



Assessment Of Chemical Toxicity in Adult *Drosophila Melanogaster*

Jessica M. Holsopple^{*,1,2}, Shannon R. Smoot^{*,1}, Ellen M. Popodi^{1,2}, John K. Colburne³, Joseph R. Shaw⁴, Brian Oliver⁵, Thom C. Kaufman¹, Jason M. Tennessen¹

¹Department of Biology, Indiana University, Bloomington, IN 47405

²Bloomington *Drosophila* Stock Center, Department of Biology, Indiana University, Bloomington, IN 47405

³School of Biosciences, University of Birmingham, Birmingham, United Kingdom, B15 2TT

⁴O'Neill School of Public and Environmental Affairs, Indiana University, Bloomington, IN, USA.

⁵Section of Developmental Genomics, Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Kidney and Digestive Diseases, National Institutes of Health, Room 3339, 50 South Drive, Bethesda MD 20892

Abstract

Human industries generate hundreds of thousands of chemicals, many of which have not been adequately studied for environmental safety or effects on human health. This deficit of chemical safety information is exacerbated by current testing methods in mammals that are expensive, labor-intensive, and time-consuming. Recently, scientists and regulators have been working to develop New Approach Methodologies (NAMs) for chemical safety testing that are cheaper, more rapid, and reduce animal suffering. One of the key NAMs to emerge are the use of invertebrate organisms as replacements for mammalian models to elucidate conserved chemical modes of action across distantly related species, including humans. To advance these efforts, here we describe a method that uses the fruit fly, *Drosophila melanogaster*, to assess chemical safety. The protocol describes a simple, rapid, and inexpensive procedure to measure the viability and feeding behavior of exposed adult flies. In addition, the protocol can be easily adapted to generate samples for genomic and metabolomic approaches. Overall, the protocol represents an important step forward in establishing *Drosophila* as a standard model for use in precision toxicology.

SUMMARY:

The protocol describes an efficient and inexpensive method that uses liquid media to assess the effects of chemical toxicants on the viability of adult *Drosophila melanogaster*.

Contacts: Jason M. Tennessen (jtenness@iu.edu).

*Equal contributions

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INTRODUCTION:

Humans are constantly exposed to chemicals from a variety of sources, including the air¹, food², water^{3,4}, medications⁵, cleaning agents⁶, personal care products⁷, industrial chemicals⁷, and building materials⁷. Moreover, thousands of new chemicals are introduced each year⁸, many of which are not properly vetted for health and environmental safety. This lack of adequate chemical safety testing stems, in part, from an over-reliance on mammalian models, such as mice and rats. While such rodent models are informative, chemical safety testing in these systems is expensive, time consuming, and often causes unacceptable levels of suffering to the test animal⁹.

The financial and ethical burdens associated with mammalian chemical safety testing, as well as the time-consuming nature of mammalian studies, are major contributing factors to the paucity of data surrounding new chemicals. To address this issue, the U.S. Environmental Protection Agency (EPA), the European Chemicals Agency (ECHA), Health Canada, and other agencies are implementing measures that incorporate New Approach Methodologies (NAMs) into regulatory frameworks¹⁰, thus placing North American and European policy in line with international goals to replace, reduce, and refine the use of animal (the 3Rs principal)^{11–14}. NAMs encompass a variety of assays primarily based on *in vitro* and *in silico* models that provide a mechanistic understanding of chemical toxicity instead of observing adversity inflicted on mammalian test species, thereby increasing the rate of data generation for chemical risk assessment while still producing high fidelity outputs¹⁵. However, these methods are not yet proven to safeguard against systemic toxicity including the disruption of vital biological processes involving interorgan communication and endocrine signaling, nor can they account for the bioaccumulation of chemicals within specific tissues, the ability of individual compounds to be absorbed and secreted, and the interplay between behavior and chemical exposure.

Due to the limitations of *in vitro* and computational models, successful use of NAMs to reduce or replace mammalian models should also include invertebrate *in vivo* models, such as the fruit fly, *Drosophila melanogaster*. Previous studies in the fly have demonstrated that this organism is well suited for studying the conserved genetic pathways that protect animal cells against toxic molecules^{16–22}. Moreover, the fly shows remarkable genetic similarity to humans, including functional homologs to over 65% of human diseases^{23–25} and even greater conservation of important functional pathways²⁶. These features, combined with their relatively short life cycle, low maintenance cost, and readily observable behavioral responses make *Drosophila* well-suited for use as a toxicological model^{27–30}. Moreover, flies are much higher throughput than rodent models and capture effects on metabolism, physiology, and hormone signaling that are not readily detectable by other non-organismal NAMs⁹.

The protocol described here represents a framework for testing the effects of chemical exposure on adult *Drosophila*. The method is designed to be efficient, inexpensive, and reproducible, while also minimizing the time researchers must be in contact with the test chemical and accommodating sample collection for metabolomics and other omics approaches. The protocol is optimized for testing a single chemical per experiment but can

easily accommodate other experimental parameters, such as varied solvents or combinations of chemicals.

