Video Article

Experimental Protocol for Using *Drosophila* As an Invertebrate Model System for Toxicity Testing in the Laboratory

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Abstract

Emergent properties and external factors (population-level and ecosystem-level interactions, in particular) play important roles in mediating ecologically-important endpoints, though they are rarely considered in toxicological studies. *D. melanogaster* is emerging as a toxicology model for the behavioral, neurological, and genetic impacts of toxicants, to name a few. More importantly, species in the genus *Drosophila* can be utilized as a model system for an integrative framework approach to incorporate emergent properties and answer ecologically-relevant questions in toxicology research. The aim of this paper is to provide a protocol for exposing species in the genus *Drosophila* to pollutants to be used as a model system for a range of phenotypic outputs and ecologically-relevant questions. More specifically, this protocol can be used to 1) link multiple biological levels of organization and understand the impact of toxicants on both individual- and population-level fitness; 2) test the impact of toxicants at different stages of developmental exposure; 3) test multigenerational and evolutionary implications of pollutants; and 4) test multiple contaminants and stressors simultaneously.

Video Link

The video component of this article can be found at https://www.jove.com/video/57450/

Introduction

Every year, approximately 1,000 new chemicals are introduced by the chemical industry[1,2]; however, the environmental impacts of only a small percentage of these chemicals are tested before distribution[2,3]. Although large-scale catastrophes are uncommon, sublethal and chronic exposure to a large variety of pollutants are widespread in both humans and wildlife[4,5]. The historical focus of ecotoxicology and environmental toxicology was to test lethality, single chemical exposure, acute exposure, and the physiological effects of exposure, as a means of measuring the impact of pollutants on survival[6,7,8,9,10]. Although there is a shift towards ethical and non-invasive approaches to animal testing, current approaches are limiting because of the role that development, emergent properties, and external factors (such as population-level and ecosystem-level interactions) play in mediating ecologically-important endpoints[8]. Therefore, there is a need for methods that incorporate a more holistic approach without sacrificing wildlife and/or vertebrates in the laboratory.

Invertebrate model systems, such as *Drosophila melanogaster*, are an attractive alternative to address the need for a more holistic approach to toxicity testing. *D. melanogaster*, was originally developed as an invertebrate model system for human-related genetic research about a century ago[11].*D. melanogaster* is now prominently used as a vertebrate model alternative for several reasons: 1) the conservation of genes and pathways between *D. melanogaster* and humans; 2) short generation time compared to vertebrate models; 3) inexpensive cost of maintenance; 4) ease in generating large sample sizes; and 5) plethora of phenotypic- and ecologically-relevant endpoints available for testing[11,12,13,14,15,16,17].

Several laboratories[11,15,16,17,18,19,20,21,22,23,24,25] are now using *D. melanogaster* as a vertebrate model alternative for toxicity testing to understand the impacts of pollution on humans. Local wild species of *Drosophila* can be utilized, as well, as toxicity models for wildlife (and humans) to answer ecologically-, behaviorally-, and evolutionarily-relevant questions at multiple biological levels of organization. Using species within the *Drosophila* genus as a model, several measurable endpoints are possible[11,15,16,18,19,20,21,22,23,24,25]. In addition, using the *Drosophila* model, toxicologists can: 1) ethically link effects at multiple biological levels of the organization; 2) incorporate the role of emergent factors and development; 3) study ecologically-important endpoints (in addition to medically-important endpoints); 4) test multiple stressors simultaneously; 5) and test long-term multigenerational (*e.g.* evolutionary and transgenerational) implications of stressors. Therefore, using *Drosophila* as a model system enables a multitude of approaches, not limited to studying mechanistic approaches with inbred strains of *D. melanogaster* in the laboratory.

In this paper, we present the methods for rearing and collecting *Drosophila* to answer various toxicological questions. More specifically, we describe the methodology for 1) rearing *Drosophila* in medium laced with one or more pollutants; 2) collecting *Drosophila* throughout development (*e.g.* wandering third-instar larvae, pupal cases, newly-eclosed adults, and mature adults); and 3) rearing *Drosophila* in the

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contaminated medium to test intergenerational and transgenerational transmission, as well as evolutionary implications of long-term toxicant exposure. Using this protocol, previous authors[18,19,20,21,22,23,24,25] have reported different physiological, genetic, and behavioral effects of developmental lead (Pb^{2+}) exposure. This protocol enables toxicologists to use a more holistic toxicological approach, which is essential to understanding how pollutants are risk factors for both humans and wildlife in an ever increasingly polluted environment.

