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TITLE:

Methods to Test Endocrine Disruption in *Drosophila Melanogaster*

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SUMMARY:

Endocrine disruptor chemicals (EDCs) represent a serious problem for organisms and for natural environments. *Drosophila melanogaster* represents an ideal model to study EDC effects in vivo. Here, we present methods to investigate endocrine disruption in Drosophila, addressing EDC effects on fecundity, fertility, developmental timing, and lifespan of the fly.

ABSTRACT:

In recent years there has been growing evidence that all organisms and the environment are exposed to hormone-like chemicals, known as endocrine disruptor chemicals (EDCs). These chemicals may alter the normal balance of endocrine systems and lead to adverse effects, as well as an increasing number of hormonal disorders in the human population or disturbed growth and reduced reproduction in the wildlife species. For some EDCs, there are documented health effects and restrictions on their use. However, for most of them, there is still no scientific evidence in this sense. In order to verify potential endocrine effects of a chemical in the full organism, we need to test it in appropriate model systems, as well as in the fruit fly, *Drosophila melanogaster*. Here we report detailed in vivo protocols to study endocrine disruption in Drosophila, addressing EDC effects on the fecundity/fertility, developmental timing, and lifespan of the fly. In the last few years, we used these Drosophila life traits to investigate the effects of

exposure to 17-α-ethinylestradiol (EE2), bisphenol A (BPA), and bisphenol AF (BPA F). Altogether, these assays covered all Drosophila life stages and made it possible to evaluate endocrine disruption in all hormone-mediated processes. Fecundity/fertility and developmental timing assays were useful to measure the EDC impact on the fly reproductive performance and on developmental stages, respectively. Finally, the lifespan assay involved chronic EDC exposures to adults and measured their survivorship. However, these life traits can also be influenced by several experimental factors that had to be carefully controlled. So, in this work, we suggest a series of procedures we have optimized for the right outcome of these assays. These methods allow scientists to establish endocrine disruption for any EDC or for a mixture of different EDCs in Drosophila, although to identify the endocrine mechanism responsible for the effect, further essays could be needed.

INTRODUCTION:

Human activities have been releasing into the environment a massive amount of chemicals, which represent a serious problem for organisms and for natural ecosystems[1]. Of these pollutants, it is estimated that about 1,000 different chemicals may alter the normal balance of endocrine systems; according to this property, they are classified as endocrine disrupting chemicals (EDCs). Specifically, based on a recent definition by the Endocrine Society, the EDCs are "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action"[2]. Over the last three decades, there has been growing scientific evidence that EDCs can affect the reproduction and development of animals and plants[3–8]. Further, EDC exposure has been related to the increasing prevalence of some human diseases, including cancer, obesity, diabetes, thyroid diseases, and behavioral disorders[9–11].

General mechanisms of EDC

Due to their molecular properties, EDCs behave like hormones or hormone precursors[3–12]. In this sense, they can bind to a hormone's receptor and disrupt endocrine systems either by mimicking hormone activity or by blocking endogenous hormones binding. In the first case, after binding to the receptor, they can activate it as its natural hormone would do. In the other case, binding of the EDC to the receptor prevents the binding of its natural hormone, so the receptor is blocked and can no longer be activated, even in the presence of its natural hormone[3]. As a consequence, EDCs can affect several processes, such as the synthesis, secretion, transport, metabolism, or peripheral action of endogenous hormones that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior of the organism. Receptor binding is not the only way of action described so far for the EDCs. It is now clear that they can also act by recruiting coactivators or corepressors in enzymatic pathways or by modifying epigenetic markers deregulating gene expression[10–14], with consequences not only for the current generation but also for the health of generations to come[8].

Drosophila hormones

The potential effects of selected EDCs have been studied widely, both in wildlife species and in several model systems in which endocrine mechanisms are reasonably well known. For invertebrates, endocrine systems that influence growth, development, and reproduction have been extensively characterized in insects for several reasons, involving their extensive use in the

field of biological research, their economic importance, and finally the development of insecticides able to interfere specifically with the hormone system of pest insects.

In particular, among insects, the fruit fly *D. melanogaster* has proven to be a very powerful model system to evaluate the potential endocrine effects of EDCs. In *D. melanogaster*, as well as in vertebrates, hormones play an important role throughout the entire life cycle. In this organism, there are three main hormonal systems, which involve the steroid hormone 20-hydroxyecdysone (20E)[15,16], the sesquiterpenoid juvenile hormone (JH)[17], and the neuropeptides and peptide/protein hormones[18]. This third group consists of several peptides discovered more recently but clearly involved in a huge variety of physiological and behavioral processes, such as longevity, homeostasis, metabolism, reproduction, memory, and locomotor control. 20E is homologous to cholesterol-derived steroid hormones such as estradiol, while JH shares some similarities with retinoic acid; both of them are the better-known hormones in Drosophila[19,20]. Their balance is vital in coordinating molting and metamorphosis, as well as in controlling several postdevelopmental processes, such as reproduction, lifespan, and behavior[21], thus offering different possibilities for testing endocrine disruption in Drosophila. Further, ecdysteroid hormones and JHs are the main targets of the so-called third-generation insecticides, developed to interfere with developmental and reproductive endocrine-mediated processes in insects. The agonist or antagonist mode of action of these chemicals is well known, and thus they can serve as reference standards for evaluating the effects of potential EDCs on the growth, reproduction, and development of insects[22]. For example, methoprene, which has been widely used in controlling mosquitoes and other aquatic insects[23,24], works as a JH agonist and represses 20E-induced gene transcription and metamorphosis.

In addition to hormones, the nuclear receptor (NR) superfamily in Drosophila is also well known; it consists of 18 evolutionarily conserved transcription factors involved in controlling hormone-dependent developmental pathways, as well as reproduction and physiology[25]. These hormone NRs belong to all six NR superfamily subtypes, including those involved in neurotransmission[26], two for retinoic acid NRs, and those for steroid NRs that, in vertebrates, represent one of the primary targets of EDCs[27].