PROTOCOL:

NOTE: Wear nitrile gloves for all steps in this protocol. Wear laboratory coat, eye protection, and/or respirators per safety data sheets for each chemical being evaluated.

1. Vial and Humidity Chamber Preparation

NOTE: Steps 1.1 – 1.5 can be completed at any time prior to beginning the other experimental sections.

NOTE: Nitrile gloves must be worn at all times during vial preparation to prevent contamination.

1.1 Stack 4 sheets of grade 1 cellulose chromatography paper and cut into 2-inch-wide strips. Punch out flower-shaped filter paper inserts using a 1.5-inch paper punch containing a flower-shaped die.

1.2 Use a 22 mm × 220 mm unvarnished wooden dowel to push filter paper to the bottom of a 28.5 mm diameter polypropylene vial. Confirm that the stack of filter paper is securely located at the bottom of the vial.

1.3 Store prepared vials in plastic or cardboard trays and place trays in large (approximately 280 mm × 240 mm) plastic bags until use.

1.4 Construct a humidity chamber by cutting a 120 mm × 280 mm hole in the plastic lid of a 606.24 mm × 225.42 mm × 403.22 mm plastic tub. Glue mesh over the hole to allow airflow.

1.5 Cut a section of plastic grid from louvered ceiling light panels (originally 610 mm × 1220 mm) to fit in the bottom of plastic tub used in step 1.4.

NOTE: A variety of different plastic tubs and plastic/metal grid materials can be used build a humidity chamber.

2. Fly husbandry

2.1 Start cultures of adult flies (minimally 30 adults) in glass milk bottles containing standard Bloomington *Drosophila* Stock Center (BDSC) media³¹. Close the bottles with a rayon plug wrapped in a delicate task wipe. Do not crowd bottles.

NOTE: The number of required bottles will depend on the number of chemical exposures being conducted and the genotype of the test strain. Normally, several hundred flies can be obtained from a single bottle when using robust and fecund stocks. The standard assay uses Oregon R wild-type flies (BDSC stock #2057), but any genotype(s) of interest can be used with this protocol. Keep in mind that compromised genotypes with low fecundity and/or viability will require an increased number of bottle cultures.

2.2 Incubate trays of culture bottles at 25 °C with approximately 60% humidity and a 12h:12h light:dark cycle until the third larval instar or early pupal stages are observed. This stage is identifiable by the presence of larvae wandering up the sides of the bottle and the appearance of pupae on bottle walls.

NOTE: Only handle flies during the lights on period of the 12h:12h light:dark cycle.

NOTE: For robust stocks, bottles will reach this stage after 3–4 days under the described conditions.

2.2.1 Remove adult flies and determine the liquidity of the media. If the media flows along the side of the bottle when inverted, the medium is too liquid. Insert a delicate task wipe or a rayon ball into the bottom of the bottle to solidify the fly food.

2.3 When flies begin to eclose, clear all adults from the bottles and allow pupae to continue to eclose for 48 h. At this point, any adults cleared from the culture bottles can be either transferred to new bottles to propagate the stocks (see step 2.1) or discarded.

2.4 Transfer flies that eclose in the 48 h period to new bottles containing standard BDSC media. Age for 3 days.

NOTE: The adults in these bottles will be 3–5 days old and can be used in the lethality and feeding experiments. The bottles used to age adult flies will contain eggs and larvae. As a result, these bottles can be used to propagate the next generation of flies.

3. Preparation of flies for chemical exposure

3.1 Anesthetize 5–7-day old flies with CO₂. Sort anesthetized flies by sex using their genitalia. For assistance with anesthetizing and sexing flies, see reference²⁸.

3.2 Place groups of either 20 male or 20 female flies into vials containing standard BDSC media. Close the vial with a rayon plug. Store male and female vials separately. Mark vials containing female flies with a stripe. Vials with male flies are unmarked to avoid accidentally mixing sexes.

NOTE: The number of vials that must be prepared in step 3.2 is dictated by the size of the exposure experiments. As described in steps 5.3 and 5.6, a typical range finding experiment for a single test chemical will require a minimum of 40 vials of males and 40 vials of females. A standard dose response curve experiment described in steps 7.4 and 7.8 requires a minimum of 63 vials of males and 63 vials of females.

3.3 Store the vials for 48 h in an incubator at 25 °C at approximately 60% humidity and a 12h:12h light:dark cycle. During this time, prop the tray of sorted vials at a 60° angle to prevent flies from getting stuck in the food while recovering from anesthesia.

NOTE: This step allows flies to recover from the CO₂ anesthesia. Do not anesthetize flies again during the remainder of the exposure protocol.

3.4 After the 48 h recovery period, add 0.75 ml of sterile purified water to vials prepared in step 1.3. The number of vials prepared in this step should be identical to the number of vials setup in step 3.2.

