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Effects of atrazine exposure on male reproductive performance in *Drosophila melanogaster*

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Abstract

Atrazine is a commonly utilized herbicide to control broadleaf weeds in the agricultural setting. It can, however, have negative effects on male reproductive performance in a variety of vertebrate species. Much less is known, however, about the effects of atrazine on invertebrates. In this study, we investigated the effects of several different concentrations of larval atrazine exposure on measures of reproductive performance in adult male *Drosophila melanogaster*. Atrazine exposure had significant effects on a male's mating ability and the number of eggs his partner lays when he was successful at mating. Exposed males also sired a smaller proportion of the offspring under competitive conditions when they were the first male to mate to a doubly mated female. Atrazine exposure had no measurable effect on a male's ability to prevent a mated female from mating to another male or on the proportion of offspring sired when the exposed males were the second male to mate. Exposure upregulated expression of one male reproductive gene, *ovulin*, but had no effect on expression of another, *sex peptide*. Exposed males produced and transferred more sex peptide protein to the female during mating but *ovulin* protein levels were not affected. In general, we observed non-monotonic responses such that the intermediate exposure levels showed the largest reduction in male reproductive performance. This study suggests that atrazine exposure affects male reproductive performance in insects and future studies should aim to understand the molecular mechanisms underlying the fitness effects of exposure.

Keywords

sperm competition; toxicology; non-monotonic; seminal fluid protein; sexual selection; exposure

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1. Introduction

The herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), although banned in the European Union (Sass and Colangelo, 2006), is one of the most commonly applied herbicides in the United States (Scribner et al., 2005). It is persistent in soils (Ma and Selim, 1996) and can enter streams through rainwater run-off (van Dijk and Guicherit, 1999). Atrazine levels of greater than 200 ppB have been detected in streams (Blanchard and Lerch, 2000; Lerch and Blanchard, 2003; Scribner et al., 2005) and it has been estimated that levels up to 500 ppB can be considered ecologically relevant (Rohr and McCoy, 2010).

Given the potential for atrazine to enter aquatic systems, many of the toxicology studies have focused on aquatic organisms. Evidence suggests that atrazine causes numerous reproductive effects in male African clawed frogs (*Xenopus laevis*), (Hayes et al., 2002; Hayes et al., 2010), as well as smaller body size in Northern leopard frogs (*Lithobates pipiens*), (Paetow et al., 2012). Problematically, these effects occur at low dosage levels, as low as 1 ppB (Hayes et al., 2002) which is below the US EPA allowable levels of 3 ppB in drinking water (EPA, 2012). Atrazine also appears to affect reproductive traits in both zebrafish, *Danio rerio*, (El-Amrani et al., 2012) and fathead minnows, *Pimephalus promelas* (Bringolf et al., 2004; Tillitt et al., 2010). Furthermore, rainbow trout (*Oncorhynchus mykiss*) show a decrease in free plasma testosterone when exposed to even a low (2 ug/kg) dose of atrazine (Salaberria et al., 2009). Atrazine also affects aquatic invertebrates. Exposure has been shown to affect gene expression in *Chironomus tentans* (Londono et al., 2004; Miota et al., 2000) as well acetylcholinesterase activity (Campero et al., 2007a), predation (St Clair and Fuller, 2014) and physiology in odonates (Campero et al., 2007b). In addition, atrazine has been shown to influence diversity in aquatic insect communities (Dewey, 1986).

Although more research has focused on impacts to aquatic systems, atrazine is also a potential environmental contaminant for terrestrial systems if it escapes the agricultural setting (Basta et al., 1997). Laboratory studies in rats show decreases in testosterone 2 α -hydroxylase and oestradiol 2-hydroxylase (Hanioka et al., 1998), along with decreases in testicular sperm number, epididymal sperm number, sperm motility, and increases of dead and abnormal sperms after atrazine exposure (Abarikwu et al., 2010). Atrazine was found in farm workers' houses in higher concentrations than in non-farmers' houses (Curwin et al., 2005), which leads to higher dosages in children of farmers than children of non-farmers (Curwin et al., 2007). Correlations have been observed between the amount of atrazine in drinking water and an increase in non-Hodgkin's lymphoma in farm workers in the Midwestern U.S. (Schroeder et al., 2001), decreased sperm quality in men in agricultural areas (Swan, 2006; Swan et al., 2003), and irregularities in female menstrual cycles (Cragin et al., 2011).

