A, Hassan M, Shariff M, 2001 DNA fragmentation, an indicator of apoptosis, in cultured black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus (WSSV). *Dis. Aquat. Organ* 44, 155–159. [PubMed: 11324818]
- Sandeman RE, Clarke D, Sandeman DC, Manly M, 1998 Growth-related and antennular amputation-induced changes in the olfactory centers of crayfish brain. *J. Neurosci* 18, 6195–6206. [PubMed: 9698313]
- Sandeman RE, Sandeman DC, 2003 Development, growth, and plasticity in the crayfish olfactory system. *Microsc. Res. Techniq* 60, 266–277.
- Santos T, Martinez C, 2012 Atrazine promotes biochemical changes and DNA damage in a Neotropical fish species. *Chemosphere* 89, 1118–1125. [PubMed: 22739540]
- Schmidt A, Sengupta N, Saski C, Noorai R, Baldwin W, 2017 RNA sequencing indicates that atrazine induces multiple detoxification genes in *Daphnia magna* and this is a potential source of its mixture interactions with other chemicals. *Chemosphere* 189, 699–708. [PubMed: 28968576]
- Steele A, Belanger R, Moore P, 2018 Exposure through runoff and ground water contamination differentially impact behavior and physiology of Crustaceans in fluvial systems. *Arch. Environ. Con. Tox* 75, 436–448.
- Sullivan S, Rodewald A, 2012 In a state of flux: the energetic pathways that move contaminants from aquatic to terrestrial environments. *Environ. Toxicol. Chem* 31, 1175–1183. [PubMed: 22605620]
- Tierney AJ, Thompson CS, Dunham DW, 1984 Site of pheromone reception in the crayfish *Orconectes propinquus* (Decapoda, Cambaridae). *J. Crustacean Biol* 4, 554–559.
- Tierney AJ, Thompson CS, Dunham DW, 1986 Fine-structure of aesthetasc chemoreceptors in the crayfish *Orconectes propinquus*. *Can. J. Zool* 64, 392–399.
- Tierney KB, Sampson JL, Ross PS, Sekela MA, Kennedy CJ, 2008 Salmon olfaction is impaired by an environmentally realistic pesticide mixture. *Environ. Sci. Technol* 42, 4996–5001. [PubMed: 18678039]
- Tierney KB, Singh CR, Ross PS, Kennedy CJ, 2007 Relating olfactory neurotoxicity to altered olfactory-mediated behaviors in rainbow trout exposed to three currently-used pesticides. *Aquat. Toxicol* 81, 55–64. [PubMed: 17145086]
- Tong S, Chen W, 2002 Modeling the relationship between land use and surface water quality. *J. Environ. Manage* 66, 377–393. [PubMed: 12503494]
- Walther JS, 2003 Surface water pesticide contamination in the upper Terrebonne Basin of Louisiana Master's thesis. Louisiana State University, Baton Rouge, LA, p. 55.

- Wongprasert K, Khanobdee K, Glunukarn S, Meeratana P, Withyachumnarnkul B, 2003 Time-course and levels of apoptosis in various tissues of black tiger shrimp *Penaeus monodon* infected with white-spot syndrome virus. Dis. Aquat. Organ 55, 3–10. [PubMed: 12887248]
- Wu X-G, Xiong H-T, Wang Y-Z, Du H-H, 2012 Evidence for cell apoptosis suppressing white spot syndrome virus replication in *Procambarus clarkii* at high temperature. Dis. Aquat. Organ 102, 13–21. [PubMed: 23209074]
- Zhu LS, Dong XL, Xie H, Wang J, Wang JH, Su J, Yu CW, 2011 DNA damage and effects on glutathione-S-transferase activity induced by atrazine exposure in zebrafish (*Danio rerio*). Environ. Toxicol 26, 480–488. [PubMed: 20549606]
- Zuur A, Ieno E, Walker N, Saveliev A, Smith G, 2009 Mixed effects models and extensions in ecology with R Springer Science & Business Media, New York.

HIGHLIGHTS:

- Crayfish were exposed to 0, 10, 40, 80, 100 and 300 ppb atrazine for 10 days.
- TdT mediated dUTP nick-end labelling (TUNEL) was used to evaluate DNA damage.
- DNA damage increased in cells of the lateral antennules in a dose-dependent manner.
- Atrazine exposure causes impairments in chemosensory cells and chemoreception.

Figure 1:

Micrographs of the lateral antennules with aesthetasc sensilla (a) were obtained following negative (A) and positive (B) procedural controls. These micrographs show that TUNEL-positive cells were absent in the negative control sections, only the DAPI counterstain (blue) was visualized (A). When DNase I was added to the sections, an abundance of TUNEL-positive cells were noted (see fluorescent green cells and arrows in B). Scale bar is 100 μm .

Figure 2:

Sagittal sections of the lateral antennule of crayfish exposed to 0, 10, 40, 80, 100, and 300 ppb (A-F) ATR for 10 days. A TUNEL assay was performed along with DAPI staining. TUNEL positive cells (arrows) were noted in the antennules of crayfish exposed to all concentrations of ATR tested. TUNEL-positive cells (green) can be seen throughout the lateral antennules and associated with aesthetasc sensilla (a). Scale bar is 100 μ m.

Figure 3:

Percentage of TUNEL-positive cells in the lateral antennules of crayfish following a 10-day exposure to 0, 10, 40, 80, 100 and 300 ppb ATR. The percentage of TUNEL-positive significantly increases when crayfish treated with 0 ppb ATR were compared to those exposed to concentrations of 10 ppb ATR and higher. Control and ATR-treated groups are assigned different letters a, b, c, d when they are significantly different ($P < 0.001$).