3.5 Transfer sorted, sex-matched flies to the starvation vial by opening the glass vial containing the flies from step 3.2, putting it into the mouth of the prepared plastic vial, and then tapping the bottom of the plastic vial against a benchtop. Close the plastic vial with a cellulose acetate plug (commonly known as a flug).

3.5.1 Record any flies lost in this transfer directly onto the vial (transfer to records of starting number of flies for each vial later).

3.5.2 Mark starvation vials containing female flies with a stripe. Vials with male flies are unmarked to avoid accidentally mixing sexes.

3.6 Prepare the humidity chamber for the overnight exposure. See steps 1.4–1.5 for a description of how to build this chamber.

3.6.1 Place 6 standard paper towels in the bottom of the humidity chamber. Soak the paper towels with 100 ml of water.

3.6.2 Put the plastic grid cut (step 1.5) over the wet towels to ensure vials do not contact the saturated paper towels.

3.7 Place trays of starvation vials in a horizontal position within the humidity chambers. Place humidity chambers in a 25°C incubator (at approximately 60% humidity) overnight.

NOTE: Ideally, the overnight starvation period is approximately 16 hrs, however, the timing can be adjusted for individual lab schedules.

4. Preparation of stock solutions

NOTE: Flies are fed test chemicals in a yeast-sucrose liquid media. This section describes how to prepare stock solutions of concentrated feeding media and test chemical.

4.1 Prepare a 4x yeast-sucrose solution containing 16% sucrose and 6% yeast extract (m/v) dissolved in sterile purified water. Autoclave the solution on a liquid cycle for the appropriate sterilization time, e.g. 40 min for 1L

NOTE: The solution can be made in bulk and stored in aliquots at –20 °C. Thaw individual aliquots 1 day prior to use by placing them in a 4 °C refrigerator.

4.2 Prepare a stock of the test chemical. For the initial experiment, the stock solution should be made at the highest concentration that the chemical can be completely dissolved in water.

NOTE: Solvents other than water can be used to dissolve the test chemical. See Discussion regarding caveats for using alternative solvents.

4.3 Prepare a 100x Blue Dye stock solution by dissolving 1 g of FD&C Blue No. 1 (CAS 3844-45-9) in 10 mL of sterile purified water.

NOTE: The Blue Dye stock solution can be made in bulk and stored in aliquots at 4 °C.

5. Preparation of Exposure Vials – Range Finding Experiment

NOTE: Sections 5 and 6 of the protocol are designed to identify the lowest dose of test chemical that induces 100% lethality and the highest dose that fails to induce a lethal phenotype. If these concentrations are already determined by previous experimentation, see sections 7 and 8 for calculating a dose-response curve.

NOTE: Exposure media should be prepared immediately prior to adding flies to the exposure vials.

5.1 Label eight 15 mL centrifuge tubes as follows: (i) no chemical, (ii) highest concentration, (iii) 1:2, 1:10, 1:20, 1:100, 1:200, and 1:1000.

5.2 Prepare the exposure media in labeled centrifuge tubes from step 5.1. by first adding 2.5 mL of 4x yeast/sucrose stock solution to all eight labeled tubes.

5.2.1 Add 7.5 mL of sterile purified water to the tube labeled “no chemical.” This dilution is the negative control.

5.2.2 Add 7.4 mL of the test chemical stock solution to the tube labeled “Highest Concentration.” Add 100 µL of sterile purified water to this tube so the final volume is 10 mL. Calculate and record the molarity of the test chemical in this solution.

NOTE: The addition of 100 µL of sterile purified water to the tube ensures that the chemical concentration in this step is identical to that used in step 5.5, where a blue dye stock solution is added to the exposure media as a method for assessing feeding behavior.

5.2.3 Add the appropriate amount of test chemical stock solution and sterile purified water to the remaining tubes. The final volume of each tube should be 10 mL. The final concentration of test chemical in these tubes relative to the “Highest Concentration” tube should be 1:2, 1:10, 1:20, 1:100, 1:200, and 1:1000.

5.3 Prepare and label eight exposure vials for each individual concentration of exposure media generated in step 5.1. There should be eight sets of vials (64 vials total) containing the following labels: no chemical, highest concentration, 1:2, 1:10, 1:20, 1:100, 1:200, and 1:1000.

5.3.1 Pipette 0.75 mL of exposure media prepared in step 5.2.3. into the corresponding set of exposure vials.

5.4 Label eight 5 mL individual tubes as follows: (i) no chemical, (ii) highest concentration, (iii) 1:2, 1:10, 1:20, 1:100, 1:200, and 1:1000.

5.5 Prepare blue dye exposure media by first mixing 500 µL of 4x yeast/sucrose stock solution and 20 µL of Blue Dye stock solution to eight labeled 5 mL tubes.

5.5.1 Add 1.48 mL of sterile purified water to the tube labeled “no chemical.” This dilution is the negative control.

5.5.2 Add 1.48 mL of the test chemical stock solution to the tube labeled “Highest Concentration.” No water is added to this tube. Calculate and record the molarity of the test chemical in this solution.