Drosophila as a model system for studying EDCs

Currently, on the basis of molecular properties, several environmental agencies around the world are attributing the potential to interfere with the endocrine systems to different man-made chemicals. Given that the EDCs are a global and ubiquitous problem for the environment and for organisms, the overall goal of the research in this field is to reduce their disease burden, as well as to protect living organisms from their adverse effects. In order to deepen the understanding about the potential endocrine effects of a chemical, it is necessary to test it in vivo. To this end, *D. melanogaster* represents a valid model system. To date, the fruit fly has been extensively used as in vivo model to evaluate the effects of several environmental EDCs; it has been reported that exposure to several EDCs, such as dibutyl phthalate (DBP)[28], bisphenol A (BPA), 4-nonylphenol (4-NP), 4-tert-octylphenol (4-tert-OP)[29], methylparaben (MP)[30], ethylparaben (EP)[31,32], bis-(2-ethylhexyl) phthalate (DEHP)[33], and 17-α-ethinylestradiol (EE2)[34], influences metabolism and endocrine functions as in vertebrate models. Several reasons have led to its use as a model in

this field of research. Beyond an excellent knowledge of its endocrine systems, further advantages include its short lifecycle, low cost, easily manipulable genome, a long history of research, and several technical possibilities (see the FlyBase website, http://flybase.org/). *D. melanogaster* also provides a powerful model for easily studying transgenerational effects and population responses to environmental factors[8] and avoids ethical issues relevant for in vivo studies in higher animals. In addition, the fruit fly shares a high degree of gene conservation with humans which might make it possible for Drosophila EDC assays to help in predicting or suggesting potential effects of these chemicals for human health. Besides expanding the understanding about human health effects, Drosophila can help to assess risks of EDC exposure to the environment, such as biodiversity loss and environmental degradation. Finally, the fruit fly offers the additional advantage of being used in laboratories, where the factors potentially affecting its development, reproduction, and lifespan can be kept under control in order to attribute any variation to the substance to be tested.

With this in mind, we have optimized simple and robust fitness assays for determining EDC effects on some Drosophila hormonal traits, such as fecundity/fertility, developmental timing, and adult lifespan. These assays have been widely used for some EDCs[23–27]. In particular, we have used the following protocols to evaluate the effects of the exposure to the synthetic estrogen EE2[34] and to BPA and to bisphenol AF (BPA F) (unpublished data). These protocols may be easily modified to investigate the effects of a given EDC at a time, as well as the combined effects of multiple EDCs in *D. melanogaster*.

PROTOCOL:

1. Food preparation

1.1. For stock maintenance and for larval growth, use a cornmeal medium containing 3% powdered yeast, 10% sucrose, 9% precooked cornmeal, and 0.4% agar, hereafter called cornmeal medium (CM).

1.1.1. Put 30 g of yeast into 200 mL of tap water, bring it to a boil, and let it boil for 15 min.

1.1.2. Separately, mix well 90 g of precooked cornmeal, 100 g of sugar, and 4 g of agar into 900 mL of tap water.

1.1.3. Bring the solution to a boil, lower the heat, and cook it for 5 min, stirring continuously.

1.1.4. After 5 min, add the hot yeast solution and let it simmer for another 15 min.

1.1.5. Turn off the heat source and allow the solution to cool to about 60 °C.

1.1.6. Add 5 mL/L of 10% methyl 4-hydroxybenzoate in ethanol, mix thoroughly, and let it sit for 10 min.

NOTE: Be careful with the amount of methyl 4-hydroxybenzoate, given that a high concentration of fungicide could be lethal for larvae.

1.1.7. Dispense the medium into vials/bottles: 8 mL into each fly vial of 25 mm x 95 mm, 3 mL into each fly vial of 22 mm x 63 mm, and 60 mL into each fly bottle (250 mL).

1.1.8. Cover the vials with cheesecloth and allow them to dry at room temperature (RT) for 24 h prior to storage.

1.1.9. Calibrate experimentally right consistency and hydration of the MC by modifying either the amount of agar used and/or the cooling/drying times.

NOTE: Unplugged, boxed, and wrapped vials are stable for about 15 days at 4 °C.

1.2. For Drosophila adults, use a medium containing 10% powdered yeast, 10% sucrose, and 2% agar, hereafter called adult medium (AM).

1.2.1. Mix 10 g of powdered yeast, 10 g of sucrose, and 2 g of agar into 100 mL of distilled water.

1.2.2. Bring this mixture to a boil 2x, with a 3 min interval, or until the agar is dissolved, by using a microwave.

1.2.3. Once the solution cools to 60 °C, add 5 mL/L of 10% methyl 4-hydroxybenzoate in ethanol, mix thoroughly, and dispense the mixture in vials (10 mL per vial).

1.2.4. Cover the vials with cheesecloth and let them dry at RT for 24 h before storing.

NOTE: Unplugged, boxed, and wrapped vials are stable for about 15 days at 4 °C.

1.3. For the fecundity/fertility assay (see section 5), use Drosophila tomato juice-cornmeal medium.

1.3.1. Pour off 70 mL of warm cornmeal food into a 100 mL beaker and add 30 mL of tomato juice (30% v/v).

1.3.2. Mix them thoroughly with a food processor and pipet 3 mL into small vials.

1.3.3. Cover the vials with cheesecloth and allow them to dry at RT for 24 h prior to storage.

NOTE: Unplugged, boxed, and wrapped vials are stable for about 15 days at 4 °C.

1.4. For embryo collection, use agar plates containing 3% agar, 30% tomato sauce, and 3% sugar.

NOTE: Be careful not to make any bubbles when pouring the medium in the plates.

2. Drosophila EDC dosing

2.1. Prepare an appropriate stock solution dissolving the selected EDC in a suitable solvent. For the EE2 (molecular weight 296.403), dissolve 1.48 g in 10 mL of 100% ethanol to make a 0.5 M stock solution and store it at -80 °C.

CAUTION: EDCs are considered environmental pollutants and precautions should be taken in handling them.

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

NOTE: It is recommended to keep the final concentration of the solvent as low as possible, bearing in mind that the final concentration of ethanol should not exceed 2% in fly food.

2.3. Add the solution containing the right dilution of the selected EDC to the cornmeal-based food before solidification, mix it thoroughly with a food processor, dispense 10 mL per vial, cover them with cotton gauze, and let them dry at RT for 16 h before use.

NOTE: Use this medium immediately after its preparation.

2.4. For the adult rearing, prepare different working EE2 solutions (10 mM, 50 mM, and 100 mM) 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, respectively). For the control, use the same volume of the solvent alone.

NOTE: Alternatively, add the solution containing the right dilution of the selected EDC to a small amount of AM in a 50 mL conical tube, vortex thoroughly, and stratify 1 mL of it onto the surface of each AM vial.

