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Atrazine exposure affects longevity, development time and body size in *Drosophila melanogaster*

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Abstract

Atrazine is the one of the most widely used herbicides in the United States and non-target organisms may encounter it in the environment. Atrazine is known to affect male reproduction in both vertebrates and invertebrates but less is known about its effects on other fitness traits. Here we assessed the effects of five different chronic exposure levels on a variety of fitness traits in *Drosophila melanogaster*. We measured male and female longevity, development time, proportion pupated, proportion emerged, body size, female mating rate, fertility and fecundity. Atrazine exposure decreased the proportion pupated, the proportion emerged and adult survival. Development time was also affected by atrazine and exposed flies pupated and emerged earlier than controls. Although development time was accelerated, body size was actually larger in some of the exposures. Atrazine exposure had no effect on female mating rate and the effects on female fertility and fecundity were only observed in one of the two independent experimental blocks. Many of the traits showed non-monotonic dose response curves, where the intermediate concentrations showed the largest effects. Overall this study shows that atrazine influences a variety of life history traits in the model genetic system, *D. melanogaster*, and future studies should aim to identify the molecular mechanisms of toxicity.

Graphical abstract

Keywords

hormesis; pesticide; ecotoxicology; reproduction; monotonicity; survival

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1 Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is one of the most widely used herbicides in the United States with 70 million pounds applied annually to agricultural fields and turf (Barbash et al., 2001; Buchholz et al., 1999; Meyer et al., 2007). Atrazine has been banned in the European Union since 2003 due to some reported negative fitness effects and remains a controversial chemical in the United States (Sass and Colangelo, 2006). According to the US Geological Survey, atrazine has been detected in surface water, ground water and rainfall (Barbash et al., 2001) and human drinking water supplies occasionally exceed the US Environmental Protection Agency (US EPA) safe level of 3 parts per billion (3 ppB) (Barbash et al., 2001).

Many studies show that atrazine has detrimental effects on a number of different vertebrate taxa (Alvarez and Fuiman, 2005; Am-in and Tuntasuparuk, 2012; Belloni et al., 2011; Dodson et al., 1999; Hayes et al., 2011; Hayes et al., 2002; Hayes et al., 2010; Hayes et al., 2006). For example, when exposed as tadpoles to 0.1 ppB atrazine, genetic male African Clawed frogs (*Xenopus laevis*) experienced demasculinization and feminization; their larynx size is reduced, nuptial pads were lighter in color and 10% of these males produced viable eggs (Hayes et al., 2002; Hayes et al., 2010; Hayes et al., 2006). Other work has shown that atrazine affects some developmental, immunological and reproductive traits in several different fish species (Alvarez and Fuiman, 2005; Blahova et al., 2012; Botelho et al., 2012; Kreutz et al., 2012). Additional experiments with fish have shown that atrazine exposure alters plasma testosterone, 17 β -estradiol and 11-ketotestosterone, as well as causes the over expression of *cyp19A1*, the gene that encodes for aromatase and controls the conversion of testosterone to estradiol (Spano et al., 2004; Suzawa and Ingraham, 2008).

Although much of the work on atrazine has focused on aquatic organisms, atrazine is also a potential toxicant for terrestrial systems. Laboratory experiments have shown that rats exposed to atrazine experience a broad array of physiological effects including body tremors (Campos-Pereira et al., 2012), reduced body weight (Abarikwu et al., 2010; Campos-Pereira et al., 2012; Victor-Costa et al., 2010), insulin resistance (Lim et al., 2009), liver cell abnormalities (Campos-Pereira et al., 2012), reduced female uterine weight (Quignot et al., 2012), decreased male testosterone (Friedmann, 2002; Hanioka et al., 1998) and decreased testicular sperm number (Abarikwu et al., 2010; Kniewald et al., 2000). Furthermore, atrazine increases aromatase expression in mammalian and human cell lines (Fan et al., 2007; Sanderson et al., 2002).