5.5.3 Add the appropriate amount of test chemical stock solution and sterile purified water to the remaining tubes. The final volume of each tube should be 2 ml. The final concentration of test chemical in these tubes relative to the “Highest Concentration” tube should be 1:2, 1:10, 1:20, 1:100, 1:200, and 1:1000.

5.6 Prepare and label two exposure vials with each individual concentration of blue exposure media. There should be eight sets of vials (16 vials total) containing the following labels: no chemical, highest concentration, 1:2, 1:10, 1:20, 1:100, 1:200, and 1:1000. The word “blue” should also be written on these vials.

5.6.1 Pipette 0.75 mL of blue exposure media into the corresponding set of blue exposure vials.

6. Fly Chemical Exposure – Range Finding Experiment

6.1 Prepare chemical exposure vials using the vials of overnight starved flies from step 3.7 as follows:

6.1.1 Transfer four vials of starved female flies (twenty flies per vial) into four exposure vials of each chemical concentration. Label these vials “Female”. Use the same transfer method described in step 3.5.

6.1.2 Transfer four vials of starved male flies (twenty flies per vial) into four exposure vials of each chemical concentration. Label these vials “Male”. Use the same transfer method described in step 3.5.

6.2 Prepare chemical exposure vials containing blue dye using the vials of overnight starved flies from step 3.7 as follows:

6.2.1 Transfer one vial of starved female flies (twenty flies per vial) into one blue exposure vial for each chemical concentration. Label this vial “Female”. Use the same transfer method described in step 3.5.

6.2.2 Transfer one vial of starved male flies (twenty flies per vial) into one blue exposure vial for each chemical concentration. Label this vial “Male”. Use the same transfer method described in step 3.5.

6.3 Record the number of flies that are present in each vial after transfer and note the number that died or escaped. Normally, all 20 flies should survive the overnight starvation and transfer.

6.4 Place exposure vials horizontally in freshly prepared humidity chambers (See step 3.6 for humidity chamber preparation). Place chambers in a 25 °C incubator with approximately 60% humidity and a 12 h:12 h light:dark cycle.

6.5 Examine the exposure vials at 24 and 48 hrs after the start of the chemical exposure. Count and record the number of dead flies in each vial at each time point.

NOTE: Death is used as the readout for this assay, but the protocol can be adapted to examine other phenotypes. Exposed flies are commonly collected at these timepoints for transcriptomic and metabolomic studies.

6.6 Examine the blue exposure vials at 24 hrs after the start of the chemical exposure. Use the following techniques to determine if exposed flies consumed the blue exposure media:

6.6.1 Examine the vial walls for indications of blue feces, which will appear as small dots on the side exposure vial as well as on the flug.

6.6.2 Anesthetize flies with CO₂ and examine the abdomens for the presence of blue dye.

NOTE: We normally analyze blue exposure vials after 24 hrs. However, this step could be performed at 48 hrs, instead.

NOTE: Flies eating normally have a blue stripe through the abdomen, indicating that the exposure media has entered the gut.

6.6.3 Examine flies for abnormal feeding behaviors, such as regurgitation, crop distension, and breakdown of intestinal barrier function (indicated by the appearance of blue dye throughout the organism rather than just limited to the GI tract, commonly referred to as smurfing^{32,33}).

6.7 Discard all contaminated vials, filter paper, flugs, and flies in appropriate chemical waste containers. If there are live flies remaining in vials, freeze the vials to kill flies prior to discarding in the proper waste container.

NOTE: Disposal of exposure vials and flies will be dictated by the chemical(s) being analyzed in the experiment. Always follow chemical safety procedures outlined on the chemical safety data sheet.

NOTE: If concentrations used in the range-finding experiment kill flies at the lowest dose, repeat Step 6 using a dilution series beginning with the lowest concentration that killed 100% of animals.

7. Preparation of Exposure Vials – Generating a Dose Response Curve

NOTE: The protocol outlined in Sections 5 and 6 are designed to broadly determine the chemical concentration required to elicit a phenotype. Sections 7 and 8 of the protocol are used to calculate an accurate dose response curve.

7.1 Calculate the test chemical concentrations that must be analyzed to generate a dose response curve using the following method:

7.1.1 Determine the lowest concentration of the test chemical that kills 100% of exposed flies at 48 hrs.

7.1.2 Determine the highest concentration of test chemical that has no effect on viability at 48 hrs.

7.1.3 Calculate an additional four concentrations that are equally distributed between the concentrations determined in steps 7.1.1 and 7.1.2.

7.2 Label nine individual 15 mL centrifuge tubes with the molarity of the following concentrations: (i) no chemical, (ii) concentration determined in step 7.1.1, (iii) concentration determined in step 7.1.2, (iv) concentrations determined in 7.1.3, (v) twice the concentration determined in step 7.1.1, (vi) a 1:2 dilution of concentration determined in step 7.1.2.