Recently, the potential effects of environmental toxicants on terrestrial invertebrates have been gaining attention as these toxicants may contribute to colony collapse disorder in honeybee colonies (Chauzat et al., 2009). Atrazine has been shown to affect expression of stress response genes (Le Goff et al., 2006) and protein production (Thornton et al., 2010) in *Drosophila melanogaster*, egg production in *Orchesella cincta* (Badejo and Vanstraelen,

1992) and mating choices in *Tenebrio molitor* (McCallum et al., 2013). However, an investigation of the effects of atrazine across all stages of male reproduction has never been conducted for an invertebrate species. The goal of this study is to utilize the model genetic system, *D. melanogaster*, to study the effects of larval atrazine exposure on male reproductive performance and measure whole organism phenotypes that are important components of overall fitness.

2. Methods

2.1 *Drosophila* lines and husbandry

Several different standard *Drosophila* lines were used in this study. Oregon-R (ORE-R) is a wild type (red-eyed) robust inbred line. Three recessive white-eyed mutants (*cn bw-1*, *cn bw-2*, and *cn bw-3*) with different genetic backgrounds were used as either the females or the competitor males. All flies were maintained on a 12 h light/dark cycle. Virgin collection bottles were maintained in incubators at 25°C and virgin collections and all mating experiments were conducted at 22°C (room temperature). Atrazine (Sigma-Aldrich; 45330) was dissolved in dH₂O and this stock solution was used in the appropriate volume to make the *Drosophila* media. Standard agar-dextrose-yeast media (McGraw et al., 2007) was used for all lines with atrazine dissolved in the experimental food at four concentrations (2 ppB, 20 ppB, 2 ppM or 20 ppM). The virgin males and females used in the experiments were produced as follows. Males and females from bottles with no history of exposure were stocked at medium density (~75 total flies depending upon the line) in 177 mL bottles containing 75 mL of the appropriate food. Females were allowed to oviposit for 4 days and then removed to keep larval densities low. Virgin males and females were collected over light CO₂, and maintained on control food in single sex vials (25 × 95 mm filled with 10 mL of food) with 1 – 13 live flies until 3–7 days old. Thus experimental males were exposed to atrazine from the time they were eggs until less than 8 hours after emergence and were then aged for 3 to 7 days on food lacking atrazine. Females used for the experiments were reared and maintained only on untreated food. Exposure level was treated as a factor and standard errors are reported throughout.

2.2 Mating rate and refractoriness to remating

Males from all five exposure levels (unexposed, 2 ppB, 20 ppB, 2 ppM and 20 ppM) were tested for their ability to mate with virgin females (*mating rate*) and then prevent that female from remating to a second male (*refractoriness*). Males were also tested for their ability to mate with a previously mated female (*remating rate*). To estimate *mating rate* and *refractoriness*, a single virgin ORE-R male (either unexposed or from one of the exposure treatments) was paired with a single virgin *cn bw-1* female in vial 1 beginning at 0700 hrs and the approximate time each vial was set up was recorded. Vials were observed for mating at least once every 10 minutes until 1300 hrs and observed copulations were recorded to the nearest 15 minutes. The male was removed from the vial after mating finished while the female remained in vial 1. Pairs that did not mate by 1300 hrs were left in the vial until 1700 hrs, at which time the male was removed. Females remained alone in vial 1 until 0700 hrs on day 3 (48 hrs after setting up the first mating) and then two competitor *cn bw-1* males were tapped into vial 1. Mating observations and male removal proceeded as described above and