2.4.1. Cover the vials with cotton gauze, allow them to dry at RT for 12–16 h under gentle agitation, and use them immediately.

NOTE: The drying process should be adjusted experimentally because it depends on the ambient humidity.

2.5. For the feeding assay, add both the solution containing the right dilution of the selected EDC (EE2 0.1 mM, 0.5 mM, and 1 mM) and a coloring food (e.g., the red dye no. 40 at 1 mg/mL)[35] to the CM before solidification, mix them strongly with a food processor, and then, dispense the mixture into vials.

3. Rearing flies

3.1. Use a robust isogenic strain, such as Oregon R, maintained for several generations in the laboratory.

3.2. Keep the flies in a humidified, temperature-controlled incubator, with a natural 12 h light:12 h dark photoperiod at 25 °C in vials containing CM food.

3.3. In each assay, use vials at RT.

4. Feeding assay

NOTE: This assay is recommended to test if the presence of the selected EDC in the medium could affect the feeding of the flies.

4.1. Put 15 young flies in vials containing CM supplemented with different concentrations of the selected EDC and a coloring food. Allow the flies to feed on the media for 1 day.

NOTE: For example, use red dye no. 40[35] (1 mg/mL).

4.2. Put 15 young flies in vials containing CM supplemented with the solvent alone and a coloring food for control. Allow the flies to feed on the media for 1 day.

4.3. Anesthetize individually each group of flies with ether.

4.3.1. Transfer the flies of each group to a cylindrical glass container (etherizer) with a funnel inserted into the open end, inverting the vial over the funnel and gently tapping the two containers together to make the flies fall into the etherizer.

NOTE: The funnel will prevent them from getting out of the etherizer.

4.3.2. Knock the flies down by gently tapping the etherizer on a soft surface, such as a mouse pad, and quickly replace the funnel with an ether-soaked cotton-and-gauze plug.

4.3.3. Wait about 1 min until the flies fall to the bottom and stop moving.

NOTE: Do not exceed the time or the flies will die.

4.4. Put the immobilized flies under a stereomicroscope and compare the abdominal coloring of each treatment group with respect to the control group.

5. Fecundity/fertility assay

5.1. For each EDC concentration, prepare three vials of flies, hereafter called parental vials, each

with eight females and four males in 10 mL of CM/EDC food; for the control, prepare six vials of flies, each with eight females and four males in 10 mL of CM food supplemented with solvent. Rear flies in an incubator at 25 °C.

NOTE: Avoid overcrowding larvae during their development and try to use consistent larval densities across treatments.

5.2. After 4 days, remove the parents and return the vials to the incubator for 9 days.

5.3. In the late afternoon of day 9, remove all newly emerged flies from the vials and put the vials in an incubator at 18 °C overnight.

NOTE: This removal must be done very carefully, checking the surface of the medium well.

5.3.1. On the morning of day 10, for each treatment group, collect virgin females and young males into two groups, under light CO_2 anesthetization. Randomly subdivide each group of flies in small subgroups (10 females or 20 males per vial) in independent vials filled with fresh corresponding CM.

5.3.1.1. Repeat steps 5.3 and 5.3.1, taking care both to carefully remove all newly emerged flies from the vials 8–10 h before collection and to leave the vials at 18 °C until at least 30 virgin females and 30 males for each EDC concentration and at least 90 virgin females and 90 males for the control are obtained.

5.3.2. House these groups of flies at 25 °C until they are aged 4 days posteclosion, transferring them into new vials containing fresh corresponding medium every 2 days.

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

5.3.3. After 2 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. Use 20 single flies of each sex for each treatment group to set up 20 single crosses into small vials containing fresh CM-tomato medium without EDC, as described below.

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

5.4.2. Make a fertility spreadsheet to record the different series, each corresponding to a treatment group.

5.4.3. For each sex, anesthetize all the flies belonging to each treatment group under light CO_2

and randomly transfer them as follows.

5.4.3.1. Transfer one solvent-treated female into a small vial containing fresh CM-tomato medium without EDC and add one solvent-treated male for the control cross.

NOTE: Tomato juice should be added to the medium during its preparation because dark medium increases the contrast with the white embryos.

5.4.3.2. Transfer one EDC-treated female into a small vial containing fresh CM-tomato without EDC and add one solvent-treated male for each treatment.

5.4.3.3. Transfer one EDC-treated male into a small vial containing fresh CM-tomato medium without EDC and add one solvent-treated female for each treatment.

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

5.5. Transfer each mating pair to fresh CM-tomato vials without EDC every day for the subsequent 10 days. Label the replicated vials of each series sequentially (e.g., 1-a, 1b, 1c … 20a, 20b, 20c) and report these numbers on the fertility spreadsheet.

5.6. Visually inspect each vial every day for the eggs and report their number on the fertility spreadsheet.

5.7. Save each vial and, when newly emerged flies start to emerge, also record the daily number of adult progenies over the 10 day period. After 10 days from the initial mating, remove the parents.

NOTE: Discard the vial in which one or both parents died; in case one or both parents escape, include all data in the analysis until the day they were lost.

5.8. Sum the daily number of eggs and the daily number of adult progenies from each treatment group to obtain the total fecundity/fertility, the mean egg and adult progeny production by a fly for 10 days, and the ratio of total progeny to total number of eggs laid. Calculate the differences in percentage of each treatment values with respect to the control.

5.9. Carry out three independent experiments for each group of flies by using a minimum of 10 flies for each treatment group.

5.10. Perform statistical analysis to compare the different groups.

6. Developmental timing

NOTE: In the two following alternative protocols, the developmental timing is evaluated by counting both the number of pupae that form per day and the amount of adult progeny eclosing

per day.

6.1. Eclosion assay protocol 1

6.1.1. For each treatment group, set up 10 vials of young (<2 days), healthy flies, each with six females and three males in 10 mL of cornmeal food without EDC.

6.1.2. Rear the flies on food for 24 h, and allow them to mate.

6.1.3. Prepare 10 parallel vials per treatment group with 10 mL each of fresh cornmeal food supplemented with different EDC concentrations or the solvent alone for the control. Transfer the mated flies to these new vials.

NOTE: For each treatment group, assign a different series of sequential numbers which uniquely identifies it, and label the respective vials.