Protocol

The following protocol is an experimental protocol used to rear species in the *Drosophila* genus on contaminated medium when oral ingestion of a toxin is appropriate; other forms of exposure are possible using the *Drosophila* model[11,15,16,26]. The methods described in this protocol have been previously described by Hirsch *et al.*[19] and Peterson *et al.*[23,24,25].

1. Set Up Stock Populations of *Drosophila* in the Research Laboratory

1. Set up an environmentally-controlled incubator (or small room) to house stock populations of *Drosophila* by setting the incubators for a constant temperature, light:dark cycle, and humidity, depending upon the preferences of the test species.
   NOTE: Preferred environmental conditions will vary depending upon the native ecology of the species chosen for the study. For example, *D. melanogaster* is native to sub-Saharan Africa[27] and is typically maintained at 25 °C, 12:12 light:dark cycle, and approximately 60% humidity[16,18,19,20,21,22,23,24,25,28,29,30]. On the other hand, *D. montana* range extends throughout most of Canada and the midwest USA, a much colder region; therefore, *D. montana* is typically maintained at 19–20 °C and sometimes a 24 h light regime to simulate conditions during the mating season[31]. For a more detailed description of the geographic ranges of various species of *Drosophila*, see the *Drosophila* Speciation Patterns website[32].
2. **Obtain a preferred *Drosophila* species and/or inbred line from either a stock center (see Table of Materials), another research laboratory upon request, or collect wild, genetically variable populations from the field.**
   NOTE: The following steps explain the methods to collect wild, genetically variable populations of *Drosophila* to maintain in the research laboratory. These methods have been modified from Markow and O'Grady[33] and Werner and Jaenike[34] to collect the widest diversity of species at once, rather than target particular species with one bait source.
   1. Freeze half a dozen ripe bananas in the freezer overnight and defrost before setting bait traps.
   2. Prepare multiple 1–2 L plastic bottles by cutting a u-shaped slit in the front of the bottle to allow flies to be captured in the bait bottle and not escape. Cap the plastic bottles with their bottle caps so the flies do not escape via the lids.
   3. Add the defrosted banana to the bottom of the bottles so that the bottom of the bottles contains approximately one-inch of banana. Place a slice of ripe tomato in the bottle. Add a yeast slurry (the leftover yeast from the beer making process) to the banana at the bottom of the bottle so that the banana gets to soak in the yeast slurry.
   4. Add wooden sticks (in an upright vertical position) to the bottle so the flies have a clean substrate to walk on away from the yeast slurry and banana.
      Figure 1 illustrates the final product of these methods.
   5. Hang bait bottles in trees overnight and check every 24 h. Mouth aspirate flies out of bottles and individually place females in vials with medium to create iso-female lines.
      NOTE: Multi-female lines can be created, however, only if the females of each species can be clearly identified. In addition, flies within the genus *Drosophila* occupy different ecological niches and will have different dietary requirements depending upon those niches (Werner and Jaenike[34]); see Werner and Jaenike[34] for diet recommendations and food recipes.
   6. Examine the adult F_1 offspring under the dissection microscope to identify the species of the collected *Drosophila* (see Markow and O'Grady[33] and Werner and Jaenike[34] for assistance in identifying various species).

Figure 1: Pictorial representation of traps and bait used to collect wild populations of *Drosophila* in the field. (A) Fly traps set at a local field site in Colorado. **(B)** A closer view of the fly traps set at this field site. Please click here to view a larger version of this figure.

3. Maintain the iso-female or the multi-female lines in an environmentally-controlled incubator or room with constant temperature, light:dark cycle, and humidity. To do this, house flies in vials or bottles in preferred medium and allow the gravid females to lay eggs in the medium. Monitor the vials for the presence of larvae and pupae.
   NOTE: Flies within the genus *Drosophila* occupy different ecological niches and will have different dietary requirements and environmental abiotic preferences depending upon those niches[33,34]. Environmental preferences and dietary recommendations (and further instruction on fly husbandry) can be found in Elgin and Miller[28], Shaffer *et al.*[29], Stocker and Gallant[30], Markow and O'Grady[33], and Werner and Jaenike[34]. If using wild-caught species, local environmental conditions can be simulated in the incubators until the species can be identified.
4. Transfer stocks frequently to fresh medium, discarding old vials, to maintain healthy lines and avoid infection from mites.
   NOTE: The frequency of transfer will depend on the life cycle of the species. For example, transfer *Drosophila melanogaster* every 2 weeks to fresh medium.*For further information on maintaining lines in the laboratory,* see Rand *et al.*[16], Elgin and Miller[28], Shaffer *et al.*[29], Stocker and Gallant[30], Greenspan[35], and Science Education Database[36].