females were transferred to vial 2 at 1700 hrs. Females were removed from vial 2 on day 5. Upon emergence 12 days later, the adult progeny were counted and their eye color recorded to determine which females had remated. Females with progeny with both red and white eyes were determined to have mated with both males. *Remating rate* was estimated similarly except the competitor *cn bw-1* male was the first male to mate and the experimental ORE-R males were the second. Estimates of *mating*, *remating* and *refractoriness* were repeated in three different blocks. Females were categorized as either having mated (1) or not (0) and the time the mating occurred was recorded based on the following time windows (within 30, 45, 60, 90, or 120 minutes for observed copulations or 600 minutes based on presence of progeny). A time of 601 minutes was recorded for females that did not mate and thus the data was considered right truncated for the Cox regression. Effects of block and treatment were tested using Cox regression with the *survival* package (Fox, 2002; Therneau, 2014; Therneau and Grambsch, 2000) in R version 3.1.0 (R Core Team, 2014). In this analysis, mating is treated as the event and the time it happens is also incorporated to fit a proportional hazards model. When the effects of treatment were significant, Tukey post hoc comparisons were completed using the *multcomp* package (Hothorn et al., 2008).

2.3 Proportion of offspring sired

The experimental males in the above experiment sired nearly 100% of the offspring when competing against the *cn bw-1* males, regardless of treatment or mating order. Therefore we repeated the competitive mating experiment using competitor males that were F1s from the mating of *cn bw-2* males and *cn bw-3* females. F1s were used to ensure recessive white-eyed competitors that were outbred and had a high probability of siring offspring under competitive conditions. Matings were conducted as described above. *PI'* was estimated when the experimental males were the first to mate to the female and *P2'* was estimated when the experimental males were the second to mate. *PI'* and *P2'* were estimated using only progeny from vial 2, after the female had the opportunity to mate to both males. *PI'* and *P2'* were completed in two different blocks and estimated only for unexposed males and males reared on 2 ppM atrazine food as this condition often showed a significant effect on the other phenotypes. Effects of block and treatment were tested using generalized linear models of the binomial family using a chi-squared test and correcting for overdispersion by setting the dispersion factor to the residual deviance divided by the residual degrees of freedom. Analyses were done in R version 3.1.0 (R Core Team, 2014).

2.4 Egg production, offspring production and egg to adult survival

The number of eggs laid, the number of offspring produced and egg to adult survival were estimated from females mated to males exposed to different levels of atrazine. All five exposure levels: unexposed, 2 ppB, 20 ppB, 2 ppM and 20 ppM, were investigated. This experiment was completed in six (6) different blocks and the effect of treatment and block was included in the analyses. As before, single virgin experimental males (from the appropriate treatments) were mated to virgin *cn bw-1* females starting at 0700 hrs. The exact minute when copulation began was recorded, and males were removed immediately after mating. Any females that were not observed to mate by 1300 hrs were discarded. Females were tapped into a new control food vial every 24 hours for 7 days total and then discarded. The number of eggs laid in the previous 24 hours was counted soon after the female was

transferred to the next vial. Vials were dyed with a small amount of green food coloring (220 μ l of 50% concentration) to increase the visibility of eggs. All the progeny that emerged from each vial were counted 11 days after laying. All counts were excluded if the female died within the seven days or if she laid fewer than five eggs. The identity of the researcher who counted the eggs and adults was recorded but this effect was not significant and was not included in subsequent analyses. The number of eggs laid and the number of progeny that emerged were analyzed as the sum across all 7 days and for each day individually using ANOVA. Egg to adult survival (within each day and overall) was analyzed using generalized linear models of the binomial family including the number of eggs laid and the number of offspring that emerged. Analyses were done in R version 3.1.0 (R Core Team, 2014). Because the larvae burrow into the food, attempts to count them were not reliable and therefore not reported.

2.5 Seminal fluid protein production and transfer to females

The effects of atrazine exposure on the production and transfer of two male seminal fluid proteins (SFPs), sex peptide and ovulin, were investigated using enzyme-linked immunosorbent assays (ELISAs) according to protocols in Sirot et al. (2009). Three exposure levels were studied: unexposed, 20 ppB, and 2 ppM. For measures of SFP production, the male reproductive tract (accessory glands, ejaculatory bulb, and ejaculatory duct) was dissected out of virgin males in 10% Dulbecco's Phosphate Buffering Solution (DPBS) with protease inhibitors (DPBS), ground individually with 20 μ L DPBS, brought up to 400 μ L DPBS, and then stored on ice. Thirty males per treatment (three replicate blocks each with ten males) were analyzed. For measures of SFP transfer, virgin experimental males were mated to virgin *cn bw-1* females and females were flash-frozen in liquid nitrogen 25 minutes after the start of mating. The female lower reproductive tract (uterus, sperm storage organs, common oviduct, and parovaria) was dissected in DPBS, ground individually with 20 μ L DPBS, brought up to 200 μ L DPBS, and stored on ice. Sixty females per treatment (three replicate blocks each with 20 females) were analyzed. One-way ANOVAs in SPSS were used to test for differences between treatments in the amount of SFPs produced or transferred. Post-hoc comparisons were based on Least Significant Differences estimates.

