Asymmetrical positive assortative mating induced by developmental lead (Pb$^{2+}$) exposure in a model system, *Drosophila melanogaster*

Elizabeth K. Peterson$^a$,*, Roman Yukilevich$^b$, Joanne Keelbeck$^c$, Kelly M. LaRue$^d$, Kyle Ferraiolo$^a$, Kurt Hollocher$^e$, Helmut V.B. Hirsch$^a$, and Bernard Possidente$^f$

Abstract

Anthropogenic pollutants have the potential to disrupt reproductive strategies. Little is known about how lead (Pb$^{2+}$) exposure disrupts individual-level responses in reproductive behaviors, which are important for fitness. *Drosophila melanogaster* was used as a model system to determine the effects of: 1) developmental lead exposure on pre-mating reproductive behaviors (i.e., mate preference), and 2) lead exposure and mating preferences on fitness in the $F_0$ parental generation and $F_1$ un-exposed offspring. Wild-type strains of *D. melanogaster* were reared from egg stage to adulthood in control or leaded medium (250 μM PbAc) and tested for differences in: mate preference, male song performance, sex pheromone expression, fecundity, mortality, and body weight. $F_0$ leaded females preferentially mated with leaded males (i.e., asymmetrical positive assortative mating) in 2-choice tests. This positive assortative mating was mediated by the females (and not the males) and was dependent upon context and developmental exposure to Pb. Neither the courtship song nor the sex pheromone profile expressed by control and leaded males mediated the positive assortative mating in leaded females. Leaded females did not incur a fitness cost in terms of reduced fecundity, increased mortality, or decreased body weight by mating with leaded males. These results suggest that sublethal exposure to lead during development can alter mate preferences in adults, but not fitness measures once lead exposure has been removed. We suggest that changes in mate preference may induce fitness costs, as well as long-term population and multi-generational implications, if pollution is persistent in the environment.

Key words: cuticular hydrocarbons, positive assortative mating, random mating, species recognition system.
The lethal effects of lead exposure are well documented (Demayo et al. 1982; Mateo et al. 2003) and have resulted in the population declines of several wildlife species (Eisler 1988), including the California condor (Gymnogyps californianus, Finkelstein et al. 2012). However, the sublethal effects of lead exposure on wildlife are less known, particularly those that alter complex behavioral systems necessary for reproduction and survival. These sublethal effects on behavior include: learning and cognitive function (Burger and Gochfeld 1985; Rice 1993), motor skills (Burger and Gochfeld 2005), individual recognition (Burger and Gochfeld 2005), and locomotion and movement (Burger and Gochfeld 1988; Burger 1990; Burger and Gochfeld 1993). Despite these observations, little is known about how lead exposure disrupts both pre-mating and post-mating reproductive behaviors in field populations, which are necessary for individual fitness and impact population growth (Hansen and Johnson 1999; Dell’Omo 2002; Clotetéter et al. 2004; Kane et al. 2005; Weis 2014).

A comprehensive understanding of lead-induced alterations on reproduction is essential for conservation efforts but can be challenging. Little (1990) noted, “behavioral toxicosis is neither frequently nor readily observed in the field because of the difficulty and expense associated with observations of organisms in natural environments.” Therefore, invertebrate model systems, such as Drosophila melanogaster, are an alternative to field research given the ease of sampling and manipulation, reduced cost, and technological tools available (Rubin et al. 2000; Burke and Rose 2009; Pandey and Nichols 2011). Our research group has established D. melanogaster as an invertebrate model alternative to understand lead-induced impacts on the nervous system, genetics, and behavior (Hirsch et al. 2003, 2009, 2012; Morley et al. 2003; He et al. 2009; Ruden et al. 2009).

Drosophila melanogaster is useful for these studies because they exhibit a wide range of complex behaviors, including mating and reproductive behaviors (Sokolowski 2001, 2010; Dickson 2008; Markow and O’grady 2008). Prior to mating, adults must be both fertile and behaviorally mature (Markow and O’grady 2008). Males and females will assess each other first using chemosensory cues [sex pheromones, called cuticular hydrocarbons (CHCs)] to determine that their potential mate is of the appropriate sex and species (Greenspan and Ferveur 2000). CHCs are long-chain hydrocarbons that produce a wax-like covering on the adult fly cuticle (Everaerts et al. 2010). CHCs are both species- and sex-specific and are exchanged during courtship via both gustatory and chemosensory systems (Everaerts et al. 2010).