NOTE: Including the concentrations that are twice the concentration determined in 7.1.1 and a 1:2 dilution of that determined in step 7.1.2 is important for accurately calculating the dose response curve.

7.3 Prepare the exposure media by first adding 2.5 mL of 4x yeast/sucrose stock solution to nine labeled individual 15 mL centrifuge tubes prepared in step 7.2.

7.3.1 Add 7.5 mL of sterile purified water to the tube labeled “no chemical.” This dilution is the negative control.

7.3.2 Add the appropriate amount of test chemical stock solution and sterile purified water to the remaining tubes. The final volume of each tube should be 10 mL. The final concentration of chemical within an individual tube should be equal to that written on the outside of the tube.

7.4 Prepare and label twelve exposure vials for each concentration of exposure media generated in step 7.2. There should be nine sets of vials (108 vials total).

7.5 Pipette 0.75 mL of exposure media into the corresponding set of exposure vials.

NOTE: Steps 7.6 to 7.8 are optional. If the test chemical concentrations prepared in step 7.2 are known to not affect feeding behavior, these steps can be skipped.

7.6 Prepare and label nine different 5 mL tubes for blue exposure media with the same series of concentrations used in step 7.2.

7.7 Prepare blue dye exposure media by first mixing 500 μ L of 4x yeast/sucrose stock solution and 20 μ L of Blue Dye stock solution.

7.7.1 Add 1.48 mL of purified sterile water to the tube labeled “no chemical.” This dilution is the negative control.

7.7.2 Add the appropriate amount of test chemical stock solution and purified sterile water to the remaining tubes. The final volume of each tube should be 2 ml. The final concentration of chemical within an individual tube should be equal to that written on the outside of the tube.

7.8 Prepare and label two exposure vials for each individual concentration of blue exposure media. There should be nine sets of vials (18 vials total), with each set of vials labeled with the concentrations listed in step 7.2. The word “blue” should also be written on these vials.

7.8.1 Pipette 0.75 mL of blue exposure media into the corresponding set of blue exposure vials.

8. Fly Chemical Exposure – Generating a Dose Response Curve

8.1 Prepare chemical exposure vials using the vials of overnight starved flies from step 3.7 as follows:

8.1.1 Transfer six vials of starved female flies (twenty flies per vial) into six exposure vials of each chemical concentration. Label these vials “Female”. Use the same transfer method described in step 3.5.

8.1.2 Transfer six vials of starved male flies (twenty flies per vial) into six exposure vials of each chemical concentration. Label these vials “Male”. Use the same transfer method described in step 3.5.

8.2 Prepare chemical exposure vials containing blue dye using the vials of overnight starved flies from step 3.7 as follows:

8.2.1 Transfer one vial of starved female flies (twenty flies per vial) into one blue exposure vial for each chemical concentration that was prepared in step 7.6. Label this vial “Female”. Use the same transfer method described in step 3.5.

8.2.2 Transfer one vial of starved male flies (twenty flies per vial) into one blue exposure vial for each chemical concentration that was prepared in step 7.6. Label this vial “Male”. Use the same transfer method described in step 3.5.

8.3 Record the number of flies present in each vial after transfer. Normally, all 20 flies should survive the overnight starvation and transfer but ensure any that died or escaped during transfer are subtracted from the total.

8.4 Place exposure vials horizontally in freshly prepared humidity chambers (See step 3.6 for humidity chamber preparation). Place chambers in a 25 °C incubator with approximately 60% humidity and a 12 h:12 h light:dark cycle.

8.5 Examine the exposure vials at 24 and 48 hrs after the start of the chemical exposure. Count and record the number of dead flies in each vial at each time point.

8.6 Examine the blue exposure vials at 24 hrs after the start of the chemical exposure. Use the method outlined in step 6.6 to assess changes in feeding behavior.

8.9 Discard all contaminated vials, filter paper, flugs, and flies in appropriate chemical waste containers. If there are live flies remaining in vials, freeze the vials to kill flies prior to discarding in the proper waste container.

NOTE: Disposal of exposure vials and flies will be dictated by the chemical(s) being analyzed in the experiment. Always follow chemical safety procedures outlined on the chemical safety data sheet.

9. Calculating a dose-response curve.

9.1 Use the Environmental Protection Agency Benchmark Dose Software (BMDS) version 3.2 or other similar software to analyze the data³⁴. The following describes the workflow using the BMDS software.

9.2 **Click the Data tab** of BMDS and **click insert new data set**. Input the number of samples in the dataset, **click dichotomous**, and then **click create dataset**. Enter each replicate as an individual row in the dataset.

9.3 Input the dose in the first column of the generated table, the starting number of flies (excluding any that died or were lost prior to step 8.3) in the second column, and the number of flies that died from chemical exposure in the third column.

9.4 **Click the Main tab** of BMDS.

9.5 **Click the drop-down arrow** for the Select Model Type menu and **click “Dichotomous”**.

9.6 **Click enable** for the dataset from step 9.2 in the Data Sets table.

9.7 **Click the boxes for the desired models** for analysis in the MLE and Alternatives table.