6.1.4. Make a developmental spreadsheet to record the different series.

6.1.5. Allow the flies to lay eggs for 16 h. Then, remove the parents from the vials.

NOTE: The parent flies can be used to repeat step 6.1.5 by transferring them to other corresponding vials.

6.1.6. Incubate the vials for 3–4 days at 25 °C, or until third instar larvae are visible. Every day, count the number of newly emerged pupae in each vial and report it on the developmental spreadsheet. To avoid counting the same pupa 2x, write a number in sequence by each pupa with a permanent marker on the outside of the vial.

6.1.7. Starting from day 9, count the number of emerging adults daily until no more adults emerge, and report it on the developmental spreadsheet.

6.1.8. From these raw data, calculate the mean larval period, the mean pupal period, as well as the differences in percentage of each treatment with respect to the control.

6.1.9. Carry out three independent experiments for each group of flies by using a minimum of five flies for each treatment group.

6.1.10. Perform statistical analysis to compare the different groups.

6.2. Eclosion assay protocol 2

6.2.1. Rear young and healthy female (about 150) and male (about 50) flies in a collection cage (**Table of Materials**) with agar-tomato medium supplemented with fresh baker's yeast paste (3 g of baker's yeast in 5 mL of water), hereafter called the laying tray, for 2 days at 25 °C.

6.2.2. During these 2 days, allow the flies to acclimate to the cage in a dark, quiet place, before beginning the egg collection, and change the laying tray 2x a day.

6.2.3. On the third day, change the laying tray early in the morning. After 1 h, replace the laying tray, discarding these laid eggs.

6.2.4. Allow the flies to lay eggs for 2 h and replace the laying tray with a fresh one.

NOTE: By day 3, a good laying tray should produce 100–200 eggs in 2 h.

6.2.5. For each treatment group, prepare a series of three 60 mm dishes containing tomato cornmeal food supplemented with the corresponding EDC concentration or with solvent alone and report each series in the developmental timing spreadsheet. Alternatively, if preferred, use vials instead of dishes.

6.2.6. Gently pick up eggs under a microscope by using a paintbrush or a probe and transfer them to the top of the medium in each dish/vial. In order to facilitate counting, arrange the eggs in five groups of 10 each on the laying tray and transfer them one at a time.

NOTE: Repeat steps 6.2.4−6.2.6 as many times as is necessary to obtain enough embryos.

6.2.7. House all these dishes/vials at 25 °C. Also, store each laying tray at 25 °C and count the total number of laid eggs.

6.2.8. After 24–30 h, check each dish/vial under a stereomicroscope and count both the number of white, unfertilized eggs and the number of dark dead embryos.

6.2.9. Subtract the number of white, unfertilized eggs from the 50 transferred eggs value in order to obtain the total embryos value per dish/vial. The number of dark dead embryos can be used to determine potential EDC toxic effects during embryogenesis.

6.2.10. Repeat steps 6.1.6–6.1.10.

7. Lifespan protocol

7.1. Set up 20 vials of flies with eight females and four males and house them at 25 °C in CM (10 mL each).

7.2. After 4 days, discard the flies and place the vials back into the incubator.

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

7.3. In the late afternoon of day 9, remove all newly emerged flies from the vials and return the

vials to the incubator.

NOTE: A few adults should begin to eclose as early as the ninth day; discarding these flies makes it possible to collect a maximum number of synchronized flies, avoiding the careless selection of early emergents.

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

7.5. Maintain the flies at 25 °C for 2–3 days to allow them to mate.

NOTE: The day of the transfer to AM food vials corresponds to the first day of adulthood.

7.6. After 2–3 days, sort each cohort of flies by sex into two groups under light CO_2 anesthetization. Randomly subdivide each group into five vials per treatment at a density of 20 individuals per vial, until there are three replicates of five parallel vials for each sex per each treatment.

NOTE: Work with small groups of flies in order to prevent possible long-lasting health issues due to a long exposure time to CO_2.

7.7. Prepare a lifespan spreadsheet in which the number of dead flies is subtracted from the number of surviving flies from the previous transfer so that the number of survivors at each transfer is automatically obtained.

7.8. Transfer the flies to new vials containing the corresponding food every 3 days at the same time and check for death.

NOTE: The transfer must take place without anesthesia that could have a long-term negative effect on fly longevity.

7.8.1. At each transfer, record the age of the flies and the number of dead flies.

NOTE: The number of surviving flies is automatically calculated in the spreadsheet, but it is recommended to check it visually. Flies that accidentally escape or die during the transfer should not be considered. Be careful not to count dead flies 2x when they are carried into the new vial, reporting this with a note in the spreadsheet.

7.8.2. Repeat steps 7.8 and 7.8.1 until all flies die.

7.9. For each treatment group, create a survival curve in order to display the survival probability of a fly at any particular time.

7.10. Perform three independent experiments for each treatment group of flies by using 100 newly eclosed flies for each experiment.

7.11. Prepare a table in which to report the mean lifespan (mean survival days of all flies for each group), the half-death time (period of time in days required to reach 50% mortality), and the maximum lifespan (maximum amount of days needed to reach 90% mortality).

7.12. Calculate the differences in percentage between each treatment group with respect to the control group.

7.13. Perform statistical analysis to compare the different treatment groups.

REPRESENTATIVE RESULTS:

In this section, key steps of the above protocols are reported in the form of simplified schemes. Given that flies tend to avoid unpalatable compounds, the first thing to do is to assay the taste of the selected EDC. This can be done by mixing a food coloring (for example, red food dye no. 40)[35] with the food supplemented with the selected EDC at various doses or with the solvent alone. Flies fed on these media are examined under a stereomicroscope and the food intake is estimated by their abdominal coloring (**Figure 1**). A typical desired situation is shown in **Figure 1** with two adult females: one fed on medium containing the selected EDC and one on medium not containing the EDC, both presenting the same coloration in their abdomens.

It has been widely accepted that EDCs, like natural hormones, have effects at extremely low doses and that there is not a simple, linear relationship between dose and effect[29], with higher doses not necessarily having a larger effect[36,37]. So, rather than a dose-response approach, in order to assess fully their impacts, it is advisable to use more doses, starting from relevant concentrations for the environment or for other organisms. In any case, it is important to assay the EDC taste at each used concentration to make sure that flies digest comparable quantities of EDC in each treatment group (**Figure 2**).