Although male choice is less understood, males will initiate courtship via an elaborate courtship dance (Greenspan and Ferveur 2000). During courtship, males and females exchange acoustic, tactile, chemosensory, visual, and gustatory signals (Sokolowski 2001, 2010; Dickson 2008). During the courtship dance, males produce a courtship song (Greenspan and Ferveur 2000), which includes both a pulse song (a high frequency song) and a sine song (a low frequency, rhythmic song) (Dickson 2008). The courtship song is an important component of the courtship dance, as the interpulse interval (time between pulses within a pulse train) is important in species recognition (Dickson 2008). Previous studies found that the cacophony gene is down regulated by lead exposure (Ruden et al. 2009). The cacophony gene is an ion channel gene (Chakravorty et al. 2012) that functions as a voltage-sensitive Ca^{2+} channel (Sokolowski 2001); mutants exhibit polycyclic pulse songs and higher than normal interpulse intervals (Yamamoto et al. 1997).

Very little is known regarding lead-induced changes in pre-mating or post-mating reproduction in D. melanogaster, particularly mate choice. One study (Hirsch et al. 2003) has shown that sublethal doses of lead acetate (10 μM PbAc) increased fecundity (the total number of sexually mature offspring produced by each female) and the number of pairs mating within a 20-min period (Hirsch et al. 2003). Copulation latency is shortened in females developmentally exposed to Pb (Swinton 2003). Therefore, developmental lead exposure has the potential to disrupt both pre-mating reproductive strategies and post-mating reproduction.

In this study, we used D. melanogaster as a model system to delineate the effects of developmental lead exposure on pre-mating and post-mating reproduction. The overall aim of this research was to evaluate whether developmental lead exposure disrupts normal pre-mating reproduction. In particular, to determine: 1) whether females and males preferentially select mates based on their developmental exposure; 2) whether changes in mate preference were mediated by differences in the species recognition system between control- and lead-treated males; and 3) whether there are implications of differential mate preference on different measures of fitness (as measured by fecundity, mortality, and body weight) in both the exposed generation (F0) and the first generation of unexposed offspring (F1).

Materials and Methods

Rearing

In all experiments, we used a wild type, genetically variable population of Canton-S Drosophila melanogaster obtained from Dr Bernard Possidente (Department of Biology, Skidmore College, Saratoga Springs, NY). Flies were maintained in an incubator with a 12:12 light:dark cycle at 24°C (±0.5°C) ambient temperature and humidity in control medium (Carolina Biological Instant Drosophila Medium).

Canton-S adults were placed in either control or leaded medium [prepared by substituting lead acetate (250 μM PbAc) solution for distilled water in medium] for 4 days to lay eggs (e.g., experimental subjects) before being discarded (Figure 1A). Density was controlled for by limiting the number of males and females that laid eggs in the medium to rear experimental subjects. Experimental subjects were exposed to control or PbAc medium from egg stages to age 5 days post-eclosion. All experimental subjects were virgins collected within 6 h of eclosion using light CO2 anesthesia, housed in sex-specific vials in groups up to 10 individuals until testing, and tested 6 days post-eclosion after 24 h exposure to clean medium. This depuration period allowed them to groom any excess lead off their bodies and ensure that behavioral results are not due to the presence of lead.

To rear the F1 generation, F0 adults were collected after 24-h depuration and mated in homotypic and heterotypic pairings (depending on the experiment) in control medium for 4 days (Figure 1B). F0 parents were discarded after 4 days of mating and the F1 generation was reared in control medium from egg stage to eclosion, collected within 6 h of eclosion, and reared in control medium until 6 days post-eclosion without depuration.

Experiments were sequentially replicated, unless otherwise noted.

Accumulation of lead loads in F0 and F1 generations

Methods for determining lead loads in adults were derived from Hirsch et al. (2003). In each experiment performed, as reported below, experimental subjects were collected, placed in 15-mL Falcon tubes, and frozen at −20°C. Each tube was blinded to ensure
that lead load processing would be conducted blindly without knowledge of treatment. Samples were transported and tested at Union College (Schenectady, NY) using Inductively Coupled Plasma Mass Spectrometry with nitric acid and hydrogen peroxide. Detection limits were 0.0003, 0.0004, or 0.0005 ng Pb per tube, depending upon the experiment.