NOTE: We primarily use the Frequentist Restricted Dichotomous Hill model.

9.8 **Click Run analysis**. The program will generate an output file with the lethality curve(s) and additional details about the model(s).

REPRESENTATIVE RESULTS:

The fly has long served as a model in studies for determining sodium arsenite (NaAsO_2) toxicity^{35–38}. To demonstrate the efficacy of the protocol, male and female flies were exposed to NaAsO_2 with the goal of comparing these results with earlier studies. Using the methodology described above, adult Oregon-R (BDSC stock #2057) males and females were exposed to a range of NaAsO_2 concentrations (0, 0.01, 0.02, 0.1, 0.2, 1, and 2 mM) and scored for lethality 48 hours after the start of exposure (Figure 1A,B).

The purpose of this initial analysis was to identify the approximate range of concentrations that would allow a more precise characterization of NaAsO_2 toxicity. In subsequent experiments, concentrations were selected (0, 0.2, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 5 mM) that more precisely defined the NaAsO_2 dose-response curve (Figure 1C, D). Note that the

resulting analysis examined several concentrations that induced 100% lethality. Data were analyzed using the Environmental Protection Agency's publicly available Benchmark Dose Software version 3.2.0.1²⁵. The data were modeled as "dichotomous" and the Dichotomous Hill model was used for subsequent analyses. Based on this model, the final LD₁₀, LD₂₅, and LD₅₀ of male flies fed NaAsO₂ were 0.30 mM, 0.50 mM, and 0.65 mM, respectively. For female flies, these values were slightly higher, with an LD₁₀ of 0.30 mM, an LD₂₅ of 0.65 mM, and an LD₅₀ of 0.90 mM. Overall, the values obtained using this method are similar to those previously reported for arsenic toxicity in the *Drosophila melanogaster*^{35–38}, thus validating the methodology.

In addition to the six replicates used to calculate the dose response curve, male and female flies were also fed NaAsO₂ exposure solutions that contained 1% FD&C blue, which is easily visible in the digestive tract using light microscopy. Based on the presence of blue dye within the intestine of NaAsO₂-fed flies, both male and female flies continued to feed 24 hours after the beginning of the chemical exposure, regardless of the NaAsO₂ concentration present within the liquid media (Figure 2). However, the ingested food was observed to be occasionally regurgitated at doses above 0.2 mM for females and 0.5 mM for males (Figure 2). These findings suggest that regurgitation could serve a key role in the *Drosophila* response to arsenic poisoning.

DISCUSSION:

The fruit fly *Drosophila melanogaster* is emerging as a powerful system for NAMs^{16,18,19,21}. By leveraging the unparalleled genetic resources available to the fly community combined with recent advances in genomics and metabolomics, chemical safety studies using *Drosophila* are capable of quickly identifying the molecular mechanisms by which individual compounds interfere with metabolism, physiology, and cell signaling (for example, see³⁹). This inexpensive protocol is designed to rapidly define dose-response curves and subsequently generate samples for RNA-seq and metabolomics analysis. Moreover, this flexible protocol can be adapted for use with any genotype and can accommodate many classes of chemicals.

A notable aspect of this protocol is the choice of liquid food used in the chemical exposure, which is based on a previous study, but differs from the solid media used by most toxicological studies of *Drosophila*^{18,22}. This specific liquid media was selected to reflect the nutritional content of the standard, solid BDSC media that the flies are also fed in this protocol, to ensure the flies receive consistent nutrition. The simplicity of liquid feeding media has many advantages. Liquid media is easier to handle than solid food, which needs to be either melted and resolidified or reconstituted from powder. Liquid media also increases the system's throughput, ensures even chemical distribution throughout the feeding media, and decreases the time spent working with hazardous compounds. Additionally, the media does not require solutions to be heated, which facilitates the testing of volatile test compounds. Finally, because of the relatively few components included in the food solution, undesirable side reactions are minimized between the test chemical and other dietary components. The yeast used in the food is also inactive, further limiting reactivity

of the feeding medium. However, please note that the method is not suitable for testing developmental or larval toxicity.

Some of the materials used in the protocol can be substituted, such as using glass fly vials rather than polypropylene. However, the materials used were selected to be both inert and disposable to avoid unwanted chemical reactions between reagents and chemical exposures that could result from cleaning glassware.

The use of liquid food necessitates a vehicle for food delivery. Cellulose acetate filter paper was selected for this purpose due to its flexibility and inert nature²⁸. Other researchers used similar protocols but with other vehicles, such as delicate task wipe or glass fiber filter^{29, 30}. The cellulose acetate filter paper suited these needs because it was an inert vehicle which can be cut to the ideal shape to fit it into the bottom of the fly vials without large gaps between the paper and vial wall to prevent death due to flies becoming stuck in media or the vehicle itself.