2. Rear *Drosophila* in the Contaminated Medium

NOTE: If testing *Drosophila* in the laboratory for the first time or with a new contaminant(s), identify the lethal dose (see Castaneda *et al.*[37] and Massie *et al.*[38] for methods) and the LD50 (see Castaneda *et al.*[37] and Akins *et al.*[39] for methods) first. Then, run a dose-response curve to identify biologically-relevant concentrations for the desired phenotypic output; see Hirsch *et al.*[19] and Zhou *et al.*[40] for methods.

1. Prepare stock solutions of the contaminated medium at the desired concentration(s), depending upon the chemistry of the contaminant.
   NOTE: For example, to prepare stock solutions of PbAc: Prepare stock solutions of lead acetate (PbAc) medium by adding contaminant to distilled water (dH_20) until medium made with contaminant water reaches desired concentration. For example, a stock solution of 1,000 µM PbAc, can be prepared by adding PbAc to dH_20 until it reaches 1,000 µM PbAc. Further, dilute the stock solution (*e.g.* the 1,000 µM PbAc solution) to the desired concentration (such as 500 µM PbAc) and maintain these solutions as stock as well.
2. Prepare medium, following manufacturer's guidelines to serve as the control medium. Prepare additional medium, following manufacturer's guidelines; however, supplement prepared contaminant solution for dH_20.
   NOTE: For example, if using an instant *Drosophila* medium, add approximately one teaspoon instant medium to a plastic vial. Add approximately 5–5.5 mL dH_20 to the medium. Sprinkle a few grains of live baker's yeast to prepare control medium. To prepare experimental medium, supplement the stock solution (such as 500 µM PbAc) for dH_20.
3. **Transfer reproductively viable mature males and females from stock populations into the control and the experimental medium.**
   NOTE: The time post-eclosion to reproductive maturity is different between the *Drosophila* species[41].
   1. Gently tap the vial of stock flies down with the dominant hand. Ensure that the flies automatically move to the bottom of the vial. With the other hand, remove the cap of the vial while tapping the vial and place a fresh vial of control or contaminated medium on top of the vial with the flies. Hold the vials together and flip them over, gently tapping, so that the flies automatically are transferred to the fresh vial of control or contaminated medium. While still tapping the vial with the flies, cap the vial.
   2. Repeat with more vials, making sure to standardize the number of flies in each vial.
      NOTE: The total number of adults transferred *via* single transfer or anesthesia will depend on the size of the vials used to avoid overcrowding.
   3. Incubate adults in a standard environmental condition (*i.e.* an incubator) and allow the adults to mate and lay eggs in the medium for 24–96 h.
   4. After 24–96 h, discard adults in a morgue (a flask filled with mineral oil and capped with a tight-fitting funnel) leaving behind fertilized eggs (which will later become the experimental subjects) to mature for testing. Place the vials in the incubator to allow the eggs to develop.

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5. Monitor the vials for wandering-instar larvae by looking for larvae that are emerging from the medium.

3. Collect Experimental Subjects at Various Developmental Stages

NOTE: Experimental subjects can be collected at any developmental stage, placed in the blind coded 15-mL conical tubes, and tested for accumulation. Methods for testing the accumulation of contaminants will depend on the contaminant being studied. For example, accumulation of PbAc can be tested using Inductively-Coupled Plasma Mass Spectrometry (ICP-MS)[42]. In addition, experimental subjects can be collected at any developmental stage to be tested for a variety of phenotypic effects of contaminants. **Figure 2** illustrates the *Drosophila* life cycle[43]. **Figure 3** illustrates the experimental protocol for exposure and the different developmental stages for collection.