In all experiments, data were normalized for the number of flies in each tube. Data on lead loads in each experiment were pooled for analysis. Differences in the accumulation of lead loads in both the F₀ and F₁ generations when treated with control medium or leaded medium were analyzed using an analysis of variance (ANOVA) with Bonferroni correction (SPSS v. 24.0). Sex and experiment were used as additional fixed factors to determine whether there was an interaction between treatment and sex or treatment and experiment. Data were not corrected for weight differences between males and females, regardless of the sexual size dimorphism in D. melanogaster (Hirsch et al. 2003; Testa et al. 2013). Instead, the sexes were analyzed separately, unless analysis indicated that there was not an interaction between treatment and sex on lead loads.

Mating preferences
We studied female and male mate preference using either 2-choice tests or no-choice tests. Mating chambers in all experiments were polystyrene plastic vials (23-mL, 75 × 23.5 mm) set up side by side. A cotton ball was pushed down the vial so that there was only a 3-mm space between the cotton ball and the bottom of the vial to stimulate mating. Females were mouth aspirated and allowed to acclimate for at least 5 min before mouth aspirating males into each vial. Pairs were observed for 60 min for copulation. All mating tests were blinded to avoid observer biases.

Two-choice mating trials to test female and male mate preference
Two-choice mating tests were run to determine either female or male mate preference for conspecific (same treatment) or heterospecific (opposite treatment) partners. In 2-choice mating tests, males or females (depending on the experiment) were painted with different colored nontoxic acrylic paint (males: white, red, or blue; females: white or red) on their dorsal thorax under CO₂ anesthesia at least 24 h prior to testing for identification [paint color did not influence mate preference (data not shown; similar studies: see Yukilevich et al. 2016 and Wu et al. 1995)].

Female 2-choice mating tests were replicated 5 times; in 1 replicate, rearing was described as above, except that adults were transferred 4 days post-eclosion and tested for mate choice 5 days post-eclosion. To test the effects of maternal exposure on F₁ female mate choice, F₀ control females and F₁ leaded females were mated with F₀ control males in control medium. F₁ offspring were reared, as described above, in control medium until 7 days post-eclosion when they were painted. All F₁ adults were tested for mate choice 8 days post-eclosion. This experiment was not replicated.

Two-choice mating trials testing male mating preference were replicated 3 times. We did not test for differences in male mate preference due to maternal exposure in the F₁ generation.

No-choice mating trials
In no-choice mating tests, single virgin male–female homotypic and heterotypic pairs were tested for copulation in mating chambers; this was replicated 4 times.

Data analyses
For each replicate in both 2-choice and no-choice mating trials, we calculated the frequency of focal females or males that copulated with conspecific or heterospecific partners. To normalize for differing sample sizes between replicates, the frequency in each replicate was converted to percent (%) mating success, calculated as the total number mated divided by the total N for pairing for that group. Data were analyzed by comparing means for percent mating success across replicates, unless otherwise specified, using Chi-square test (Prism 7).
Mechanism for mating preferences

Differences in CHC expression between control- and lead-treated adults

After the 24-h depuration period, adults were anesthetized using a carbon dioxide plate, placed individually in blinded 1.5-mL microcentrifuge tubes, immediately frozen in dry ice (freezing in dry ice does not alter CHCs [Yukilevich et al. 2016]), and stored in a −20°C freezer. Whole CHC expression in whole flies was assayed using gas chromatography with mass spectrometry at Union College using methods described in Yukilevich et al. [2016]. These methods can comprehensively identify all CHCs expressed by males and females. This experiment was replicated 3 times; males were assayed in all 3 replicates whereas females were assayed in only 1 replicate.

To test for statistical differences in CHC profiles between control-treated and lead-treated individuals, total CHC variation across individuals were analyzed using a principle component analysis (PCA, JUMP v.4.0 software) and ANOVA t-tests (to test for differences between treatments for each sex).

Differences in courtship song between control- and lead-treated males

Methods to test for differences in courtship song between treatments were derived from Arthur et al. (2013). Adults were mouth aspirated into courtship chambers in single homotypic pairings. Male courtship songs were recorded using a 32-channel song recording apparatus and MATLAB (Mathworks, Inc.) automated analysis. Courtship songs were recorded 2–4 h post lights on (Zeitgeber time) at ambient temperature and humidity for 1 h. Any files with less than 10 s of song were eliminated from analysis. To correct for noise, pulse and sine calls from noise before song statistical analysis. This experiment was not replicated.

MATLAB was used to calculate the following variables for each male: mean interpulse interval (time between pulses within a pulse train with a threshold of 100 milliseconds [ms]), median bout duration (duration of each bout), median pulse frequency (wavelet pulse frequency), median pulse number (the number of pulses per bout of singing), median sine duration (duration of each sine song in ms for each singing bout), median sine frequency (frequency of each sine bout), pulse start rate (ratio of bouts started with pulses instead of sine song), and pulse to sine transition ratio (number of transitions from pulse to sine as ratio over the total). For each variable, control- and lead-treated males were compared using ANOVA analyses in SPSS (v. 24.0).