Endocrine disruption affects many important traits of animal physiology such as fertility, longevity, and development that, therefore, are useful endpoints to test EDCs. For the above protocols, which we optimized to measure the EDC effects on these Drosophila life traits, primary considerations are that it is imperative to use young and healthy flies that should be correctly manipulated before testing. With this in mind, great attention must be paid to the production, handling, and storage of the used food. In addition, care should be taken in using the best solvent for the selected EDC at appropriate final concentrations (i.e., less than 1% for dimethyl sulfoxide [DMSO] and less than of 2% for ethanol)[30].

Fecundity and fertility have been used to evaluate the reproductive success in *D. melanogaster*. **Figure 3** shows a scheme of the used protocol. Fecundity is experimentally measured as the total number of laid eggs, while fertility is measured as total adult offspring. Based on the consideration that egg production in the first 10 days of adult life is a good reference for the whole adult life egg/progeny production of an organism[38,39], fertility and fecundity assays can be

carried out for 10 days by using 4 day-old flies hatched from the exposed larvae. It is important to try to obtain similar values in the parallel vials of each group; otherwise, it would be difficult to imagine which tube to remove from the analysis. So, it is advisable to examine daily each replicate vial to avoid a stressful environment, such as dried or liquefied food; starting from 20 single crosses, it is recommended to obtain at least 10 vials in good conditions in which both parents were alive for 10 days. Numbers of eggs and adult progeny, collected daily, must be reported on a table as shown in **Figure 3** and used to calculate the mean egg and adult progeny production of a fly for 10 days. Then, the fecundity/fertility change percentage of EDC-treated flies compared to control flies can be obtained applying the following formula: fecundity/fertility change % = [(control - treatment)/control] x 100. At least three independent replicates have to be obtained for each group.

Hormones play essential roles in developmental transitions in the life of *D. melanogaster*[40], for which it is likely that these phases of growth are particularly vulnerable to the adverse effects of EDCs. At 25 °C, both larval growth and the pupal stage each span approximately 4 days. Following EDC exposure, the mean duration of these stages can be affected[41]. Based on this consideration, the developmental timing protocols were optimized to determine the percentage and time of transition from larvae to pupae and from pupae to adults following EDC exposure with respect to untreated flies. Two alternative protocols could carry out this assay. Both of them were valid and based on the EDC chronic exposure to larvae over a 4 day period. Based on this larval treatment, the first protocol did not take into account the age of the embryos, which were collected over a 16–18 h period (overnight). The consequent age variability among the larvae should, however, be present in all the vials of each treatment, thus increasing the within-treatment variance but without significantly affecting the estimates of developmental timing through treatments. Instead, the second protocol used a fixed number of synchronous early embryos, making it possible to evaluate also the potential effects of the selected EDC during embryogenesis[42,43]. Further, minimizing the age differences between the larvae reduced the within-treatment variance and increased the ability to estimate true differences between treatments. **Figure 4** reports a scheme of the eclosion assay. In both protocols, plates/vials had to be checked daily, and the numbers of both the pupae and adults from those exposed and nonexposed to the EDC were to be reported separately on a table as in **Figure 4**. All formed pupae and adult flies had to be counted, whether dead or alive. Then, these raw data were used to calculate percentages and times of transition from larvae to pupae and from pupae to adults and to calculate the change percentage of these values of the EDC-treated flies compared to control flies. Following EDC exposure, an overall developmental advance or delay with respect to the control flies was to be expected. The chosen protocol had to be performed in triplicate for each group of flies. It was advisable that, for the eclosion assay protocol 2, each series of a replicate for a given EDC concentration were seeded with embryos from the same laying trail in order to maintain reliability and accuracy in embryo staging throughout the EDC concentrations.

Finally, **Figure 5** shows the key steps for measuring lifespan. For this protocol, it was essential that all the flies under analysis were synchronous by age and sex, coupled, and kept at a density low enough to allow free circulation. It is important to carry out lifespan experiments in both sexes separately because it is well known there are significant differences in lifespan between

males and females[44].

Food had to be changed every 3 days to maintain a healthy population, and mortality had to be assessed also every 3 days. On a lifespan spreadsheet, as in **Figure 5**, the number of dead flies were reported, and that number would automatically be subtracted from the number of surviving flies from the previous transfer. For each EDC concentration and for the solvent alone, the cumulative survivorship versus the elapsed days was plotted to obtain lifespan curves. A typical survivorship curve is reported in **Figure 6**; after a long initial period in which the survivorship curve remained relatively high, it declined exponentially after about 60 days. Following EDC exposure, the survivorship curve of the treated flies could be affected significantly. In order to determine if this effect is due to the selected EDC, it was advisable to carry out at least two, or better yet, three independent, noncontemporaneous replicate experiments.

In each of the above protocols, it was possible to have anomalous vials (for example, with no eggs or anomalous deaths); these vials could have been originated by different causes, such as poor food quality or infection, and could significantly alter the values of the measures. The best way to manage these anomalous situations was to avoid them through good experimental practice. So, it must be emphasized that, for all the above protocols, great and careful work was required in replicating the vials, keeping flies healthy, and in handling flies that, once exposed to an EDC, might become delicate, thus increasing the risk of death when manipulated.

FIGURE LEGENDS:

Figure 1: Feeding assay. Adult flies in vials containing CM/dye supplemented with a selected EDC (top) or solvent alone (bottom) are left to feed for 24 h. Two flies fed on medium supplemented with an 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 concentrations of an EDC (top) or solvent alone (bottom). *N* = the reference concentration of the EDC.

Figure 3: Fertility assay. Schematic of the protocol, depicting the steps from fly growth in appropriate media to the virgin collection and up to single crosses. Step 1: Adults (10 vials with eight females and four males each) from an isogenic strain are transferred to vials with CM/EDC (top) or CM/solvent alone (bottom). (Note that the scheme, for simplicity, refers 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 the 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: Setup of 40 single crosses for EDC-treated flies in CM-tomato medium without an EDC, 20x one EDC-treated male with one control female and 20x one control male with one EDC-treated female (top); setup of 20 single crosses for control flies, 20x one control male with one control female (bottom). Yellow medium is a CM supplemented with an EDC (top) or solvent as control (bottom), and red medium is a tomato/CM without an EDC or solvent.