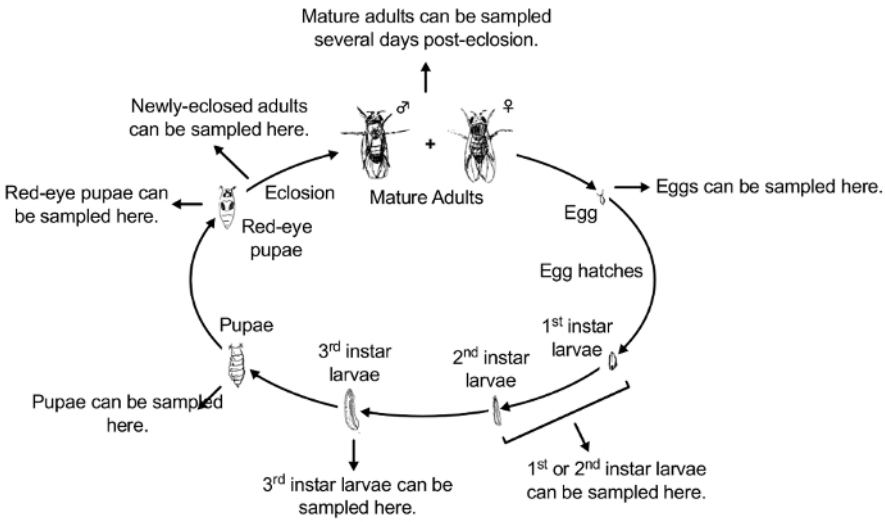

Figure 2: Conceptual overview of the life cycle of *D. melanogaster* (the most commonly used *Drosophila* model system). The stages of *Drosophila* life cycle are: 1) egg, 2) first-instar larva, 3) second-instar larva, 4) third-instar larva, 5) wandering third-instar larva, 6) white-eye pupa, 7) red-eye pupa, 8) newly-eclosed adult, and 9) mature adult. Please click here to view a larger version of this figure.

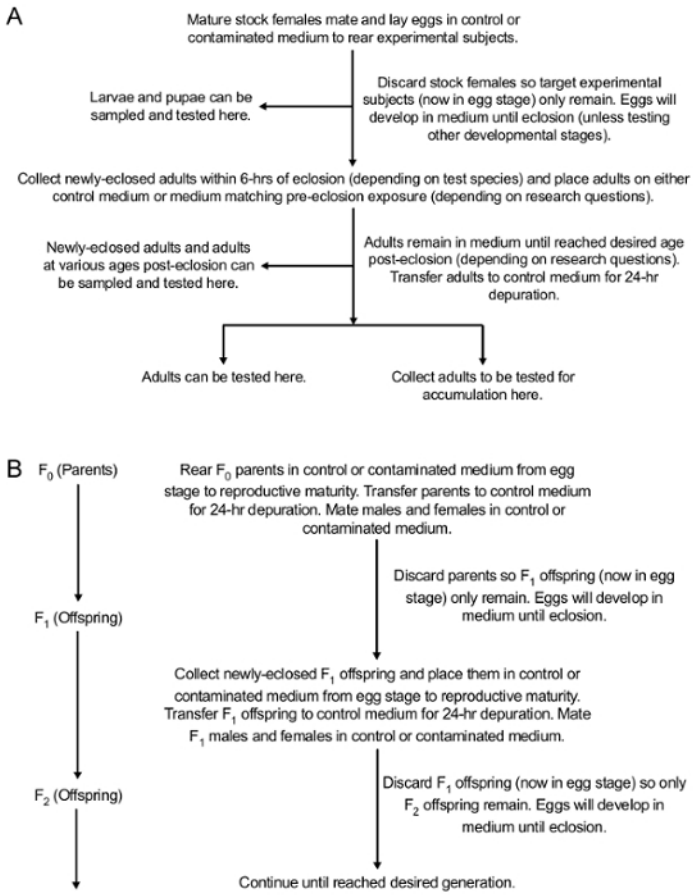

Figure 3: Conceptual overview of the methods for orally exposing *Drosophila* to contaminated medium in both the parental (F_0) and subsequent generations (F_1 and onward). (A) Methods for oral exposure during development in the exposed generation. **(B)** Methods to test the transfer of contaminants to offspring (F_1 to the desired generation). This figure has been modified from Peterson *et al.*[24] Please click here to view a larger version of this figure.