Effect of mating preference on body weight in F₀ and F₁ generations

To test for body weight differences between control- and lead-treated adults, F₀ flies were reared in control or lead medium, as described above, except that experimental subjects were maintained in groups up to 20 post-eclosion. Flies were anesthetized 6 days post-eclosion and placed in microcentrifuge tubes with 10% EtOH for preservation. Additionally, F₀ adults were mated in homotypic pairings in control medium for 4 days to rear the F₁ generation experimental subjects. F₁ adults were maintained in control medium from egg stage to adult day 6 post-eclosion, collected, and placed in 10% EtOH to be tested for differences in body weight due to parental exposure.

F₀ and F₁ adults were transferred to empty microcentrifuge tubes after being patted dry (to remove the EtOH) and dried overnight at 50°C. Adults were weighed and data were normalized for the weight of the microcentrifuge tube and the number of flies in each tube. Data were analyzed using ANOVA with Bonferroni corrections (SPSS v. 24.0).

Effect of mating preference on fecundity in F₀ and F₁

To test for differences in fecundity (the total number of adult offspring produced by each female), F₀ experimental subjects were reared as described above. Fecundity in the F₁ generation was tested separately from experiments testing fecundity in the F₀ generation and sequentially replicated 3 times. To rear F₁ experimental subjects, F₀ females were mated in groups using virgin male–female homotypic and heterotypic pairings, discarded after 4 days, and reared in control medium from egg stage to 6 days post-eclosion, as described above.

Methods for testing fecundity in F₀ and F₁ females were modified from Hirsch et al. (2003). In brief, females were mated using single virgin male–female homotypic and heterotypic pairings in polystyrene plastic vials (23-mL, 75 × 23.5 mm) with control medium. Pairs were monitored for copulation and males were discarded after 1 copulation. Females were housed individually, allowed to lay eggs in control medium for 24 h, and transferred to a new vial of control medium every 24 h for 5 days. On the fifth day, females remained in that vial for an additional 7 days before being discarded. Adult offspring were collected 15–18 days post-transfer to control medium, placed in empty 23-mL plastic vials, and frozen in individual vials.

The offspring of F₀ females were counted using an automated object counting software, SpotAFly, using MATLAB (Mathworks, Inc.) (see Supplementary Materials). Images of the offspring were post-eclosion. To rear the F₁ experimental adults, F₀ adults were mated in homotypic pairings in control medium, F₁ offspring were reared in control medium as described above, and monitored for mortality beginning 6 days post-eclosion.

To test for mortality in the F₀ and F₁ generations, mature control- and lead-treated adults were maintained in control medium in groups of up to 10 individuals during testing. Every 5 days, the number of adults who were deceased was counted and the remaining adults were transferred to new vials of control medium. Mortality was monitored until most adults in both treatment groups were deceased. Percent mortality was calculated by dividing the number of deceased flies by the initial population size in the vial. The age of onset of 50% and 80% mortality in each vial was calculated. Differences between control- and lead-treated F₀ and F₁ adults were analyzed using ANOVA analyses in SPSS (v. 24.0); sexes were analyzed separately.
photographed using a Nikon Coolpix waterproof 10 m/33ft Shockproof 1.5 m/5ft Full HD (with Nikon 5× wide optical zoom 5.0–25.0 mm 1:3.9–4.8 ED VR) camera on 8.5 × 11” pieces of paper under overhead fluorescent lighting. The SpotAFly program uses an object counting algorithm to count the number of flies in the image and generate a binary output image file, as well as an excel file with the data. Thresholds were set to either (depending on lighting and the size of the flies in the sets of images): 1) 0.85, 8, 1,000; 2) 0.85, 15, 1,000; or 3) 0.75, 50, 1,000. All “counted” binary filtered images (the “counted” images) were manually checked for accuracy and adjusted if needed so that percent error was 0.

The offspring of F₁ females were manually counted using a handheld tally counter twice by 2 independent parties to account for count biases and averaged; offspring were manually counted here because this experiment was run prior to the development of SpotAFly.

Fecundity (in both the F₀ and F₁ generations) was analyzed for overall differences in treatment groups in each generation using ANOVA (SPSS v. 11.5 or 24.0) with Bonferroni corrections (F₁ generation only).