Numerous recent toxicology studies have focused on characterizing the sublethal effects of pesticides on arthropods but only a small portion of that work focused on atrazine (Desneux et al., 2007). Low doses of atrazine have been shown to alter sex ratio in favor of males in *Daphnia pulex* (Dodson et al., 1999) and cause respiratory stress in the crab, *Oziotelphusa senex senex* (Prasad et al., 1995). In *Drosophila melanogaster*, atrazine has been shown to alter the production of proteins associated with energy production and oxidative stress (Thornton et al., 2010) and affect expression of stress response genes (Le Goff et al., 2006). Larval atrazine exposure reduces *D. melanogaster* male mating rate and male fertility (Vogel et al., 2015). Understanding the effects of atrazine on invertebrates is

important because evidence suggests that pesticides are contributing to honey bee colony collapse disorder (Chensheng et al., 2014; Fauser-Misslin et al., 2014; Oliveira et al., 2014). A small number of studies have shown that atrazine has an effect on honeybee neurotransmitter signals in the heart (Papaefthimiou et al., 2003) and inhibits acetylcholinesterase activity (Boily et al., 2013). In addition, it can be found in honeybee wax as well as larvae and adults across the US and Canada (Krupke et al., 2012; Mullin et al., 2010). Little work, however, has investigated the effects of atrazine across a broad array of fitness traits in an invertebrate. The goal of this study is to investigate the effects of atrazine on the following traits; longevity, development time, body size, female mating rate, female fertility, and female fecundity using the model genetic system *D. melanogaster*.

2. Materials and methods

2.1 *Drosophila* lines, husbandry and statistical analysis

The inbred but robust, wild type Oregon-R (ORE-R) lab strain was used for all of the experiments. The ORE-R strain was collected in 1925 in Oregon and has been maintained in the lab since that time and our line was derived from the A. G. Clark stock at Cornell University in 2006. The flies were maintained on a 12 h light/dark cycle in an incubator at 25°C on a standard yeast/dextrose media [1220 ml distilled water, 12 g agar, 100 g dextrose, 100 g inactive brewer's yeast, 0.5 ml 8.3% phosphoric acid and 0.5 ml 83.6 % propionic acid (McGraw et al., 2007)]. The incubator did not control humidity and thus vials were always distributed haphazardly around the entire incubator and were moved around at least twice a day to remove environmental variation within each individual vial. Atrazine (Sigma Aldrich; 45330) was dissolved directly in the food and five different atrazine concentrations were utilized; 0 (unexposed or control), 2 ppB, 20 ppB, 2 ppM, 20 ppM for all of the experiments except female mating and remating rate where we did not test 20 ppM. These concentrations were chosen because 2ppB is just below the EPA allowable limit of 3 ppB and 20 ppM is the highest concentration that can be dissolved in water. The parents of all experimental flies were from ORE-R stocks that have never been exposed to atrazine, thus controlling for potential transgenerational effects of exposure. Each experiment was repeated in 2 to 3 independent blocks. All statistical analyses were conducted in R version 2.15.3 (R Core Team, 2014) and block was treated as a random factor. The different atrazine exposure levels were treated as fixed effect factors, and thus we are not assuming that higher concentrations will necessarily have larger effects. When treatment was significant, we used a Tukey post hoc test to compare the different treatments using Tukey HSD from the *multcomp* package (Hothorn et al., 2008). We also tested for non-monotonicity by considering treatment as a continuous covariate, rather than as a factor, and comparing a linear model to a model with a quadratic term. To provide information on sample sizes we have reported the treatment and residual degrees of freedom as subscripts with the *P*-values. All reported errors are standard errors.