1. **Collect wandering-third instar larvae**
    1. Start monitoring vials when lights turn on in the incubator, as larvae will emerge from the medium and move upwards on the side of the vial within an h after lights turn on in the incubator. Within this h, remove the wandering-third instar larvae from the sides of the vial carefully using a wooden stick or tweezers.
    NOTE: The number of larvae available for collection will depend on the number of eggs laid in "2.3.4".
    2. To remove excess medium from the larvae, place the larvae in a small beaker with dH_2O. Pour the dH_2O out of the beaker and place the larvae on a delicate task wiper. Using a delicate task wiper, gently remove the excess dH_2O from the larvae.
    3. Maintain experimental populations in an environmentally-controlled incubator.

2. **Collect newly-eclosed adults**
    1. Monitor vials for eclosion by observing the coloration of the pupae along the sides of the vials.
    NOTE: Pupae will darken during development. Developmental time, particularly pre-eclosion, depends on the species tested.
    2. When the first adults begin to eclose, dump and discard these adults into a morgue containing mineral oil.
    3. When the lights turn on in the incubator the following morning, dump and discard any adults of unknown age (or virginity) that may have eclosed overnight or during the morningbefore lights on.
    4. Approximately 4 h later, anesthetize any adults that emerged as newly-eclosed adults with a CO_2 gun in the vials. Place adults on a CO_2 plate under a dissection microscope. Sex adults by looking for sex combs on the forelimbs of males and ovipositors in females.
    NOTE: *D. melanogaster* must be collected within 6 h of eclosion to avoid mating but other species may have longer developmental times (and therefore, do not need to be collected within this time frame).
    5. Separate adults on the CO_2 plate using a wooden stick. Gently transfer adults in sex-specific groups using a wooden stick to the medium matching pre-existing history.

3. **Collect mature adults post-eclosion**
    1. Allow adults to remain on the medium matching pre-eclosion exposure from egg stage to the desired age post-eclosion in an environmentally-controlled incubator.
    2. Singly transfer adults to the control medium for 24 h prior to testing to allow adults to groom excess contaminated medium off their bodies.

4. Rear Experimental Subjects to Test the Effects of Multigenerational or Transgenerational Exposure.

1. To rear the parental generation (a.k.a the P_0 or F_0 generations), transfer adults from stock populations to control and the experimental medium following the steps in "2.1" to "2.3" and "3.1" to "3.3".
2. When the adults are reproductively mature (see Pitnick *et al.*[41]), singly transfer (as stated in 2.3.1) one vial of males to a fresh vial of control or experimental medium. Singly transfer one vial of females to the fresh vial that now contains males. Allow adults to mate and lay eggs in the medium for 24-96 h. Dump and discard adults into a morgue containing mineral oil and re-incubate vials to allow offspring to develop.
3. Repeat steps 4.1 through 4.2 depending on the desired number of generations.

Representative Results

By orally exposing *Drosophila* to a contaminant(s) throughout development, various toxicological questions can be tested by exposing *Drosophila* at different levels of biological organization. This section presents representative results obtained using this protocol in previously published papers[23,24]. In particular, this protocol was previously used to evaluate the accumulation, elimination and sequestration of lead (Pb) within the same generation of exposure and across the first generation of offspring[23]; and to study the implication of accumulation on mate choice[24].

Table 1 and **Figure 4** show representative results obtained using this protocol to determine the accumulation and elimination of Pb in both the F_0 and F_1 generations.

Table 1 shows representative results indicative of the accumulation of Pb when exposed within generation (at various doses: 0, 10, 40, 50, 75, 100, 250, and 500 µM PbAc) in samples tested at multiple developmental stages (wandering third-instar larvae, pupal cases, newly-eclosed adults, and mature females and males) in Peterson *et al.*[23] Samples were collected at various developmental stages, frozen at -20 °C, treated with nitric acid and hydrogen peroxide, and tested for Pb using ICP-MS[23,42].