2.6 Seminal fluid protein mRNA levels

We also used quantitative real-time PCR (qRT-PCR) to measure expression of the genes encoding sex peptide (*Acp70A*) and ovulin (*Acp26Aa*) for 4 to 6 day old mated males reared on control food or on 2 ppM atrazine food. RNA extractions, cDNA manufacture and *Acp26Aa* primers are described in Fiumera et al. (2005). The primers for the control gene, *RPL17*, are 5' AAGCTGCAGCGCCAAAAG-3' and 5'-GCTGGCCCCAGAACACATTTTA-3' (M. Xie, unpublished primer sequences) and the primers for *Acp70A* are 5'-TCTTGTTCTCGTTTGCGTA-3' and 5'-GGGCTTGGAATTGGAAACTT-3' (J. Minucci, unpublished primer sequences). Reactions were performed in 25 μ L volumes on ABI 7300 Real-time PCR System using Power SYBR mix (Life Technologies) according to manufacture protocols. Any reactions containing visible primer dimers were excluded. Ten biological replicates and 2 PCR replicates (averaged prior to analysis) were run for each treatment on a single plate. For each

biological replicate, the average CT of either *Acp70A* or *Acp26Aa* was regressed against the average CT of *RPL17*. Neither of the regressions was significant and thus CT values were used directly in a one-way ANOVA to determine if there was a difference in CT between the unexposed males and those reared on 2 ppM atrazine food.

2.7 Testing for Non-Monotonic Dose Responses

In cases where we had measured the phenotype for all five concentrations and there was a significant effect of treatment we formally tested for non-monotonic dose responses. We did this by comparing two hierarchical regression models, one of which included a quadratic term (which allows for a u-shaped dose response curve) and the other which did not include the quadratic term. Block was still treated as a factor but treatment was a continuous variable. The test asks whether the data fits a model that includes a quadratic term better than a model which lacks the quadratic term thus indicating a non-monotonic response.

3. Results

3.1 Mating rate and refractoriness to remating

The ability of a male to mate to a virgin female (*mating rate*) was not affected by atrazine exposure ($\chi^2 = 6.2$, d.f. = 4, $P = 0.18$; Figure 1A). Male induced female refractoriness (*refractory*) was also not affected by atrazine exposure ($\chi^2 = 4.3$, d.f. = 4, $P = 0.37$; Figure 1B). Atrazine exposure did have significant effects on a male's ability to mate with a previously-mated female (*remating rate*; $\chi^2 = 14.5$, d.f. = 4, $P = 0.006$; Figure 1C). Post hoc comparisons from the Cox regression suggested a non-monotonic response with males reared on 2 ppM atrazine food mating more slowly than either the control males ($P = 0.01$) or males exposed to 20 ppM atrazine food ($P = 0.02$) to previously-mated females. A formal test comparing models with and without the quadratic term provided strong evidence for a non-monotonic response at 30 minutes ($P = 0.03$). Males exposed to intermediate concentrations of atrazine had reduced mating rates (6% and 5% for 2 ppB and 2 ppM, respectively) while 8% of those exposed to the highest concentration (20 ppM) and 16% of unexposed males were successful (Figure 1D).

There was no significant effect of block on the ability of a male to mate to a virgin female (*mating rate*, $\chi^2 = 4.8$, d.f. = 2, $P = 0.09$). There was a significant effect of block on both male induced refractoriness (*refractory*, $\chi^2 = 22.8$, d.f. = 2, $P = 1.2 \times 10^{-5}$) and a male's ability to mate with an already mated female (*remating rate*, $\chi^2 = 96.7$, d.f. = 2, $P < 2.2 \times 10^{-16}$) and when significant, block was included in the above models to test for treatment effects.