An important limitation of this system is that the maximum testable concentration of a chemical is tied to the solubility of the chemical. Non-water-soluble compounds require an additional solvent, which can lead to additional or synergistic effects with the chemical of interest. This can also create situations in which it is not possible to prepare concentrated enough stock solutions to achieve the desired endpoint in all organisms, therefore limiting analysis of the resulting data³¹. To address this, chemicals with low water solubility can be tested by adding up to 0.5% dimethyl sulfoxide to the food solution. Other solvents could be used as well, but additional research is needed for each solvent of interest to determine the maximum acceptable solvent concentration within the solution to maximize solubility while minimizing solvent effects on the organism.

Extensive characterization of the olfaction response in *Drosophila* has described how flies avoid consuming toxic compounds^{40,41}, leading to reduced feeding on treated media. The blue dye assay addresses this phenomenon by allowing researchers to efficiently screen the feeding behaviors of the flies fed each concentration of experimental chemical⁴²⁻⁴⁴. The presence or absence of blue in the fly gastrointestinal tract indicates if the fly has been eating the toxicant-containing medium. Although more sophisticated methods of assessing fly feeding behaviors exist, such as the Fly Liquid-Food Interaction Counter⁴⁵, this qualitative method is better suited for higher-throughput screening.

A notable aspect of this protocol is that it has been optimized for a 48-hour exposure period without the need to transfer flies or add additional liquid to the exposure vial. Using a humidity chamber and placing the chambers in an incubator kept at high humidity prevented the filter paper containing the feeding media from drying out during this timeframe. The protocol can be adapted for longer exposure durations, but the method must be adjusted to ensure that the filter paper does not become dry and cause significant changes in solution concentration or lethality due to desiccation.

Finally, an important characteristic of this protocol is that it can readily accommodate genetic variants, which allows researchers to utilize the vast array of genetic tools for *Drosophila* to expand these preliminary studies on wild-type organisms to better understand

mechanisms of chemical action *in vivo*. In this regard, the protocol outlined above could be easily modified to complement a previously describe *JOVE* protocol by Peterson and Long that allows for toxicological analysis of wild-caught flies¹⁸.

Because of the wide variety of previous studies on the toxicity of sodium arsenite in *Drosophila*^{32–36}, Oregon-R flies were treated with this compound to demonstrate the efficacy of our system. Male flies exhibited an LD₅₀ of 0.65 mM and females exhibited an LD₅₀ of 0.90 mM. This aligns with previous studies of sodium arsenite-treated adult *Drosophila*. For example, Goldstein and Babich³⁷ found that 50% of flies (mixed sexes) died after 7 days of exposure to 0.5 mM NaAsO₂. Although this is a slightly lower dose than was presently observed, the differences between their methods and this method (including the use of solid exposure media, longer time scale, and mixed sexes) likely account for this difference. Importantly, both methods resulted in overall similar LD₅₀ values.

Observations from experiments using this protocol can be used to find genetic and molecular targets for subsequent behavioral or mechanistic studies. The exposure method can also be used to treat *Drosophila* for sampling for metabolomics and proteomics, making this protocol well suited to the growing field of precision toxicology (modeled from the precision medicine field⁴⁶). In this regard, exposed flies can be collected after Step 8 for subsequent genomic and metabolomics analysis. Samples collected in Step 8 can then be processed as described in the *JOVE* method described by Li and Tennesen⁴⁷, starting with Step 3.

Ultimately, the data acquired from experiments described above, as well as any subsequent metabolomics and proteomics data, would ideally be used in a cross-species comparisons. As previously noted²⁶, such cross-species studies are powerful and capable of determining how individual chemicals interfere with conserved biological pathways. Thus, the protocol described above can be used find evolutionary commonalities in response to individual toxicants across phyla and help inform chemical safety regulation.

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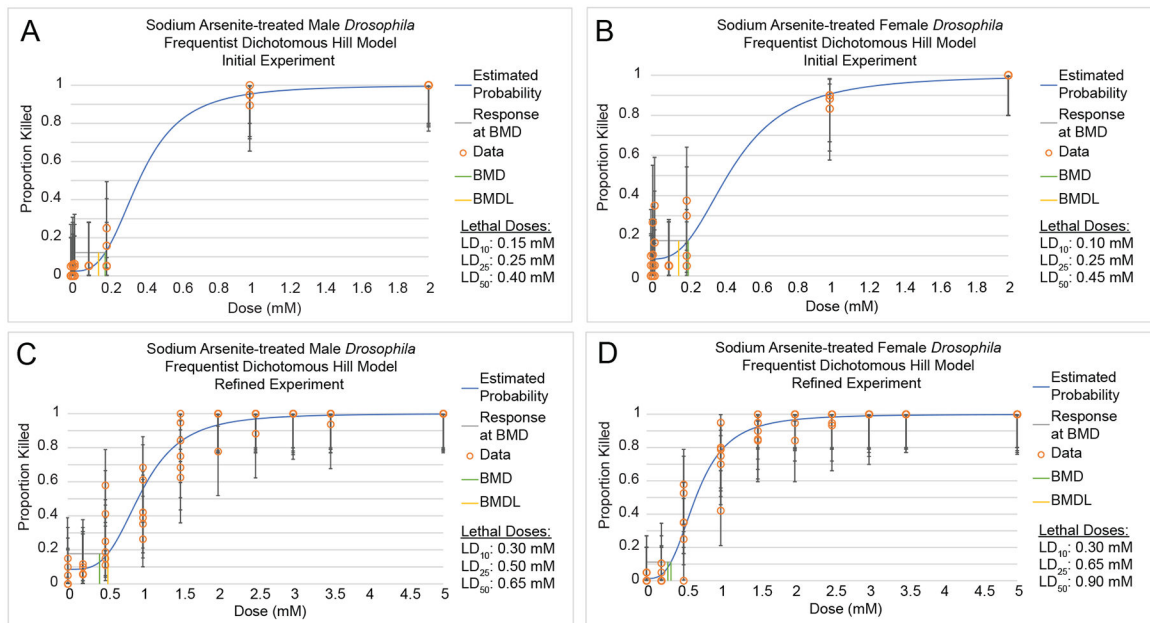