Dose (µM PbAc)	Larva	Pupal Cases	Newly-Eclosed Adults	Mature Adult Females	Mature Adult Males
0	0.009 ± 0.001	0.099 ± 0.02	0.04 ± 0.005	0.069 ± 0.031	0.0015 ± 0.003
10	0.42 ± 0.025	0.46 ± 0.03	0.12 ± 0.009	0.12 ± 0.012	0.056 ± 0.008
40	7.5 ± 0.67				
50	5.2 ± 0.71	2.77 ± 0.30	1.11 ± 0.14		
75	14 ± 1.06				
100	12 ± 0.95	4.7 ± 0.38	3.36 ± 0.58	1.20 ± 0.63	1.24 ± 0.46
250	89 ± 8.49			25.06 ± 4.72	5.46 ± 0.75
500	271 ± 41.01	334.30 ± 39.43	8.44 ± 0.84	46.50 ± 14.72	14.43 ± 1.83

Table 1: Mean Pb loads (ng/individual) tested in *D. melanogaster* during development after oral exposure to Pb from egg stage to test stage. Means (ng/fly) ± standard error of mean shown (n = 8 larva, n = 3 control-reared adults, n = 3 Pb-reared adults). Wild type *D. melanogaster* were reared on control or leaded medium (0, 10, 40, 50, 75, 100, 250 or 500 µM PbAc) from egg stage to various stages of development. Samples were collected and tested for Pb accumulation using ICP-MS.[42] This table has been modified from Peterson *et al.*[23]

In **Figure 4**, the parental generation (F_0) was exposed to Pb from egg stages to adulthood, mated in control medium, and the first generation of offspring (F_1) were reared in control medium until adulthood[24]. Methods to detect Pb accumulation and elimination were similar to Peterson *et al.*[23]. Results from this experiment indicate that parental exposure is not transmitted to the first generation of adult offspring[24]. Therefore, using this protocol, it is possible to test adaptive responses at different evolutionary scales, as well as transgenerational effects of F_0 exposure. Similar results were found in Peterson *et al.*[23]

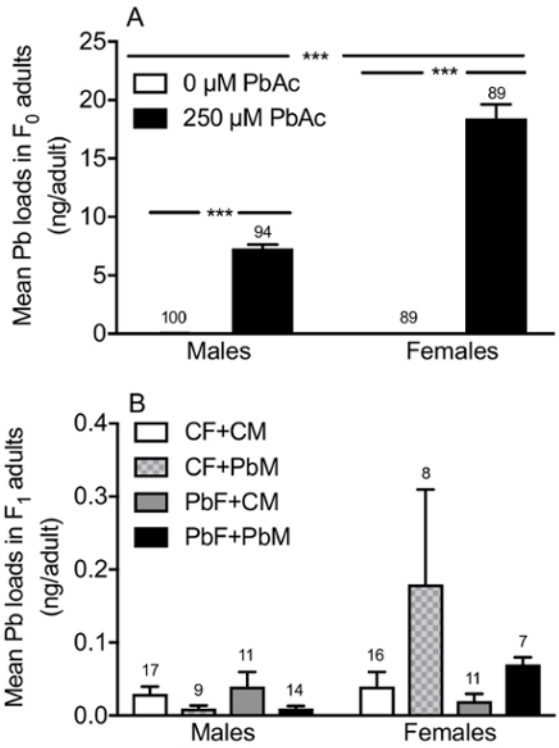

Figure 4: Pb accumulation in *D. melanogaster* (A) parents (F_0) and (B) unexposed offspring (F_1). Bars in **(A)** and **(B)** show mean (ng/adult) ±SEM. Sample sizes shown above bars in (A) and (B). *** = p <0.001. **(A)** F_0 adults were orally exposed to 250 µM PbAc using this protocol from egg stage to age 5 d post-eclosion and collected age 6 days post-eclosion (after 24 h depuration) to be tested for Pb accumulation using ICP-MS.[42] **(B)** F_0 adults were mated within treatment in control medium. Unexposed F_1 offspring were reared in control medium from egg stage to adulthood (using this protocol) and tested for Pb accumulation using ICP-MS. In (B): "CF+CM"= F_1 adults with parents reared in control medium, "CF+PbM" = F_1 adults with fathers reared in leaded medium, "PbF+CM" = F_1 adults with mothers reared in leaded medium, "PbF+PbM" = F_1 adults with parents reared in leaded medium. This figure has been modified from Peterson *et al.*[24] Please click here to view a larger version of this figure.

The results presented in **Table 1** and **Figure 4** indicate that *Drosophila* readily accumulates Pb at different doses, developmental stages, and evolutionary scales using this protocol. Therefore, this indicates the protocol's effectiveness in exposing *D. melanogaster* to an oral contaminant.