3.2 Proportion of offspring sired

Atrazine exposure affected a male's ability to sire offspring under competitive conditions (Figure 2) but only when they were the first male to mate (PI'). When mating first, unexposed males ($N = 205$) sired 41% of the progeny compared to a tester male ($PI' = 0.41 \pm 0.001$) while males exposed to 2 ppM atrazine ($N = 221$) sired only 30% of the progeny ($PI' = 0.30 \pm 0.001$; $P = 0.0006$ for treatment, $P = 0.2$ for block, Figure 2). There was no significant difference when the experimental males were the second male to mate

(unexposed $N = 177$; $P_2' = 0.84 \pm 0.001$; 2 ppM $N = 174$, $P_2' = 0.85 \pm 0.002$; $P = 0.78$ for treatment, $P = 0.66$ for block, Figure 2).

3.3 Egg production, offspring production and egg to adult survival

Significant effects of atrazine exposure on egg production are seen on the first five of the seven days post mating ($P < 0.05$; Figure 3) and when summed across all seven days ($P_{4,301} = 0.0004$). Overall the response was non-monotonic ($P = 0.006$, for the model with quadratic term) with females mated to either unexposed males or males reared on the highest concentration producing the largest number of eggs (97.0 ± 1.3 and 96.0 ± 1.0 , respectively) while females mated to males reared at intermediate concentrations tended to produce fewer eggs (76.6 ± 1.2 , 82.1 ± 1.2 , and 69.3 ± 1.1 for 2 ppB, 20 ppB and 2 ppM respectively). All days showed patterns consistent with non-monotonicity and in general, females mated to males from the 20 ppB and 2 ppM treatments laid the fewest eggs (Figure 3). This was most evident on the first day when females mated to males reared on 2 ppM atrazine laid 14.0 ± 0.6 eggs compared to females mated to unexposed males who produced 23.4 ± 0.7 eggs and females mated to males reared on 20 ppM who produced 20.6 ± 0.6 eggs. Atrazine exposure also affected the overall number of progeny that emerged ($P_{4,301} = 0.003$) and again there was a non-monotonic response ($P = 0.03$ for the model with quadratic term). There was no effect of atrazine exposure on egg to adult survival ($P_{4,301} = 0.57$) and thus the differences in the number of adults that emerged appears to be due solely to the differences in the number of eggs laid. Block had a significant effect on the number of eggs laid ($P_{5,301} = 2.5 \times 10^{-5}$) and was included in the models to test for effects of treatment.

3.4 SFP production, transfer and gene expression

Neither the amount of ovulin produced by males nor the amount transferred to females was significantly affected by atrazine treatment (ovulin production: $P_{2,83} = 0.09$; ovulin transfer: $P_{2,175} = 0.99$; Figure 4A). In contrast, both the amount of sex peptide produced by males and the amount transferred to females were affected by atrazine exposure ($P_{2,84} < 0.001$; $P_{2,173} = 0.01$, respectively; Figure 4B). Males that were exposed to 2 ppM produced significantly more sex peptide than either the unexposed or 20 ppB males (unexposed vs. 20 ppB: $P = 0.08$; unexposed vs. 2 ppM: $P < 0.001$; 20 ppB vs. 2 ppM: $P = 0.006$). Similarly, males exposed to either level of atrazine transferred significantly more sex peptide than unexposed males (unexposed vs. 20 ppB: $P = 0.007$; unexposed vs. 2 ppM: $P = 0.01$; 20 ppB vs. 2 ppM: $P = 0.84$). In contrast to the ELISA results, atrazine exposure (2 ppm) significantly upregulated ovulin mRNA production ($P_{1,18} = 0.043$), but did not change sex peptide mRNA production ($P_{1,18} = 0.49$; Figure 5).