2.2 Development and body size

Flies without any previous exposure to atrazine were placed onto grape plates [2714 ml H₂O, 2275 ml grape juice, 110 g agar, 290 g dextrose, 115 g sucrose, 90 g inactive yeast, 110 ml of 1.25M NaOH, 28 ml of 8.3% phosphoric acid and 28 ml of 83.6% propionic acid

(McGraw et al., 2007)] and allowed to lay eggs for eight hours and then discarded. The grape plates did not contain any atrazine. Twenty-four hours later, first instar larvae (based on morphology) were picked from the grape plates using dissecting probes dabbed with yeast paste. First instar larvae were collected from the grape plates and haphazardly allocated to the different treatments. Although there could be up to an 8-hour age difference between larva, this variation would be present in all the vials across all the treatments. This will increase the within treatment variance and thus reduce the power to detect true differences between treatments but it will not bias the estimates of development time across the treatments. Fifteen replicate vials (25 × 98 mm with 10 mL of fly food), each containing 30 larvae, were set up for each of the five atrazine concentrations (0 ppB, 2 ppB, 20 ppB, 2 ppM, 20 ppM). Vials were checked daily and when the pupae began developing the vials were checked and counted every 8 to 10 hours until adult flies finished emerging. Virgins were collected over CO₂ within 10 hours of emergence and the time of emergence and sex of each fly was recorded. Development time was measured over three independent blocks.

Body size was measured using the adult flies that emerged from blocks 2 and 3. The flies in block 1 emerged and were analyzed for development time but all the adults were accidentally housed on control food (rather than their appropriate treatment food) and thus those flies were not used for body size measurements. The emerged virgin flies (both males and females) from blocks 2 and 3 were housed at very low densities (< 5 flies per vial) in single sex vials of food containing the appropriate atrazine concentration. No larval activity was observed in these vials indicating that the flies were virgins. After 48 hours, the flies were frozen at -20°C prior to measuring dry weight. Flies were dried for 24 hours at 60°C and weighed individually on a Mettler Toledo MX5 scale. We measured three flies per vial and the average was taken prior to analysis. Body size was measured over two independent blocks.

The effects of treatment on total development time in hours (from the midpoint of egg laying to emerged adults), pupation time in hours (from the midpoint of egg laying to pupae) and dry weight were analyzed in R (R Core Team, 2014) using linear models (lm) and TukeyHSD for post-hoc analyses using the *multcomp* package (Hothorn et al., 2008). Larvae that failed to pupate or pupae that failed to emerge were excluded from the development time analyses. The effects of treatment on the proportion of larvae that pupated and the proportion that emerged were tested using generalized linear models (*glm*) of the binomial family with TukeyHSD for post-hoc analyses. Two vials that produced fewer than 4 flies were excluded from all analyses. We analyzed all blocks simultaneously, treating block as a random factor, and also analyzed each block separately.

2.3 Longevity

Unexposed females were allowed to oviposit in polypropylene bottles (177 mL filled with 75 mL of food) with the appropriate experimental food and discarded after 2 days. Twenty four hours after emergence, between 270 and 400 flies were transferred without CO₂ (and thus without counting) to large longevity bottles modified from (Promislow et al., 1996). The bottles were 3000 cm³ clear PET bottles (Container and Packing Supply #B100) with a 5.1 cm diameter circular hole cut near mouth and covered with screen for airflow. Another

5.1 cm diameter hole was cut close to the mouth for removing dead flies and covered with a square rubber gasket. A screen secured with a rubber band covered the mouth of the bottle. Lids (Container and Packing Supply #L465) were filled with approximately 100 mL of the appropriate food and covered with cheesecloth. The population bottles were inverted on top of the lid and attached with rubber bands. Dead flies were removed daily between 0800 h and 1100 h and sexed. Bottle lids containing fly food were changed every other day and the screens washed and dried as needed. Longevity was assayed for 50 days at which time the remaining adults were sexed and counted. The experiment was replicated in three independent blocks with each block containing two replicate bottles for each experimental treatment. We used a Cox regression in the *survival* package to test for effects of treatment on longevity (Fox, 2002; Therneau, 2000). We analyzed all three blocks simultaneously, treating block as a random factor, and also analyzed each block separately. To control for any effects of the number of flies in each bottle, we excluded two bottles that included more than 400 flies and also included the population size of the remaining bottles as a covariate.