Figure 4: Developmental timing (eclosion assay protocol 2). On the left, this figure shows 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 2 days, the old sliding tray is replaced with one with fresh food, and the eggs deposed in the next 1 h are discarded because they are asynchronous. Hereafter, eggs are collected every 2 h on a new sliding tray, counted, and placed on dishes containing tomato/CM supplemented with an EDC or with solvent alone. Data (total laid eggs, undisclosed eggs, pupae, and eclosed adults) are reported on a series of developmental timing spreadsheets (right).

Figure 5: Lifespan assay. One day synchronized flies are transferred to a 250 mL bottle with adult food (AM) supplemented with an EDC (top) or solvent (bottom) as control, to allow feeding (left in the scheme). After 2–3 days, the flies are sorted by sex and transferred to five vials (containing the corresponding medium of the starting bottle) for each sex, in groups of 20 individuals/vial. Every 3 days, the adults are transferred to fresh vials until no longer necessary (central part of the scheme). On the right is a scheme of the table in which the data are recorded for each day.

Figure 6: Lifespan curves. On the left, a representative table is reported in which the number of dead flies has been recorded every 3 days, both for the treatment group (medium + EDC [0.05 mM EE2]) 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 the control group. On the right, a typical survival curve is shown of male flies fed with medium containing EE2 (0.05 mM) or only ethanol for the control. The survivorship curve decreased more rapidly for treated flies than for the control group, with an earlier turning drop point.

DISCUSSION:

The fruit fly *D. melanogaster* has been extensively employed as an in vivo model system to investigate the potential effects of environmental EDCs such as DBP[28], BPA, 4-NP, 4-tert-OP[29], MP[30], EP[31,32], DEHP[33], and EE2[34]. Several reasons have led its use as a model in this field of research. Apart from its undisputed advantages as a model system, Drosophila shares a high degree of gene conservation with humans, for which Drosophila EDC assays may help in predicting or suggesting potential effects for human health. In addition, Drosophila belongs to the invertebrates, which are widely represented in all ecosystems and require greater protection measures against the damaging effects of EDCs. Invertebrates are located at the base of the food chain and perform very important functions for the environments in which they live. It has been reported that several EDCs released into the environment may have a harmful influence on the development and reproduction of several animal species. Drosophila offers the additional advantage of being used in the laboratory, where the factors potentially affecting development, reproduction, and lifespan can be kept under control in order to attribute any variation to the substance to be tested.

Here we provide detailed protocols for studying the effects of EDC exposure in this model system on hormonally regulated life traits such as fecundity/fertility, developmental rate, and lifespan.

We have optimized these protocols that made it possible to investigate the effects of EE2[34], BPA, and BPAF (unpublished data) exposure, but they can be easily adapted to study the effects of other EDCs. In particular, these assays can be used to investigate both pure EDCs and combinations of different EDCs, reproducing more closely what occurs in nature. Although apparently, they may seem like simple growth assays, it is important to work according to the appropriate guidelines, ensuring accuracy and reproducibility[45]. It is well known that fertility, developmental timing, and longevity in *D. melanogaster* can be affected by external and internal factors. These critical factors, including photoperiod, temperature, humidity, nutrition, population density, genetic structure, and age, have to be carefully controlled for the outcome of the reported protocols. In order to minimize the component of genetic variability, an isogenic strain should be used. This strain must be carefully reared in a humidified, temperature-controlled incubator, with a natural 12 h light:12 h dark photoperiod at 25 °C.

In addition to the maintenance of a controlled environment, larval and fly overcrowding has to be avoided. It has been reported that larval overcrowding can affect the developmental timing and induce the expression of several genes, including heat shock or immunity-related genes, that influence the total fitness of adult flies[46]. Also, adult flies have to be maintained at a low enough density to allow free movement in order to minimize adult stress. Further, it is important to make sure that flies consume comparable quantities of the EDC in each treatment group, taking in consideration the taste of the compound at different concentrations, because the flies tend to avoid unpalatable food. Another important aspect of these protocols is food quality; the food must also look good, devoid of bubbles, bacteria cracks, and so on[47,48]. Further, it is necessary to keep in mind synchronization, mating status, and gender cohabitation for the flies to be tested. In all the reported protocols, it is important that the parallel vials under analysis must be very similar; otherwise, it would be difficult to understand which to discard so that maximum attention and good experimental practice are required. Finally, by using these protocols to evaluate the endocrine effects of selected EDCs, it is important to take in account possible interactions with other EDCs present in the medium, such as methyl 4-hydroxybenzoate or the BPA of the plastic vials. In this sense, it could be useful to change the antifungal agent, use glass vials, or perform pilot assays both with and without possible contaminants.

The reported Drosophila assays can be very effective for the assessment of any chemicals or mixture of chemicals, such as EDCs, by evaluating the potential effects on hormonally regulated life traits, such as reproduction, development, and lifespan. However, these assays cannot serve to clearly identify the endocrine mechanism responsible for the EDC adverse effects. To overcome this limitation, it is possible to perform the same protocols by using reference EDCs that evoke responses representative of a certain mode of action on the insect endocrine system (e.g., third-generation insecticides working as JH or ecdysteroid agonist/antagonist)[22]. Alternately, it is also possible to use molecular endpoints, carrying out molecular analysis on specific genes that are hormonally regulated and that are considered predictive and specific biomarkers for the endocrine disruption[34].

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DISCLOSURES:

The authors have nothing to disclose.