Results

Accumulation of lead loads in F₀ and F₁ generations

We tested for lead accumulation by collecting samples of F₀ and F₁ adults in each experiment and testing for lead loads (ng/adult). When all samples from each experiment were pooled, lead loads in the developmentally exposed F₀ generation were significantly higher than controls (F₀ = 407.602, df₀ = 1, P = 0.0005, ANOVA), indicating lead accumulation in lead-reared adults (Figure 2A). We found a significant interaction between sex and treatment (F₀ = 81.758, df₀ = 1, P < 0.001, ANOVA) in the F₀ generation, likely due to higher accumulation of lead in leaded females (mean 18.44 ng/female ± 1.21 standard error of mean (SEM)) in comparison to leaded males (mean 7.32 ng/male ± 0.32 SEM). Regardless, lead loads were significantly higher in males (F₀ = 514.435, df₀ = 1, P < 0.001, ANOVA) and females (F₀ = 204.471, df₀ = 1, P < 0.001, ANOVA) treated in leaded medium than control adults (males: 0.021 ± 0.005 SEM, females: mean 0.02 ± 0.003 SEM).

To determine whether there was a difference in lead loads between experiments, data were labeled by experiment and this was used as an additional fixed factor in statistical analyses. We found an interaction between treatment and experiment (F₀ = 6.980, df₀ = 7, P < 0.001, ANOVA) in the F₀ generation. This may be due to variation in lead loads in females (Figure 2C) and males (Figure 2D).

Lead loads in unexposed F₁ generation with lead-treated parents (either lead-treated mothers, lead-treated fathers, or both parents exposed) were near background and not significantly different from F₁ adults with control-treated parents (F₁ = 1.934, df₁ = 3, P = 0.131, ANOVA; Figure 1B).

Mating preferences

Females 2-choice mating trials

We examined female mate preference for either control or leaded males when females were either developmentally exposed (F₀ generation) or their mothers were developmentally exposed (F₁ generation) to lead. There was no significant difference in the number of pairs that mated versus the number of pairs that did not mate, in either the F₀ generation (χ² = 0.3679, df₁ = 1, P = 0.5441, Chi-square test; data not shown) or the F₁ generation (χ² = 0.04268, df₁ = 1, P = 0.8363, Chi-square test; data not shown). Therefore, pairs that did not mate were omitted from further analyses.

First, female preferences for either control or leaded males in 2-choice tests were examined. When all 5 replicates were combined, we found non-random mating with females preferentially mating with conspecific males over heterospecific males (χ² = 11.95, df₁ = 1, P = 0.0005, Chi-square test; Figure 3A). Control females mated with control males approximately 60.44% (mean ± 6.6% SEM) of the time, while they mated with leaded males less frequently (mean 39.62% ± 6.68% SEM). In addition, leaded females mated more frequently with leaded males (mean 64.49% ± 2.97% SEM) over control males (mean 35.51% ± 2.97% SEM), across all 5 replicates.

When control females were analyzed separately for deviation from random mating (i.e., 50:50), there was no significant deviation in control female mate preference from random mating (χ² = 2.02, df₁ = 1, P = 0.1532, Chi-square test; data not shown). However, we found that leaded females significantly deviated from random mating (50:50) when analyzed separately (χ² = 4.604, df₁ = 1, P = 0.0319, Chi-square test; data not shown).

F₁ females did not indicate a significant preference for either F₁ males with control mothers or F₁ males with leaded mothers (χ² = 1.035, df₁ = 1, P = 0.3090, Chi-square test; data not shown).

Males 2-choice mating trials

We tested male mate preference for either control or leaded females in the F₀ generation. There was no difference in the number of males that mated or did not mate (χ² = 0.2785, df₁ = 1, P = 0.5977, Chi-square test; data not shown). In subsequent analyses, pairs that did not mate were omitted. When replicates were combined, we found random mating in males (χ² = 0.6099, df₁ = 1, P = 0.4348, Chi-square test; Figure 3B); in other words, males did not significantly prefer conspecific females over heterospecific females.

No-choice mating trials

Mate preference was tested in no-choice mating trials when singly paired in either homotypic or heterotypic single pairings. There was no significant difference in mean % mating success between homotypic and heterotypic pairs when replicates were combined (χ² = 3.643, df₁ = 3, P = 0.3027, Chi-square test, data for pairs that did not mate were included in analyses; Figure 3C).