2.4 Female mating rate

Virgin females were collected from four atrazine concentrations (0, 2 ppB, 20 ppB, 2 ppM), while virgin males were only collected from control food. All flies were aged 3 to 5 days on the same type of food as their larval environment. Between the ages of 2 to 6 days old is an ideal window for experimentation as the flies are sexually mature but show no sign of senescence. All flies (including exposed females) were transferred individually to control food a day before the matings. Beginning at 0700 h we tapped single virgin males into vials with single females. None of the vials used for mating contained atrazine. Vials were observed at least every ten minutes and the time of mating was recorded. Males were removed from the female vials as soon as copulation was complete. Any females that did not mate by 1300 h were discarded. For those females that mated, we tested re-mating rate 48 hours after the first matings were set up. Beginning at 0700 h we tapped two virgin unexposed males into vials with the singly mated females and recorded mating as described above. All flies were discarded after re-mating. We tested 100 to 200 females per treatment in each of three independent blocks. The time of mating was recorded as a binomial (0 or 1) within each of the following time points (minutes): 15, 30, 60, 90, 120, 180, 240, 300 for mating and 30, 45, 60, 90, 120, 180, 240, 300 for re-mating. Females that failed to mate were assigned a value of 0 at time 301 thus producing right truncated data. We used a Cox regression implemented in the *survival* package (Fox, 2002; Therneau, 2000) to test for differences in mating and re-mating rate between the treatments. In this case mating was considered as the event and the time it happened fit to a proportional hazards model.

2.5 Number of eggs laid and offspring produced

Virgin females were collected from all five concentrations, while virgin males were only collected from control food. Virgins were aged for 2 to 3 days on the same concentration of atrazine as their larval environment. Females were then individually transferred to control food and at 0800 h the next morning single male flies were tapped into the control vials with the single females. The pair was transferred to a new control vial every morning at 0800 h for 7 days and the number of eggs in the previous vial was immediately counted. If a male died or escaped, a replacement male that was mated and similarly aged was added to the

vial. Approximately 14 days later, once all the flies had emerged, the vials were frozen upside down and then flies were sexed and counted. Approximately 40 vials of each treatment were set up in each of 2 independent blocks. If a female died, she was excluded from the entire experiment. If a female escaped, all data until the day she was lost was included in the analysis. Females were excluded if they laid 0 eggs on at least one day during the 7 experimental days. The number of eggs laid and number of progeny emerged was analyzed as a sum across seven days and also at each day separately using a linear model in R (R Core Team, 2014).

3. Results

3.1 Development time and body size

The proportion of larvae that pupated and the proportion of larva that reached eclosion were significantly reduced by atrazine exposure ($P_{4, 6565} < 2.2 \times 10^{-16}$, $P_{4, 6565} < 2.2 \times 10^{-16}$ respectively; Figure 1; Supplemental Tables 1 to 8). The proportion pupated ranged from $0.87 (\pm 0.009 \text{ SE})$ in the control vials to $0.73 (\pm 0.013 \text{ SE})$ in the 20 ppB treatment while the proportion emerged ranged from $0.85 (\pm 0.009 \text{ SE})$ in the control vials to $0.68 (\pm 0.01 \text{ SE})$ in the 20 ppB treatment. Post hoc analysis of proportion pupated showed significant differences between control and all of the atrazine treatments and also between 20 ppB and 2 ppM. Post hoc analysis of the proportion emerged showed significant differences between all of the treatment pairs except between 2 ppM and 20 ppM and between 2 ppM and 2 ppB. Although the proportion pupated and proportion emerged appear U-shaped, neither fit the quadratic model better than the linear ($\chi^2 = 1.82$, d.f. = 1, $P = 0.18$ and $\chi^2 = 3.08$, d.f. = 1, $P = 0.08$, respectively). There was a significant effect of block on proportion pupated and proportion emerged ($P_{2, 6563} = 3.8 \times 10^{-14}$, $P_{2, 6563} < 2.2 \times 10^{-16}$ respectively) and thus block was included in the analyses.