4. Discussion

Our results strongly suggest that ecologically-relevant doses of larval atrazine exposure (Blanchard and Lerch, 2000; Lerch and Blanchard, 2003; Scribner et al., 2005) affect male reproductive performance in *Drosophila melanogaster*. Exposed males take longer to mate to previously mated females. Singly mated females lay fewer eggs and ultimately produce fewer offspring after mating to exposed males. Exposed males also sire a smaller proportion

of the offspring in doubly mated females. In addition, gene expression level and protein level of two important seminal fluid proteins are affected by larval atrazine exposure.

Understanding the mechanism of toxicity is an important step towards fully understanding a toxicant's potential ecological impacts. Based on the observed reduction in female fecundity when mated to atrazine exposed males we hypothesized that regulation of either *Acp26Aa* (ovulin) or *Acp70A* (sex peptide) might be affected by atrazine exposure. Both ovulin and sex peptide are male seminal fluid proteins that influence the physiology of the mated female, and can increase female egg laying rate. Knockout and knockdown studies have demonstrated that ovulin influences the release of mature oocytes from the ovary (Heifetz et al., 2005) while sex peptide appears to be a global regulator of female reproduction (Gioti et al., 2012), influencing factors such as egg maturation (Soller et al., 1999), feeding rate (Carvalho et al., 2006), release of stored sperm (Avila et al., 2010), remating propensity (Chapman et al., 2003; Chen et al., 1988; Liu and Kubli, 2003), and lifespan (Wigby and Chapman, 2005). We observed an effect of atrazine exposure on expression of the gene that encodes ovulin and also on production and transfer of sex peptide protein, but not in the manner that we hypothesized. Given the reduction in egg laying rate, we expected a down regulation of one or both genes and that the males would have produced and transferred less protein to the females during mating. Sex peptide production and transfer, however, was increased in the exposed males compared to the controls, while expression of the gene encoding sex peptide was unchanged. In addition, expression of the gene encoding ovulin was increased in the exposed males but the changes in expression did not correspond to significant increases in either production or transfer of the ovulin protein. There are several possible explanations for these observations. Perhaps subtle differences in production and transfer of these seminal fluid proteins do not have the same effect as knockouts or knockdowns. For example, Smith et al. (2009) observed a nonlinear relationship between sex peptide transfer and male induced refractoriness to remating. Intermediate transfer levels had a shorter refractory period between female rematings. It is also possible that the differences observed, although statistically significant, were not biologically relevant. Smith et al. (2012) observed significant differences in the quantity of sex peptide transferred among different *Drosophila* lines but these differences did not result in corresponding differences in overall female egg laying rate. Future studies should investigate the mechanisms underlying the decoupling of variation in seminal fluid mRNA and protein levels from variation in female post-mating responses. It is also important to recognize that we investigated only two of the nearly two hundred seminal fluid proteins (Avila et al., 2011) that are transferred to females during mating. We chose *Acp70A* and *Acp26Aa* because of their known functions and also because protein-specific antibodies exist (with no cross-reactives) that have well established ELISA protocols. It would be valuable to investigate how atrazine exposure affects the full set of proteins that are important in male reproduction using a proteomics approach (Baer et al., 2009; Takemori and Yamamoto, 2009).

Interestingly, many of the responses to atrazine showed evidence of non-monotonicity including mating rate, the number of eggs laid and the number of adults emerged. The intermediate doses, 20 ppb and 2 ppm, appear to have the largest effect on male reproductive

performance, while the highest exposure level often did not show phenotypic effects. Non-monotonic responses are often observed in toxicology studies (Conolly and Lutz, 2004; Vandenberg et al., 2012). For example, mating rate in *D. melanogaster* after lead exposure (Hirsch et al., 2003), growth in *V. fribrio* when exposed to some antibiotics (Zou et al., 2013), acetylcholinesterase activity in the earthworm species *Eisenia andrei* when exposed to formalin (Hackenberger et al., 2012) and mating preferences after atrazine exposure in *Chironomus tentas* (McCallum et al., 2013) all show non-monotonic responses. Numerous hypotheses have been proposed to explain non-monotonic responses (Vandenberg et al., 2012) and mechanistic studies will be needed to better understand the non-monotonic responses to atrazine we observed in *D. melanogaster* reproduction.

