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To Xiaoyan Cao, Ph.D.
Review Editor
JoVe
617.674.1888

Friday, February 01, 2019

Dear Dr. Cao,

Enclosed, please find the revised version of our manuscript entitled **"Methods to Test Endocrine Disruption in *Drosophila Melanogaster*"** by Bovier et al (JoVE59535_R1_RE).

We have modified the manuscript accordingly your comments. We feel confident that we have been able to adequately address your concerns. A point-by-point reply to the comments is included below.

In addition, we corrected Figure 3 and Figure 6, and added the EDC we used, ether and the etherizer to the material list.

We hope that this revised manuscript will meet the standards of the editorial revision.

Thank you in advance for your consideration

Sincerely yours,

Filomena Anna Digilio, Ph.D.

Ennio Giordano, Ph.D.

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Authors' revisions

We thank the Editor for the comments about this work.

Accordingly, we have amended the manuscript JoVE59535_R1_RE.

Editorial comments:

Summary. Please shorten it to no more than 50 words. **We shortened the summary to less of 50 words (49 words).**

1. **Food preparation**

1.1.1. Choose one of the many nutrient media suitable for raising *D. melanogaster* in the laboratory and prepare it according its recipe.

We decided to specify the medium we use, so reported both recipe and cooking method, from 1.1.1 to 1.1.7.

1.1.2. Once the medium cools to 60 °C, add 5 mL/L of 10% Nipagin in ethanol, mix thoroughly and dispense into containers to a depth of about 5 cm.

We reported all informations required and used the scientific name of the Nipagin (Methyl 4-hydroxybenzoate).

1.1.3. Regardless of the chosen recipe, ensure that medium has the right consistency and hydration so that flies do not drown in it; calibrate these parameters experimentally by modifying either the amount of agar used and/or the cooling/drying times.

We changed in "1.1.8. Calibrate experimentally right consistency and hydration of the MC by modifying either the amount of agar used and/or the cooling/drying times".

1.1.4. Cover vials with cheesecloth and allow them to dry for 24 h prior to storage.

We reported all required informations in 1.1.7 and 1.1.8.

1.2.1. Bring this mixture to a boil two times or until agar is dissolved, by using a microwave.

We specified a volume to prepare in 1.2.1 and reported the waiting time in 1.2.2. In 1.2.4 we also specified Room Temperature.

1.3. For feeding assay, add both the solution containing the right dilution of the selected EDC and a coloring food (e.g., the red dye no. 40 at 1 mg/mL)[35] to the AM medium before solidification, mix strongly with a food processor and then dispense into vials.

We specified type and dilution of EDC and moved this step to 2.5.

1.4.2. Mix thoroughly and pipet 3 mL in small vials.

We specified it. We added with a food processor

1.4.3. Cover vials with cheesecloth and allow them to dry for 24 h prior to storage.

We specified the temperature.

2.2. Dilute the EE2 stock solution in 10% ethanol in water (v/v) in order to obtain a 100 mM solution. Make next dilutions (0.1 mM, 0.5 mM and 1 mM) in Drosophila medium, starting with lowest concentration and using the same final concentration of solvent for each treatment group. For the control vials use same volume of the solvent alone.

We specified the medium

Commentato [A1]: Please mention the media chosen in this specific protocol.

Commentato [A2]: Is the medium heated before this step?

Commentato [A3]: Please replace it with a generic term.

Commentato [A4]: What container is used? What is the capacity?

Commentato [A5]: Please replace it with a generic term.

Commentato [A6]: It is unclear what "these parameters" refer to. Please specify.

Commentato [A7]: Are the vials the containers used in step 1.1.2? What type of vials are used, what is the capacity?

Commentato [A8]: At room temperature? Please specify.

Commentato [A9]: An approximate volume to prepare would be helpful.

Commentato [A10]: How long is the waiting period between the two boils? Please specify.

Commentato [A11]: Please specify the type and dilution of the EDC used.
Please consider moving this step to section 2 where EDC dosing is described.

Commentato [A12]: Please consider moving this step to section 2 where EDC dosing is described.

Commentato [A13]: How to mix thoroughly? By vortexing or pipetting?