Atrazine exposure accelerated adult emergence in both males and females ($P_{4, 2242} < 2.2 \times 10^{-16}$; $P_{4, 2363} < 2.2 \times 10^{-16}$ respectively; Figure 2). The intermediate exposure level (20 ppB) had the fastest development time for both males ($238 \text{ hours} \pm 0.44 \text{ SE}$) and females ($232 \pm 0.37 \text{ SE}$) compared to the controls ($248.8 \text{ hours} \pm 0.51 \text{ SE}$ for males and $243.8 \pm 0.48 \text{ SE}$ for females). Overall, there was strong evidence of a non-monotonic response for time to emergence for both males and females ($P_{1, 2456} = 5.5 \times 10^{-05}$, $P_{1, 2578} = 1.4 \times 10^{-08}$, respectively). Some of the variation in time to emergence is due to a decrease in time to pupation for individuals reared on atrazine food ($P_{4, 4972} < 2.2 \times 10^{-16}$; Figure 3), although we did not differentiate between males and females at the pupal stage. The mean time to pupation for control flies was 154 hours ($\pm 0.26 \text{ SE}$) and the mean pupation time for individuals reared on atrazine ranged from 147.9 hours ($\pm 0.16 \text{ SE}$ for 2 ppB, $\pm 0.17 \text{ SE}$ for 20 ppB) to approximately 148 hours ($\pm 0.17 \text{ SE}$ for 2 ppM, $\pm 0.19 \text{ SE}$ for 20 ppM). There was an effect of block on time to pupation ($P_{2, 5183} = 6.8 \times 10^{-06}$) and thus block was included in the analysis. We included vial as a nested factor within treatment in the models for pupa and adult development time, and vial did have a significant effect on development time ($P_{212, 4972} < 2.2 \times 10^{-16}$ for pupa, $P_{213, 2363} < 2.2 \times 10^{-16}$ for females, and $P_{212, 2242} < 2.2 \times 10^{-16}$ for males.) We also separately included the number of larvae that pupated as a covariate in the model and found that it did not affect time to pupation ($P_{I, 5183} = 0.72$)

while the number of adults that emerged had a weak but non-significant effect on the time to eclosion ($P_{1, 5033} = 0.051$) and thus neither of the covariates was included in the final models. The full ANOVA tables for development time are presented in Supplemental Tables 9 to 20.

Atrazine exposure also significantly affected adult body size ($P_{4, 140} = 0.005$ for males and $P_{4, 140} = 0.002$ for females; Figure 4; Supplemental Tables 21 to 26). Surprisingly, flies reared on the highest concentration (20 ppM of atrazine) were significantly larger than control flies in both males ($0.29 \text{ mg} \pm 0.002 \text{ SE}$ for 20 ppM versus $0.27 \text{ mg} \pm 0.002 \text{ SE}$ for control) and females ($0.45 \text{ mg} \pm 0.005 \text{ SE}$ for 20ppM versus $0.42 \text{ mg} \pm 0.004 \text{ SE}$ for control). There was no effect of block on body size ($P_{1, 140} = 0.10$ for males, $P_{1, 140} = 0.15$ for females) and there was no evidence for non-monotonicity in males ($P_{1, 142} = 0.93$) or females ($P_{1, 142} = 0.99$).