Mechanism for mating preferences

Differences in CHC expression profiles between control- and lead-treated adults

We examined whether there were differences in CHC profiles between control- and lead-treated males and females. Males produced up to 14 CHCs, whereas females produced between 10 and 22 CHCs. We generated an overall PCA for males and females (treatments combined, but sexes analyzed separately). The first 3 principal components (PCs) explained 95.9% of the total variation in CHCs in males (PC1 explained 84.0%, PC2 explained 9.2%, and PC3 explained 2.7% of the variation). The first 4 PCs explained 96.1% of the total variation in CHCs in females (PC1 explained 59.3%, PC2 explained 20.3%, PC3 explained 12.7%, and PC4 explained 3.9%). For the PCs that explained the most variation in CHCs, we did not find a significant difference between control- and lead-treated males (PC1: F = 0.2982, df₁ = 53, P = 0.5873; PC2: F = 0.0096, df₁ = 53, P = 0.9223; PC3: F = 2.6338, df₁ = 53, P = 0.1103; data not shown) or between control- and lead-treated females (PC1: F = 0.9511, df₁ = 18, P = 0.3424; PC2: F = 2.2072,
df = 18, P = 0.1547; PC3: F = 0.7290, df = 18, P = 0.4044; PC4: F = 3.7675, df = 18, P = 0.0681; data not shown).

Differences in courtship song between control- and lead-treated males
We tested for differences in the courtship song between control- and lead-treated males. We did not find a statistical difference between control-treated and lead-treated males for any of the variables of the courtship song tested: mean interpulse interval (F = 1.445, df = 1, P = 0.232, ANOVA; data not shown), median pulse frequency (F = 0.575, df = 1, P = 0.450, ANOVA; data not shown), median bout duration (F = 0.813, df = 1, P = 0.370, ANOVA; data not shown), median pulse number (F = 0.091, df = 1, P = 0.764, ANOVA; data not shown), median sine duration (F = 1.281, df = 1, P = 0.261, ANOVA; data not shown), median sine frequency (F = 0.182, df = 1, P = 0.671, ANOVA; data not shown), pulse start rate (F = 2.997, df = 1, P = 0.087, ANOVA; data not shown), or pulse to total ratio for transitions (F = 3.409, df = 1, P = 0.068, ANOVA; data not shown).

Effects of mating preferences on fitness
Effects of mating preference on mortality in F0 and F1
We determined the effect of mate preference on time to 50% and 80% mortality in the F0 and F1 generations. In the F0 generation, we did not find a difference between control- and lead-treated adults in time to 50% mortality (males: F = 1.351, df = 1, P = 0.255, ANOVA; females: F = 0.448, df = 1, P = 0.510, ANOVA; data not shown) or 80% mortality (males: F = 0.206, df = 1, P = 0.654, ANOVA; females: F = 0.190, df = 1, P = 0.667, ANOVA; data not shown). In addition, there was no difference in either 50% (males: F = 0.073, df = 1, P = 0.790, ANOVA; females: F = 0.003, df = 1, P = 0.955, ANOVA; data not shown) or 80% mortality (males: F = 0.265, df = 1, P = 0.614, ANOVA; females: F = 0.366, df = 1, P = 0.462, ANOVA; data not shown) between F1 adults with either control-treated or lead-treated parents.

Effects of mating preference on body weight in F0 and F1
We examined the effect of mate preference on body weight (measured as average weight [g] per fly) in the F0 and F1 generations. We did not find a significant difference in dry body weight between
control- and lead-treated adults ($F = 0.373, \text{df} = 1, P = 0.544$, ANOVA; data not shown) in the $F_0$ generation. In addition, we did not find a difference in dry body weight between $F_1$ adults with control-treated and lead-treated parents ($F = 2.295, \text{df} = 1, P = 0.142$, ANOVA; data not shown).

**Discussion**

We found that $F_0$ females developmentally exposed to lead preferentially mated with leaded males in 2-choice tests. This non-random mating, with a propensity for leaded females (but not control females) to mate with conspecific males, is called asymmetrical positive assortative mating (Jiang et al. 2013). This asymmetrical positive assortative mating phenomenon replicated multiple times. To our knowledge, this is the first evidence of positive assortative mating induced by lead exposure. It is now widely accepted that positive assortative mating is the general tendency of mate choice (van den Berg et al. 1984; Hirsch et al. 1995; Barth et al. 1997; Korol et al. 2000; Sharon et al. 2005, 2010; Koukou et al. 2006; Ringo et al. 2011; Jiang et al. 2013; Lizé et al. 2014). In *D. melanogaster*, flies will preferentially mate with males of similar exposure history due to diet (Ringo et al. 2011), bacterial load (Sharon et al. 2005, 2010; Koukou et al. 2006; Lizé et al. 2014), and light exposure (Hirsch et al. 1995; Barth et al. 1997).