In **Figure 5**, the protocol described here was used by Peterson *et al.*[24] to test the effects of developmental Pb exposure on mate preference. Experimental subjects were reared from egg stage to adulthood on control or leaded medium from egg stage to adulthood and tested for mate preference after 24 h of depuration. Peterson *et al.*[24] found that Pb-exposed females preferentially mated with Pb-exposed males when given the option of either a control or Pb-exposed male. These results are one representative example of the implementation of the protocol to examine the phenotypic output.

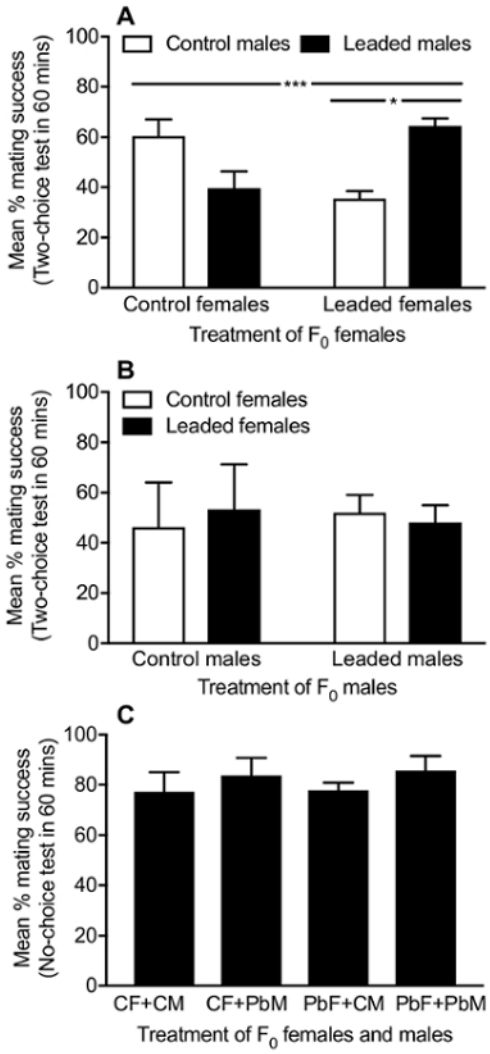

Figure 5: Mate preference in males and females exposed to 250 µM PbAc from egg stage to adulthood. Bars in (**A**), (**B**), and (**C**) show mean percent (%) mating success (in 60 mins) ± SEM. *** = p <0.001. * = p <0.05. Experimental subjects in (A), (B), and (C) were exposed to control or leaded medium (250 µM PbAc) from egg stages to mature adulthood and tested for differences in mate choice. **(A)** Female preference for either control- or Pb-reared males (i.e. two-choice test). Sample sizes were: N = 126 control-reared females and 137 Pb-reared females. **(B)** Male preference for control- and Pb-reared females (i.e. two-choice test). Samples sizes were: N = 59 control-reared males and N = 64 Pb-reared males. **(C)** Mate preference in both males and females when singly paired with one partner of either exposure (i.e. no-choice tests). In (C): "CF+CM" = one control-reared female paired with one control-reared male (N = 85 pairs), "CF+PbM" = one control-reared female paired with one Pb-reared male (N = 79 pairs), "PbF+CM" = one Pb-reared female paired with one control-reared male (N = 91 pairs), "PbF+PbM" = one Pb-reared female + one Pb-reared male (N = 98 pairs). This figure has been modified from Peterson *et al.*[24]. Please click here to view a larger version of this figure.

Discussion

Drosophila melanogaster has been established as a powerful model for a range of biological processes due to the extensive conservation of genes and pathways between *D. melanogaster* and humans[13,14]. For the same reasons that it's a powerful model for medical science, *Drosophila* has emerged as a suitable model system to study the impact of anthropogenic pollution on a range of toxicological endpoints. Several laboratories are successfully using *D. melanogaster* as a model system to study a range of compounds, including heavy metals[11,16,18-25,37,38,39,40,44,45], ethanol[46], nanoparticles[26,47], pesticides[48], and solvents[49]. Despite recent efforts to utilize *Drosophila* as a toxicology model, its use as a model system to answer the countless toxicological questions is still in its infancy. However, given its extensive use as a model for medically-related endpoints, as well as its use in ecologically[50] and evolutionary studies[17], its potential as a toxicological model system is enormous.