3.2 Longevity

Atrazine exposure significantly affected lifespan (LRT = 1283, d.f. = 7, $P = 0$; Figure 5). Compared to controls (n = 1534), lifespan was reduced for flies reared and maintained on all concentrations of atrazine food ($P < .001$ for all Tukey post hoc tests). Interestingly, flies reared and maintained on the highest concentration (20 ppM, n = 1390) lived significantly longer than flies on intermediate concentrations (2 ppB, n = 1518; 20 ppB, n = 1162 and 2 ppM, n = 1177; $P < .001$ for all Tukey post hoc tests). There was strong evidence for a non-monotonic response ($\chi^2 = 188.31$, d.f. = 1, $P < 2.2 \times 10^{-16}$). After removing the two bottles with the largest population sizes, there was still a significant effect of population size on longevity ($P = 5.5 \times 10^{-16}$), and thus it was included as a covariate. When the blocks were analyzed individually, there was a significant effect of population size on lifespan in block 1 ($P = 2 \times 10^{-16}$), but not in blocks 2 and 3 ($P = 0.35$, $P = 0.46$, respectively). This was likely because we were able to reduce the variation in population size down from a 200 fly difference in block 1 to approximately a 100 fly difference in blocks 2 and 3. Regardless of the effect of sample size, all blocks showed a strong significant and consistent reduction in lifespan for atrazine exposed flies when analyzed separately and including or excluding the two bottles with the largest population sizes. Thus the differences in sample sizes appear to have no meaningful effect on the results (Supplemental Tables 27 to 36).

3.3 Female mating rate, eggs laid and offspring produced

Overall, atrazine exposure did not consistently affect female mating ($\chi^2 = 4.22$, d.f. = 3, $P = 0.24$) or remating rate ($\chi^2 = 0.88$, d.f. = 3, $P = 0.83$). There was, however, a significant block effect (mating; $P < 2.2 \times 10^{-16}$, remating; $P = 7.6 \times 10^{-11}$) and a block by treatment interaction for both ($\chi^2 = 23.6$, d.f. = 6, $P = 0.0006$ and $\chi^2 = 20.5$, d.f. = 6, $P = 0.002$, respectively). The results of the Cox regressions for each block are shown in Supplemental Tables 37 to 42.

Across the two blocks, atrazine did have a significant effect on both the number of eggs laid (all days combined; $P_{4, 203} = 1.0 \times 10^{-06}$) and the total number of offspring produced (all days combined; $P_{4, 206} = 0.0004$). Females exposed to the highest level of atrazine, 20 ppM produced the highest number of offspring ($128.4 \pm 8 \text{ SE}$) while 2 ppB females produced the

fewest (89.1 ± 5 SE) and this is largely due to differences in the number of eggs laid (144.8 ± 6.6 SE versus 103 ± 4.5 SE, respectively). There was, however, a significant block by treatment interaction for both for the number of eggs laid ($P_{4, 203} = 0.001$), and the number of offspring produced ($P_{4, 206} = 0.0004$). Analyzing the blocks separately showed that there was a significant effect of exposure in block 2 for both the number of eggs laid and the number of offspring produced ($P_{4, 131} = 1.5 \times 10^{-9}$ and $P_{4, 132} = 9.5 \times 10^{-8}$, respectively) but not a significant effect of atrazine exposure in block 1 ($P_{4, 72} = 0.76$ and $P_{4, 74} = 0.87$, respectively). The significant effect in block 2 was observed when analyzing each day separately or combined across the 7 days ($P < 0.05$ for each day separately). Full ANOVA tables for the number of eggs laid and offspring produced are shown in supplemental tables 43 to 48.

4. Discussion

Our results strongly suggest that ecologically relevant doses of atrazine (Blanchard and Lerch, 2000; Lerch and Blanchard, 2003) have significant effects on a variety of fitness traits in *Drosophila melanogaster*. Fewer adults emerged from vials containing atrazine and those that did, emerged at an accelerated rate and had altered body sizes. In addition, longevity was decreased when those individuals were maintained on atrazine food as adults. Overall, exposed females laid more eggs and produced more offspring, however, significant effects were not observed in all blocks independently.