Female mate preference was not mediated by changes in either the male courtship song or the CHC profile in males or females; therefore, it is unclear how females are distinguishing between control- and lead-treated males. Differences in mating success may be mediated by differences in male courtship given that lead exposure decreases copulation latency (Swinton 2003) and increases the number of pairs mating within a 20-min period (Hirsch et al. 2003) at lower doses than tested in this study. Therefore, asymmetrical positive assortative mating may be mediated by differences in courtship behaviors in leaded males.

Positive assortative mating can be 1-sided (i.e., mediated by 1 sex) or dual-sided (i.e., both males and females preferentially mate) (Jiang et al. 2013). This phenomenon was 1-sided: males exhibited random mating when replicates were combined. This may indicate that females are primarily responsible for preferential, non-random mate choice, as suggested by others (Merrill 1949; Dickson 2008).

$F_1$ females with control mothers or leaded mothers randomly mated when presented with both $F_1$ males with control or lead-treated mothers in 2-choice tests. $F_1$ females with lead-treated parents exhibited lead loads that were comparable to $F_1$ females with control-treated parents. Therefore, developmental exposure to lead may be necessary for mediating the asymmetrical positive assortative mating found in the $F_0$ generation.

We found random mating in no-choice tests: females did not exhibit a preference for either control or leaded males in no-choice mating tests. In these experiments, a single female was paired with a single male and given an entire hour (60 mins) to make a choice. Lead-treatment may be altering female choice in no-choice tests, but measuring mating success in a 60-min period in a no-choice test may be masking these effects on mate choice. This is because: 1) females may opt to mate with males in no-choice scenarios, rather than forgoing reproduction altogether; or 2) no-choice tests may be more indicative of a forced mating scenario, since females are unable to escape the male’s advances. This may indicate that female choice is situation-dependent in this context and that females are soliciting several cues from their environment to maximize reproductive success.
It is possible that developmental plasticity and early experience are responsible for female mate preference in this context. Several authors (Burger and Gochfeld 1993; Hirsch et al. 1995; Barth et al. 1997; Dukas 2005) have suggested that imprinting or early experience may be responsible for incidents of positive assortative mating in Drosophila and other animals. If females rely on experience, they may improve their reproductive success (Dukas 2005). However, there is an additional hypothesis for positive assortative mating in this context.

Previous studies have shown positive assortative mating due to similar food substrates (Hurtado et al. 2012; Lizé et al. 2014), mediated by differences in bacterial composition of the medium (Sharon et al. 2005, 2010; Ringo et al. 2011). In addition, perinatal lead exposure in mice modifies gut microbiota (Wu et al. 2016). Given that Pb changes the microbial community in contaminated soil and the digestive tract (Wu et al. 2016), it is possible that the lead acetate in the medium changes the microbial community on the medium that feeds the Drosophila. Drosophila melanogaster primarily feeds during the larval stages and consume more solid food to maximize growth (Shanbhag and Tripathi 2009; Lemaire and Miguel-Alia 2013). Larvae exhibit higher lead loads compared with adults (unpublished data), which are sequestered to the digestive system (Wilson 2004), possibly for elimination. Therefore, lead exposure may modify gut microbiota, which in turn mediates the asymmetrical positive assortative mating in this context.

In conclusion, this is the first evidence that female D. melanogaster preferentially select mates based on lead exposure. Our findings indicate that sublethal exposure during development modifies female mating preferences during adulthood; however, we did not find that females engaging in asymmetrical positive assortative mating incurred fitness costs. Given that these results were tested using 1 dosage and that females were placed on control medium during mortality and fecundity tests, females may incur fitness costs if lead exposure is continual post-development or if exposed to higher doses. Given the ubiquitous nature of lead pollution and that lead can persist in the environment (Demayo et al. 1982; Caplun et al. 1984; De Vleeschouwer et al. 2007; White et al. 2007), this suggests that other species may be potentially at risk for both lead-induced changes in reproduction. In addition to potential multi-generational and long-term population implications of differential mate preference, if mate choice preferences for males similarly exposed become fixed in a population, this non-random mating could impose pre-mating isolation (Jiang et al. 2013). Drosophila could not only be used as a model system to evaluate lead-induced changes in reproduction, but also in a complementary fashion, to better understand pre-mating reproductive isolation.