Here, we present methods for rearing various species within the *Drosophila* genus on contaminated medium to test for various toxicological endpoints. Although other forms of exposure are possible using *Drosophila* as a model (*e.g.* inhalation and dermal exposure), this protocol focuses on the oral consumption of pollutants which is necessary for contaminants that would naturally be ingested (such as through the food chain). These methods can accommodate the use of multiple *Drosophila* species and contaminants. Wild, genetically variable populations of *Drosophila* can also be collected in the field and maintained in the research laboratory. There are many options of traps and bait that can be

used, depending upon the species food preferences; for field guides on field collection, see Markow and O'Grady[33] and Werner and Jaenike[34]. In addition, the methods could be altered to determine the impact of developmental exposure at various critical developmental periods and allows for long-term multigenerational testing of contaminant exposure.

The critical steps of these methods include: (1) maintaining fly stocks in environmentally-controlled conditions, (2) avoiding overcrowding of fly populations, (3) diluting the test contaminant according to its chemical properties, and (4) choosing biologically-relevant concentrations of the test contaminant. Maintaining stocks in environmentally-regulated incubators (or a small room) ensures that additional variations in environmental conditions do not confound results. In addition, seasonal variations in behavior have been previously found[51] and several *Drosophila* species enter diapause over the winter[52]. Second, larval overcrowding can have long-lasting implications for development[30], adult body size[30], and longevity[53]. In addition, dilution of the contaminant is an essential step to ensure that the contaminant is biologically available for *Drosophila* to accumulate the contaminant. For example, PbAc is dissolved in dH_2O[23,24,25], whereas other chemicals may need to be dissolved in saline water or ethanol. Choosing biologically-relevant concentrations of the contaminant can affect the direction of the results; for example, low doses of PbAc increase the mean number females mating with males (within 20 mins), whereas higher doses show significant decreases in the mean number of females mating[19]. To identify biologically-relevant concentrations of the test contaminant, readers should consider running preliminary studies to determine the lethal dose and LD50 to determine the appropriate doses to perform a dose-response curve. By performing a dose-response curve to test a range of concentrations on a particular endpoint, readers could pinpoint doses that are either "beneficial" or "hazardous" to individuals or populations for further testing.

This protocol provides an avenue to determine: 1) the interplay of multiple biological levels of organization on fitness and toxicological endpoints; 2) the role of developmental and emergent factors; 3) ecologically-important endpoints; 4) medically-important endpoints; 5) how multiple stressors interact to produce outcomes; and 6) the impact of long-term exposure that transcends generations. To illustrate the effectiveness of this protocol, evidence was provided indicating that individuals exposed throughout development accumulate Pb (**Table 1**)[23,24]. In addition, representative results show that this protocol can be used to test the implications of exposure on ecologically-important endpoints (*e.g.*, the impact of developmental Pb exposure on mate choice[24]). In addition, others have tested the effects of contaminants on multiple biological levels of organization (including physiological[18,21], genetic[20,22] and phenotypic-levels[19,23,24,25]), medically-important endpoints[18,20,21,22,23], and long-term multigenerational effects[23,24,25,54]. In addition, preliminary data indicate that developmental Pb exposure induces transgenerational epigenetic effects on fecundity in *D. melanogaster*[54]. An important limitation of this protocol is that the use of this protocol with *Drosophila* is in its infancy. Therefore, there are limited publications[18,19,20,21,22,23,24,25] to address the potential of the protocol to answer additional toxicological questions, such as the role of development and emergent factors, additional ecologically-important endpoints, multiple stressors, and evolutionary implications of exposure.

Using this protocol, readers can test contaminants that are naturally ingested using biologically-relevant methods. Continuous liquid feeding, developed by Soares *et al.*[55] is an alternative approach for oral ingestion, particularly for pesticide exposure. However, continuous liquid feeding is appropriate for adult ingestion of liquid contaminants and not applicable to contaminants where individuals may be exposed pre-eclosion. This is especially important given the potential for critical periods in development for exposure. Previous studies have shown a critical period for Pb exposure[23]. Therefore, *Drosophila* should be exposed throughout development to avoid the potential active elimination of contaminants by *Drosophila* prior to testing until critical periods can be determined.

In summary, we have provided a protocol to orally expose *Drosophila* to contaminants. Using this protocol and model system, toxicologists can shift towards ethical and non-invasive approaches to animal testing while simultaneously incorporating a more holistic approach to understanding the impact of contaminants[8].

Disclosures

The authors have nothing to disclose.

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