Figure 1. Dose-response curves for male and female *Drosophila* treated with NaAsO₂ for 48 hours.

All graphs show the estimated proportions of dead flies at each NaAsO₂ concentration tested based on the Dichotomous Hill model. (A, B) A broad range of NaAsO₂ concentrations was tested to approximate the dose at which each sex of flies begins to die. Panel A shows the male data and panel B shows the female data. n=4 vials with 20 flies per vial. (C, D) A narrower range of NaAsO₂ concentrations was tested to determine precise doses at which 10%, 25%, and 50% of each sex of flies died. These doses are indicated to the right of each graph. Panel C shows the male data and panel D shows the female data. n=6 vials with 20 flies per vial.

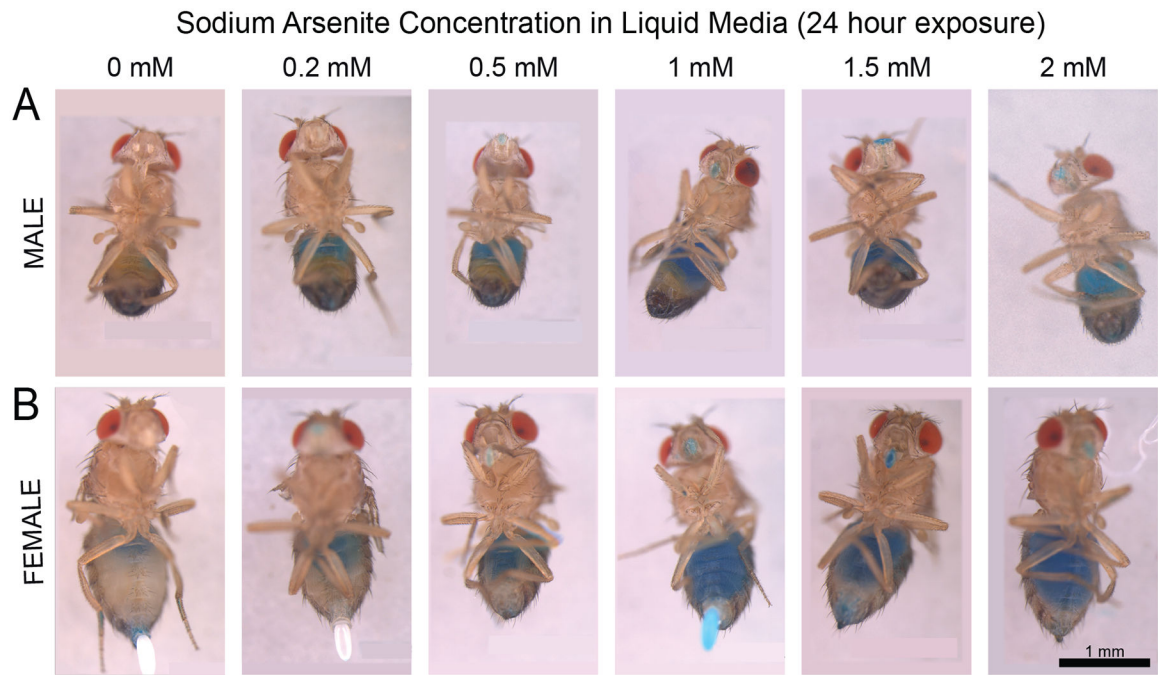


Figure 2. Representative results from the blue dye assay of male and female *Drosophila* treated with NaAsO₂ for 24 hours.

Micrographs show flies fed increasing concentrations of NaAsO₂. Row A shows male flies and row B shows female flies, with the concentration of NaAsO₂ increasing from left to right. At low concentrations, the abdomen shows a small amount of blue near the thorax, indicating that exposure media entered the gut. At higher concentrations, blue dye begins to accumulate around the mouth, suggesting that exposure media were being regurgitated. Scale bar is 1 mm.