Acknowledgments
We would like to first thank the Executive Editor of Current Zoology, Zhiyun Jia, and the guest editor of this special edition, Dr John Swaddle (College of William & Mary), for co-organizing this special edition in Behavioral Ecotoxicology. We are grateful to Dr Daniel Gertner (Kingsborough Community College), Dr Helen Ghiradella (SUNY-Albany), Dr David Lawrence (Wadsworth Institute, SUNY-Albany), and Dr Robert Osuna (SUNY-Albany) for their feedback on this project. We would like to thank the following people for their various assistance: Dr Pauline Carrico (SUNY-Albany), Kara DeSantis (SUNY-Albany), Taylor Harvey (Union College), Dr Kari Midthun, Andrew Powers (SUNY-Albany), Austin Stark (SUNY-Albany), Stephanie Topp, and Mark Waterhouse (SUNY-Albany). In addition, we would like to extend our gratitude to: 1) Dr Mala Murthy (Princeton University) for allowing us to use her laboratory to test courtship song, 2) Thomas Graziano (Bechtel) for the development of the SpotAFly program, and 3) Dr Gregory Lnenicka for his continued feedback on this project, in addition to reviewing this manuscript and providing invaluable feedback. Last, but not least, thank you to our reviewers for providing such helpful feedback.

Funding
This work was funded by: 1) a National Institute for Environmental Health Science (NIEHS) Grant (RO1 ES012933), a WSU-NIEHS Center Grant (P30 ES020957), 2) an Income Fund Reimbursable Account sponsored by Dr H.V.B.H. and Dr Helen Ghiradella, 3) the University at Albany Benevolent Association Research Grant, and 4) the University at Albany Graduate Student Association Research Grant. The first author thanks Dr H.V.B.H. and Dr Helen Ghiradella for summer financial support. Travel support to present this work at conferences was provided by the University at Albany Graduate Student Association Professional Development Grant and the National Institute for Environmental Health Science (NIEHS) Grant (RO1 ES012933).

Supplementary Material
Supplementary material can be found at https://academic.oup.com/ct.

References
Arthur BJ, Sunayama-Morita T, Coen P, Murthy M, Stern DL, 2013. Multi-channel acoustic recording and automated analysis of Drosophila courtship songs. BMC Biol 11:11.
Barth M, Hirsch HVB, Heisenberg M, 1997. Rearing in different light regimes affects courtship behavior in Drosophila melanogaster. Anim Behav 53:25–38.
Bechy A, 1991. Toxic metal uptake and essential metal regulation in terrestrial invertebrates: a review. In: Newman MC, McIntosh AW, editors. Metal Ecotoxicology: Concepts & Applications. Chelsea: Lewis Publishers, 65–90.
van den Berg MJ, Thomas G, Hendriks H, van Delden W, 1984. A reexamination of the negative assortative mating phenomenon and its underlying mechanism in Drosophila melanogaster. Behav Genet 14:45–61.
Burger J, 1990. Behavioral effects of early postnatal lead exposure in herring gull Larus argentatus chicks. Pharmacol Biochem Behav 35:7–13.
Burger J, Gochfeld M, 1985. Early postnatal lead exposure: behavioral effects in common tern chicks Sterna hirundo. J Toxicol Environ Health 16:869–886.
Burger J, Gochfeld M, 1988. Lead and behavioral development: effects of varying dosage and schedule on survival and performance of young common terns Sterna hirundo. J Toxicol Environ Health 24:173–182.
Burger J, Gochfeld M, 1993. Lead and behavioral development in young herring gulls: effects of timing of exposure on individual recognition. Fundam Appl Toxicol 21:187–195.
Burger J, Gochfeld M, 2005. Effects of lead on learning in herring gulls: an avian wildlife model for neurobehavioral deficits. NeuroToxicology 26:615–624.
Burke MK, Rose MR, 2009. Experimental evolution with Drosophila. Am J Physiol Regul Integr Comp Physiol 296:R1847–R1854.
Caplun E, Pettit D, Picciotto E, 1984. Lead in petrol. Endeavour 8:135–144.
Chadwick EA, Simpson VR, Nicholls AEL, Slater FM, 2011. Lead levels in Eurasian otters decline with time and reveal interactions between sources, prevailing weather, and stream chemistry. Environ Sci Technol 45:1911–1916.
Chakravorty S, Wajda MP, Vigoreaux JO, 2012. Courtship song analysis of Drosophila muscle mutants. Methods 56:87–94.
Cloftelder ED, Bell AM, Levering KR, 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. Anim Behav 68:665–676.