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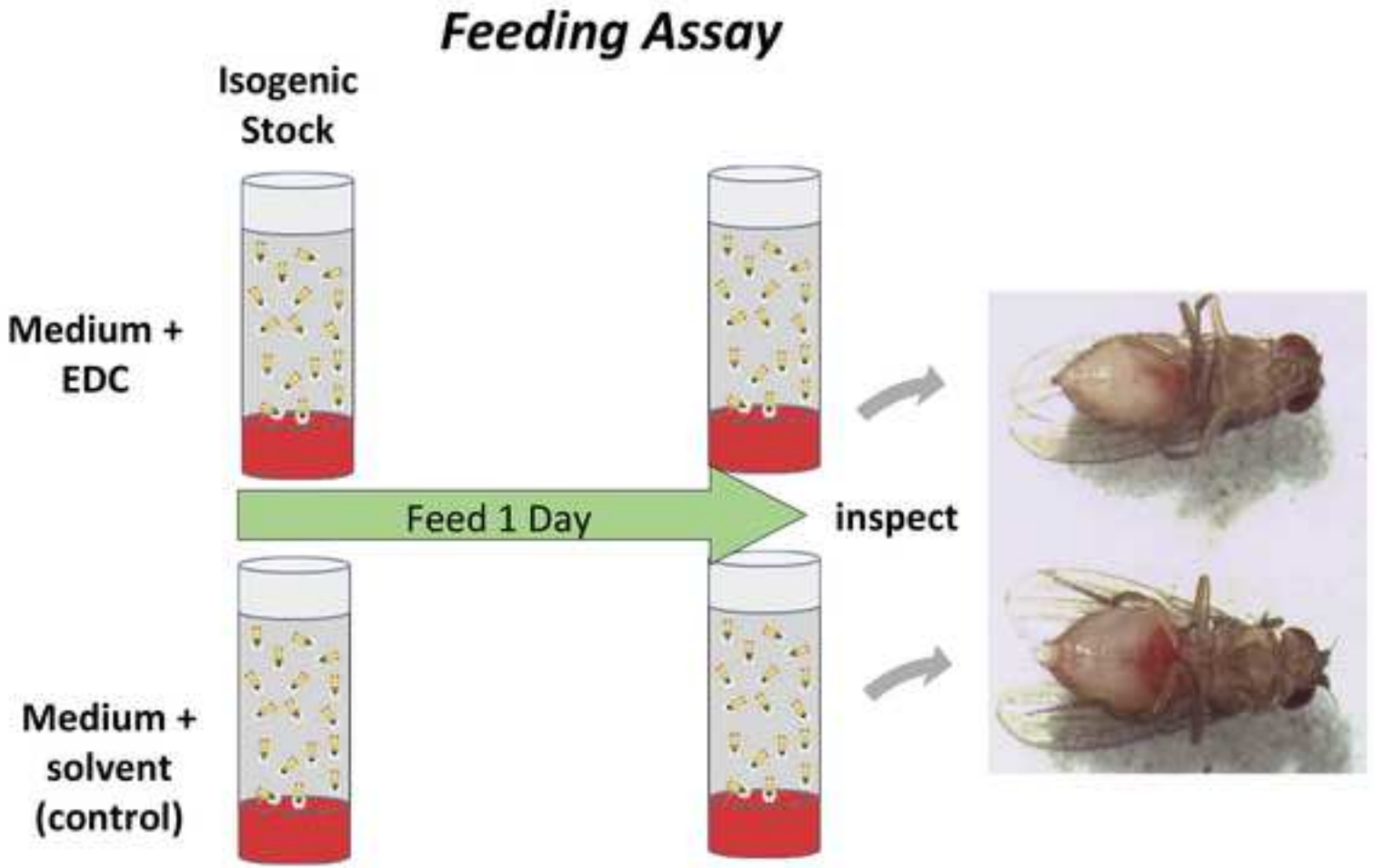
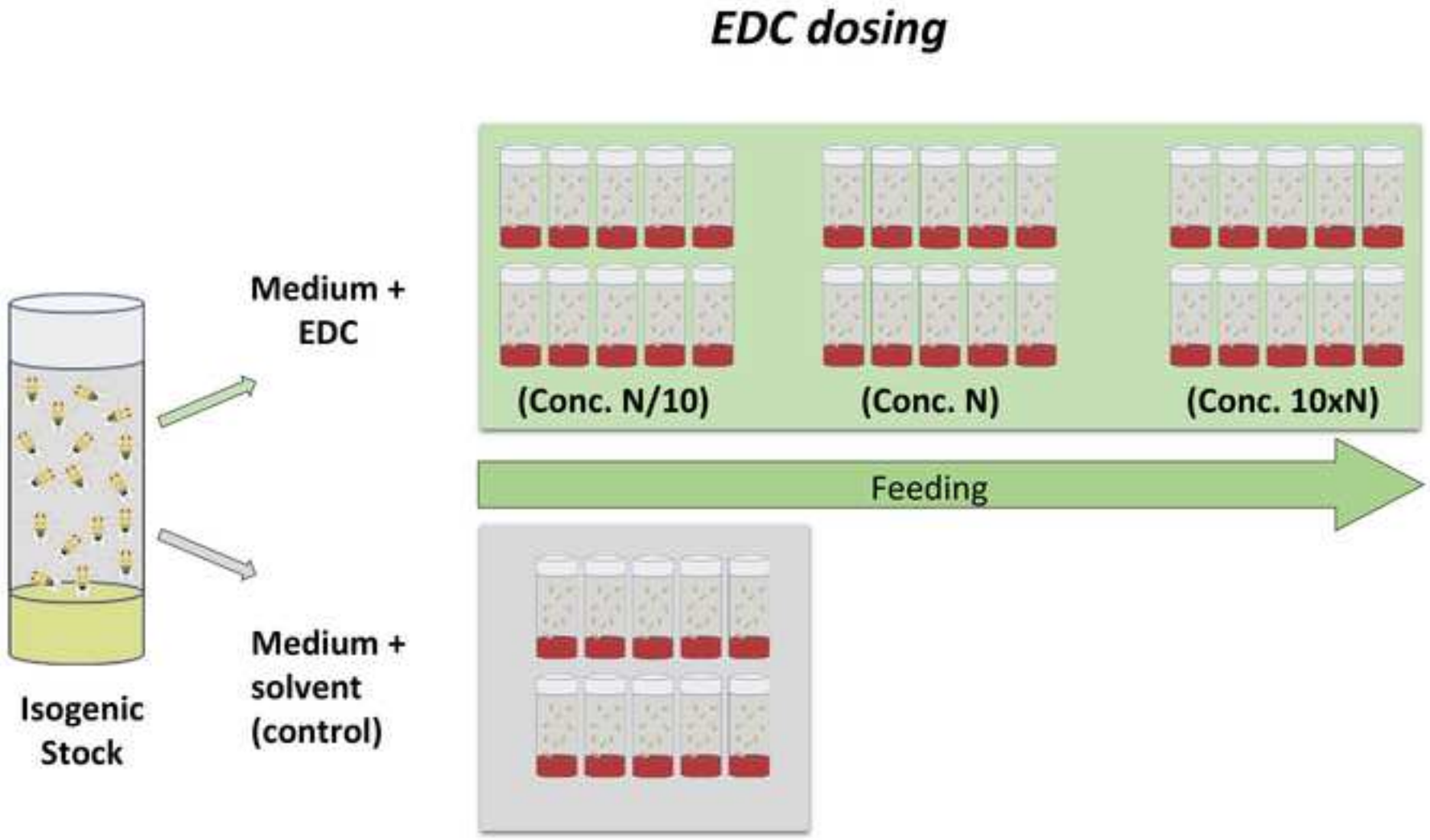
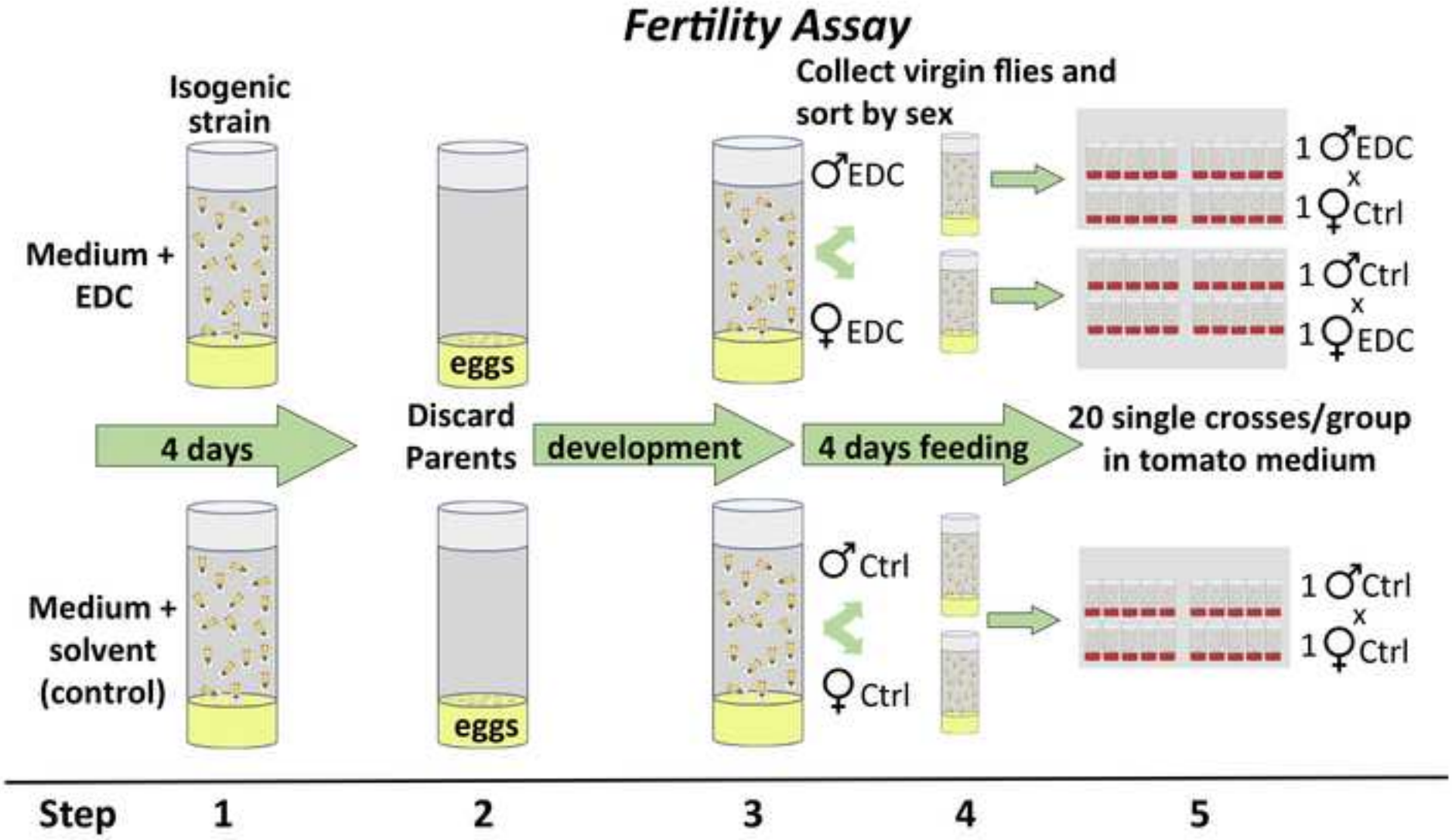