Our results are consistent with several other studies that showed that herbicide exposure can affect larval survival and adult lifespan in insects (Burdett et al., 2001; Hahn et al., 2014; Mirande et al., 2010; Russell and Schultz, 2010). For example, sethoxydim causes a 32% decrease in eclosion success of the cabbage butterfly, *Pieris rapae*, and fluzifop-p-butyl, a 21% reduction (Russell and Schultz, 2010). We also found that when adult flies were maintained on atrazine food they had a reduced lifespan and this is consistent with other studies showing a reduction in insect lifespan as a result of pesticide exposure (Bravo-Hernandez et al., 2014; Oliveira et al., 2014; Pakyari and Enkegaard, 2013). The reduction in larval, pupal and adult survival that we observed may be due to atrazine interacting with the mitochondrial electron transport system. In plants, atrazine prevents photosynthesis from occurring by binding to the plastoquinone binding sites of photosystem complex II in chloroplasts thereby inhibiting electron transport (Lim et al., 2009; Solomon et al., 2008). Animal mitochondrial electron transport chain complexes I and II have similar Q binding sites and thus it has been proposed that atrazine may affect electron transport by binding to these mitochondrial sites (Lim et al., 2009). This hypothesis is supported by work that shows that atrazine exposure causes differences in protein levels for genes known to respond to mitochondrial stress in *D. melanogaster* (Thornton et al., 2010) and work that shows that atrazine directly causes mitochondrial abnormalities in mice (Lim et al., 2009). Mitochondrial dysfunction can lead to the overproduction of reactive oxygen species which can cause oxidative stress and has been shown to be a significant component of aging and lifespan (Beckman and Ames, 1998; Dudas and Arking, 1995; Finkel and Holbrook, 2000; Harshman and Haberer, 2000; Landis et al., 2004). Furthermore, unpublished work from our lab suggests that variation in ABC transporters play a role in atrazine susceptibility (Xie, 2015). ABC transporters move a variety of molecules across cellular membranes (Kim et al.,

2013) and have been shown to regulate longevity in *D. melanogaster* (Huang et al., 2014). Based on the connection between oxidative stress and lifespan, the reduction of lifespan in *D. melanogaster* may be due to atrazine increasing reactive oxygen species and thus oxidative stress.

Several interesting observations were made regarding the effects of atrazine on development. Atrazine accelerates development time, which is consistent with other work showing a change in development time as a result of pesticide exposure (Burdett et al., 2001; Hahn et al., 2014; Mirande et al., 2010; Russell and Schultz, 2010). For example, glyphosphate causes acceleration in development time in the ladybird beetle, *Eriopis connexa germar*, (Mirande et al., 2010) while both molinate and thiobencard independently lengthen development time of *Chironomus tepperi* (Burdett et al., 2001). Interestingly, time to emergence showed a strong non-monotonic response while time to pupation seemed to show more of a threshold response to any atrazine exposure. It is possible the differences were due to our coarse time sampling of every 8 to 10 hours but it is also possible that atrazine is having different effects between larval and pupal development. In addition, at the highest atrazine exposure level, the flies developed more quickly but were larger as adults. This is contrary to past work that has asserted that faster development time is correlated with smaller adult body size (Yadav and Sharma, 2014). Development time and body size are correlated traits which are controlled by similar pathways (Colombani et al., 2005; Mirth et al., 2014; Ono, 2014) thus the mechanisms that cause an acceleration in development time may also be related to the mechanism that causes the increase in body size.

A number of possible mechanisms exist for the effects of atrazine on development. For example, the hormone ecdysone interacts with the insulin signaling pathway which regulates the timing of development and influences adult body size (Colombani et al., 2005; Thummel, 1996). Atrazine may be interacting with ecdysone and thus the insulin signaling pathway, thereby causing the acceleration in development time and change in body size. This could be mediated by through an alteration in food intake since *Drosophila* larvae will alter their food intake in response to a variety of toxins in their environment. (Mueller and Barter, 2015; Perkhulyn et al., 2015; Weiner et al., 2014). It is also possible that the gut microbiota is being affected (Broderick and Lemaitre, 2012; Han et al., 2014). The gut microbiota affects many aspects of physiology including digestion, immune function, growth, metabolism (Broderick and Lemaitre, 2012) and mating preference (Sharon et al., 2010). Specifically, the *Drosophila* gut bacteria *Lactobacillus plantarum* affects production of ecdysone and insulin (Storelli et al., 2011) and these hormones influence both the timing of development and adult body size (Colombani et al., 2005; Thummel, 1996). Thus, the acceleration of development time and the change in body size that we observed may be due to atrazine acting as an endocrine disruption chemical (EDC) and affecting some component of the endocrine system such as the insulin signaling pathway. Other chemicals that have been classified as EDCs, such as bisphenol A (BPA), alter growth and development time in *Drosophila* and mice (Nikaido et al., 2004; Weiner et al., 2014). Clearly additional work, such as tighter time point monitoring and a better understanding of how atrazine is metabolized during development, will be required to understand the mechanisms underlying the observed effects on development rate and body size.