Numerous mechanisms might underlie the response to atrazine in *Drosophila*. Since it is atrazine, rather than its common metabolites, that is likely to be toxic (Smalling and Aelion, 2006), cytochrome *P450* genes are strong candidates to contribute to the molecular response. These proteins are often involved in response to environmental stressors (Danielson et al., 1996; Frank and Fogleman, 1992) and have been implicated in pesticide resistance in *Drosophila* (Daborn et al., 2002; Schlenke and Begun, 2004; Sun et al., 2011). In addition, cytochrome *P450s* and glutathione receptors appear upregulated after exposure to atrazine in both male and female *Drosophila melanogaster* (Le Goff et al., 2006; Thornton et al., 2010). Changes in expression of cytochrome *P450s* in response to atrazine exposure could affect synthesis of hormones such as ecdysteroids and juvenile hormone (Iga and Kataoka, 2012). These hormonal changes could, in turn, affect male reproductive behavior and seminal fluid protein production (e.g. Herndon et al., 1997). Alternatively, efflux pumps, which are known to contribute to drug resistance (Davidson et al., 2008) might be important players in modulating general responses to atrazine and potentially other environmental toxicants simply by pumping the toxicant out of the cells. Future studies will hopefully begin to dissect the mechanisms underlying susceptibility to atrazine and other toxicants.

Given our observations that several male reproductive traits are affected in laboratory populations of *D. melanogaster*, it is possible that atrazine could be affecting invertebrate reproduction in the wild. For example, atrazine has been found in nearly 14% of North American bee colonies, including in the brood combs where bees lay eggs and the larvae develop (Mullin et al., 2010) and it has been found in high concentrations in honey bee colonies that were sick or dead (Krupke et al., 2012). Although numerous factors are likely contributing to colony collapse disorder (Dainat et al., 2012; vanEngelsdorp et al., 2010), the effects of herbicides, such as atrazine, and their synergistic interactions with other stressors should not be overlooked.

Several other factors should be considered for future studies. First, the *D. melanogaster* used in our experiment were chronically exposed to atrazine during egg, larval and pupal stages of life and future work should examine adult and acute exposures. Additionally, all exposed flies were taken from a single laboratory genotype and thus this study fails to address the potential for natural genetic variation to influence susceptibility. Future research could map the genetic basis to susceptibility using the *Drosophila* Genetic Reference Panel (Mackay et

al., 2012) and capitalize on the genetic tools available in *Drosophila* to understand the mechanisms underlying susceptibility.

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Highlights

- Atrazine exposure decreased male mating rate, the number of progeny produced and competitive fertilization ability
- Atrazine exposure altered gene expression and protein production and transfer of male seminal fluid proteins
- There were non-monotonic responses with intermediate exposure levels often showing the largest effects

Figure 1.

Effects of atrazine exposure on mating and refractoriness. The proportion of males that mated to either virgin females (A) or prevented those female from remating (B) or mated to non-virgin females (C) is shown for different time points. Sample sizes for each treatment are shown. Note that the final time point is 600 minutes and the lines connecting that time point are broken. Post hoc comparisons with Tukey groupings are indicated by lowercase letters for male mating rate to non-virgins at 30 minutes (D) with means and standard errors plotted.

Figure 2.

Effects of atrazine exposure on competitive fertilization rates. The proportion of offspring sired is shown when males were the first male ($P1'$) or the second male ($P2'$) to mate to a multiply mated female. Means and standard errors are plotted. *** $P < 0.001$.

Figure 3.

Effects of atrazine exposure on the number of eggs laid. The number of eggs laid by an unexposed female when she mated once to males from different atrazine exposures is shown for each of the first seven days post mating. Means and standard errors are plotted. *P*-values are reported for the overall effect of treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.S. is not significant.

Figure 4.

Effects of atrazine exposure on production and transfer of two seminal fluid proteins.

Relative ovulin production and transfer (A) and relative sex peptide production and transfer (B) are shown for three different treatments. Means and standard errors are plotted. Tukey groupings are indicated by lowercase letters for sex peptide production and transfer. N.S. is not significant.

Figure 5. Effects of atrazine exposure expression of two male reproductive genes, *ovulin* and *sex peptide*. Means and standard errors are plotted. * $P < 0.05$, N.S. is not significant.