Commentato [A14]: At room temperature?

Commentato [A15]: It is unclear what the Drosophila medium refers to (food or medium prepared in steps 1.1, 1.2, 1.3 or 1.4). Please specify.

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2.3. Add the solution containing the right dilution of the selected EDC to the cornmeal based food before solidification, mix thoroughly with a food processor, dispense 10 mL into vials, cover with cotton gauze and let dry for 16 h before using.

We specified the temperature.

2.4. For the adult rearing, prepare different working EE2 solutions in 10% ethanol in water (v/v) and layer 100 µL of each onto the surface of the AM, in order to obtain the desired concentration of the EE2 (0.1 mM, 0.5 mM and 1 mM).

We specified the different concentrations we prepared.

3.1 Use a robust isogenic strain maintained by several generation in the laboratory.

We specified the strain we used.

3.2. Keep flies in a humidified, temperature controlled incubator, with a natural 12 L: 12 D photoperiod at 25 °C in vials containing standard cornmeal medium.

We corrected it accordingly

4.1. Transfer 15 flies in vials containing cornmeal medium supplemented with different concentrations of the selected EDC and a coloring food. Allow flies to feed on these media for 1 day.

4.2. …..

We changed in "Put 15 young flies in vials containing" CM

4.1.3. Anesthetize both groups of flies with ether and compare their abdominal coloring by examining them under a stereo-microscope.

We specified this methodology

5.1.

We corrected the number of vials to be used and called them parental vials in order to avoid following misinterpretations: **"3 vials of flies, thereafter called parental vials"**

5.3. In the late afternoon of the day 9, remove all newly flies from the vials and return the containers to 18 °C; the next morning, 98% of the females will be virgins.

We corrected accordingly

5.3.1. The next morning, sort flies by sex and return the containers to 25 °C for 6 h; collect flies again and return the containers at 18 °C overnight.

5.3.2. The next morning, repeat these two steps.

Note: this procedure requires two collections of flies per day using temperature cycles and can maximize the number of virgins collected in a day. At a temperature of 18 °C, development is slowed and females do not mate for at least 12-16 h after eclosion from the pupal case.

5.3.3. Place males emerged from treated-food in vials with the same type of medium as their larval growth, avoiding overcrowding.

Note: it is recommended to make several groups of 20 males.

5.3.4. Do the same procedure of step 6 for the females, but be careful to make smaller groups (10 flies per vial).

Note: after two days ensure there are no larvae in the vials of females. If they do, the flies are not usable because they are not virgins and must be discarded.

5.4. House the collected flies at 25 °C until they are aged 4 days post-eclosion, transferring them into new vials containing fresh corresponding medium every two days.

Note: 4 days is sufficient time for the flies to become mature adults but it is very far from the beginning of the senescence.

We changed steps 5.3.1-5.4 in order to better explain these procedures.

Commentato [A16]: At room temperature?

Commentato [A17]: Please specify the different concentrations that are prepared.

Commentato [A18]: Please specify the strain used here.

Commentato [A19]: 12 h light:12 h dark?

Commentato [A20]: Prepared in section 1?

Commentato [A21]: Transfer 15 flies from the standard cornmeal medium to vials containing cornmeal medium supplemented with different concentrations of the selected EDC and a coloring food?

Commentato [A22]: Please specify how this is done.

Commentato [A23]: vials?

Commentato [A24]: 18 °C incubator?

Commentato [A25]: Should this be on the morning of day 10?

Commentato [A26]: on the morning of day 10?

Commentato [A27]: What happens to the flies after sorting?

Commentato [A28]: Do you mean removing all new flies as in step 5.3? What happens to these flies after collecting them?

Commentato [A29]: When are the flies collected?

Commentato [A30]: on the morning of day 11?

Commentato [A31]: Please specify the two steps that are repeated.

Commentato [A32]: Are males collected at different times mixed together or placed in separate vials?

Commentato [A33]: This sentence is unclear. Please revise.

Commentato [A34]: This is unclear. Please revise.

Commentato [A35]: Please update step number.

Commentato [A36]: Are females collected at different times mixed together or placed in separate vials?