Previous work has shown that atrazine exposure decreases male mating rate in *D. melanogaster* (Vogel et al., 2015). Based on this, we expected atrazine exposed males would be smaller since female flies prefer larger males (Partridge and Farquhar, 1983). We actually observed an increase in the body weight of males exposed to 20 ppM atrazine. Additionally, we found that atrazine had no effect on female mating rate which was also surprising if the changes in development time and body size are mediated via changes in insulin signaling (Wigby et al., 2011). In addition, although there was an overall effect on the number of eggs laid and offspring produced, the effects were only significant in one block, and thus other factors are also important.

The results of assays investigating development time and longevity all showed non-monotonic responses. Non-monotonicity is common in studies of EDCs as well as in studies of natural hormones and some pharmaceuticals (Cabaton et al., 2011; Shioda et al., 2006; Storrs and Kiesecker, 2004; Wozniak et al., 2005). One mechanism that is often proposed to explain non-monotonic responses to EDCs is differences in receptor affinity (Tilghman et al., 2010). For example, at low doses BPA binds to the estrogen receptor but at high doses it can also bind to other hormone receptors like the androgen receptor (Moriyama et al., 2002; Sohoni and Sumpter, 1998) and thus different effects are observed at the different concentrations. Other hypotheses, which may not be mutually exclusive, include two or more monotonic responses that overlap and appear to be non-monotonic, receptor competition between EDCs and endogenous hormones, EDC negative feedback loops and different EDC doses causing different genes to be expressed (Vandenberg et al., 2012). Taken together, it appears that different traits are more susceptible to exposure than others and we clearly need a better understanding of the underlying mechanisms involved.

Several of the effects observed here may have opposing effects on fitness and thus it may be difficult to predict how atrazine affects a wild population. For example, the reduction in lifespan is likely to reduce population sizes in the wild, however flies also develop faster and emerge significantly larger and this may increase fitness and thus offset a decrease in population size. On the other hand, previous work (Vogel et al., 2015) has shown that atrazine exposed males have a reduction in mating success and fertility and so although the males are larger they may still have a reduction in fitness and that may lead to population decline. Furthermore, there are many traits that may impact fitness that have yet to be assayed. For example, we do not know if atrazine exposure affects motor ability, immunity, cold stress or heat tolerance (just to name a few). Although it is difficult to predict how these results reflect the actual demographics of a wild population, given the growing evidence that pesticides are contributing to honey bee colony collapse disorder (Boily et al., 2013; Chensheng et al., 2014; Oliveira et al., 2014), it is possible that atrazine exposure may be one of many contributing factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Atrazine exposure affects a number of fitness traits in *Drosophila melanogaster*.

Atrazine exposure reduced pupation rate, emergence rate and adult longevity.

Atrazine exposure accelerated development time and affected body size.

Most traits showed non-monotonic dose response curves.

Figure 1. Proportion of larvae that pupated and emerged. Standard errors and Tukey groupings are shown.

Figure 2. Time to adult emergence, in hours, for males and females. Standard errors and Tukey groupings are shown.

Figure 3.
Time to pupation in hours. Standard errors and Tukey groupings are shown.

Figure 4. Adult body size as measured by dry weight for males and females. Standard errors and Tukey groupings are shown.

Figure 5.
Adult survival as a percentage of original flies still alive.