Commentato [A37]: What do these flies refer to? Please specify from which step they are obtained.

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5.5. Set up 20 single crosses for each sex and for each treatment into small vials containing fresh cornmeal-tomato medium without EDC, as described below.
We changed in
5.5. Use 20 single flies of each sex for each treatment groups to set up 20 single crosses into small vials containing fresh CM-tomato medium without EDC, as described below.

5.5.1. To each treatment group assign a different series of sequential numbers, which uniquely identifies it and label the respective vials; e.g. group1 (solvent alone) from 1 to 20, group 2 (EDC conc x) from 20 to 40, and so on.

5.5.2. Make a fertility spreadsheet in which report the different series, each corresponding to a treatment group.

5.5.3. For each sex anesthetize all the flies belonging to each treatment group under mild CO_2 and randomly transfer them in the following way:
we cannot specify the CO_2 concentration so we used light CO_2

5.5.4. Transfer one no-treated female into a small vial containing fresh cornmeal-tomato medium without EDC and add one no-treated male for the control cross. Note: tomato juice should be added to medium during its preparation because dark medium increases the contrast with the white embryos.

5.5.5. Transfer one treated female into a small vial containing fresh cornmeal-tomato without EDC and add one no-treated male for each treatment.

5.5.6. Transfer one treated male into a small vial containing fresh cornmeal-tomato medium without EDC and add one no-treated female for each treatment.

5.5.7. House all these single crosses at 25°C.

5.6. Transfer each mating pair into fresh cornmeal-tomato vials without EDC every day for the subsequent ten days.
We corrected accordingly
6.1.6. Allow flies to lay eggs for 16/18 h.
We reported 16
6.2.1. Rear young and healthy female (about 150) and male (about 50) flies on a collection cage (**Table of Materials**) with agar-tomato medium supplemented with fresh baker's yeast paste (3 g baker's yeast in 5 mL of water), thereafter called laying tray, for 2 days at 25 °C.
We specified the temperature
6.2.12. Continue from step 6.1.8 of the Eclosion assay Protocol 1.
We changed in " Repeat steps 6.1.8-6.1.13 of the Eclosion assay Protocol 1."

7.1.1. After 4 days discard flies and place vials back in the incubator.

Note: these flies can be used to start again to obtain other age-synchronized cohorts of the flies.

Commentato [A38]: Please specify the concentration of CO2 used.

Commentato [A39]: From steps 5.5.4-5.5.6?

Commentato [A40]: Should these be sub-steps of the transferring step 5.5.3? If so, they should be numbered as 5.5.3.1, 5.5.3.2, 5.5.3.3, etc.

Commentato [A41]: 16-18 h?

Commentato [A42]: At what temperature?

Commentato [A43]: Repeat steps 6.1.8-6.1.13?

Commentato [A44]: Repeat steps 6.1.8-6.1.13?

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7.1.2. In the late afternoon of the day 9, remove all newly flies from the vials 16-24 h before adult collection and return vials to the incubator.

Note: a few adults should begin to eclose as early as the ninth day; discarding these flies allows to collect a maximum number of synchronized flies, avoiding the careless selection of early emergent.

7.1.3. 16-24 h later, transfer the adult flies (1 day old) of both sexes into four groups of 250 mL bottles containing AM food supplemented with three different EDC concentrations and one with the solvent alone. If needed, collect another batch the next day.

We corrected accordingly.

7.8.2. Repeat steps 9-10 until all flies die.

We corrected accordingly.

Figure 1: Feeding assay. Two flies (dx) fed on medium supplemented with EDC (top) or solvent alone (bottom) show similar coloring on their abdomens.

Figure 2: EDC dosing. Schematic of the EDC administration to Drosophila by diet.

Figure 3: Fertility assay. Schematic of the protocol depicting the steps from flies growth in appropriate media to virgin collection up to single crosses.

Figure 4: Developmental timing. On the left is shown the egg collection cage and the sliding trays. On the right a table scheme has been reported.

Figure 5: Lifespan assay. Scheme depicting the key steps of the protocol. On the right it is reported a table scheme.

Figure 6: Lifespan curves. Typical lifespan curve of an EDC treated flies cohort in comparison with no treated flies.

We described all the figure in more details:

Figure 1: Feeding assay. Adult flies in vials containing CM/dye supplemented with selected EDC (top) or solvent alone (bottom) are left to feed 24hrs. Two flies (dx) fed on medium supplemented with EDC (top) or solvent alone (bottom) show similar coloring on their abdomens.

Figure 2: EDC dosing. Schematic of the EDC administration to Drosophila by diet. Adult flies from an isogenic stock are exposed to different concentration of EDC (top) or solvent alone (bottom). N= reference concentration of EDC.

Figure 3: Fertility assay. Schematic of the protocol depicting the steps from flies growth in appropriate media to virgin collection up to single crosses. Step 1: Adults (10 vials with 8 females and 4 males each) from an isogenic strain are transferred in CM/EDC (top) or CM/solvent alone (bottom) (note that the scheme in figure 3, for simplicity, is referred to just one of the three vials). Step 2: After 4 days, adults are discarded, and laid eggs are left to develop for 9 days until adult stage. Step 3: Newly eclosed adults are sorted by sex and collected in vials (max 20 males/vial and 10 virgin-females/vial). Step 4: Adults are left to age for 4 days on the corresponding medium of the larval growth. Step 5: (top) set up 40 single crosses for EDC-treated flies in CM-tomato medium without EDC, 20x [1 EDC-treated males with 1 control females] and 20x [1 control male with 1 EDC-treated females]; (bottom) set up 20 single crosses for

Commentato [A45]: These steps are performed after step 7.1, correct? If so, they should be numbered 7.2-7.4.

Commentato [A46]: Please update step numbers.

Commentato [A47]: Please describe this figure in more details.

Commentato [A48]: Please describe this figure in more details.

Commentato [A49]: Please describe it in more details.

Commentato [A50]: Please describe this figure in more details.

Commentato [A51]: Is Figure 6 cut off? There is no panel A. Please describe this figure in more details. Describe the left part of the figure.

Commentato [A52]: Please describe this figure in more details.

Commentato [A53]: Please describe this figure in more details.

control flies 20x [1 control male with 1 control female]. Yellow medium is a CM supplemented with EDC (top) or solvent as control (bottom), and red medium is a tomato/CM with no EDC or solvent.

Figure 4: Developmental timing: Eclosion assay Protocol 2. On the left, a scheme of the collection cage in which adults (about 150 females and 50 males) are placed to acclimate and mate in the dark before the deposition step (see protocol). After two days, the old sliding tray is replaced with one with fresh food and eggs deposed in the next one hour are discarded because asynchronous. Hereafter, eggs are collected every 2 h on a new sliding tray, counted, and placed on dishes containing tomato/CM supplemented with EDC or with solvent alone. Data (total laid eggs, undisclosed eggs, pupae, and eclosed adults) are reported on a series of developmental timing spreadsheet (right).

Figure 5: Figure 5: Lifespan assay. One days synchronized flies are transferred into 250 ml bottle with adult food (AM) supplemented with EDC (top) or solvent (bottom) as control, to allow feeding (left in the scheme). After two-three days flies are sorted by sex and transferred in 5 vials (containing the corresponding medium of the starting bottle) for each sex, in groups of 20 individuals/vial. Every three days, adults are transferred in fresh vials until necessary (central part of the scheme). On the right it is reported a scheme of the table in which the data are recorded for each days.

Commentato [A54]: Please describe this figure in more details.

Figure 6: Lifespan curves. On the left it is reported a representative table in which the number of dead flies has been recorded every three days both for the treatment group (medium + EDC: EE2 0.05 mM) and for the control group (medium + solvent), throughout the entire experimental period. The mean lifespan of each group has been calculated using the "MATRIX SUM.PRODUCT"; the treated group shortened the mean lifespan compared to control. On the right, it is shown a typical survival curve of male flies fed with medium containing EE2 (0.05 mM) or only ethanol for the control. The survivorship curve decreased more rapidly in treated flies than control group, with a turning drop point earlier.

DIscussion: **Please break this long paragraph into two or three paragraphs.**
We splitted Discussion in tree subparagraphs