Figure



Developmental timing

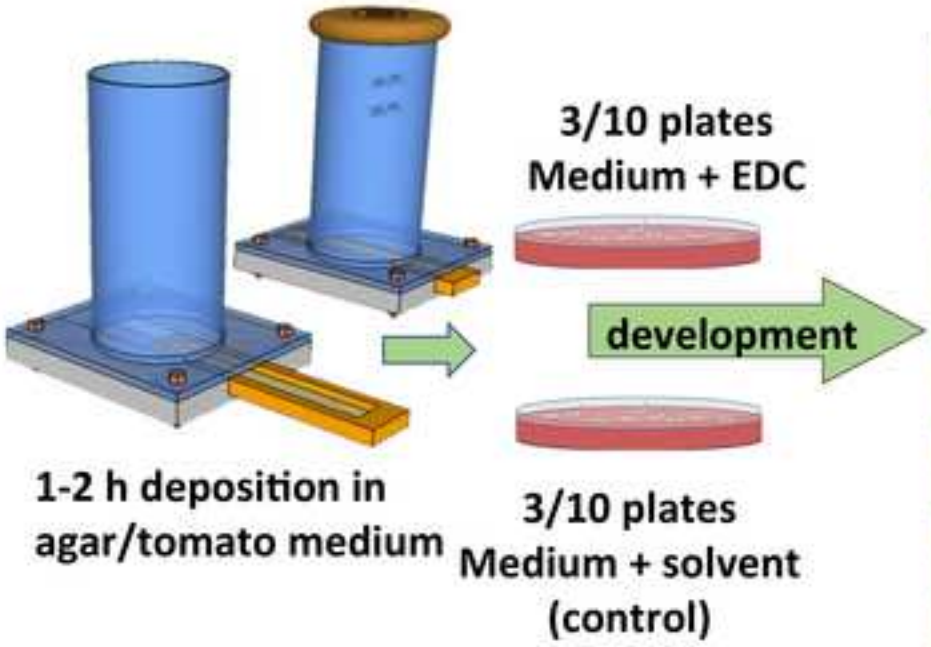
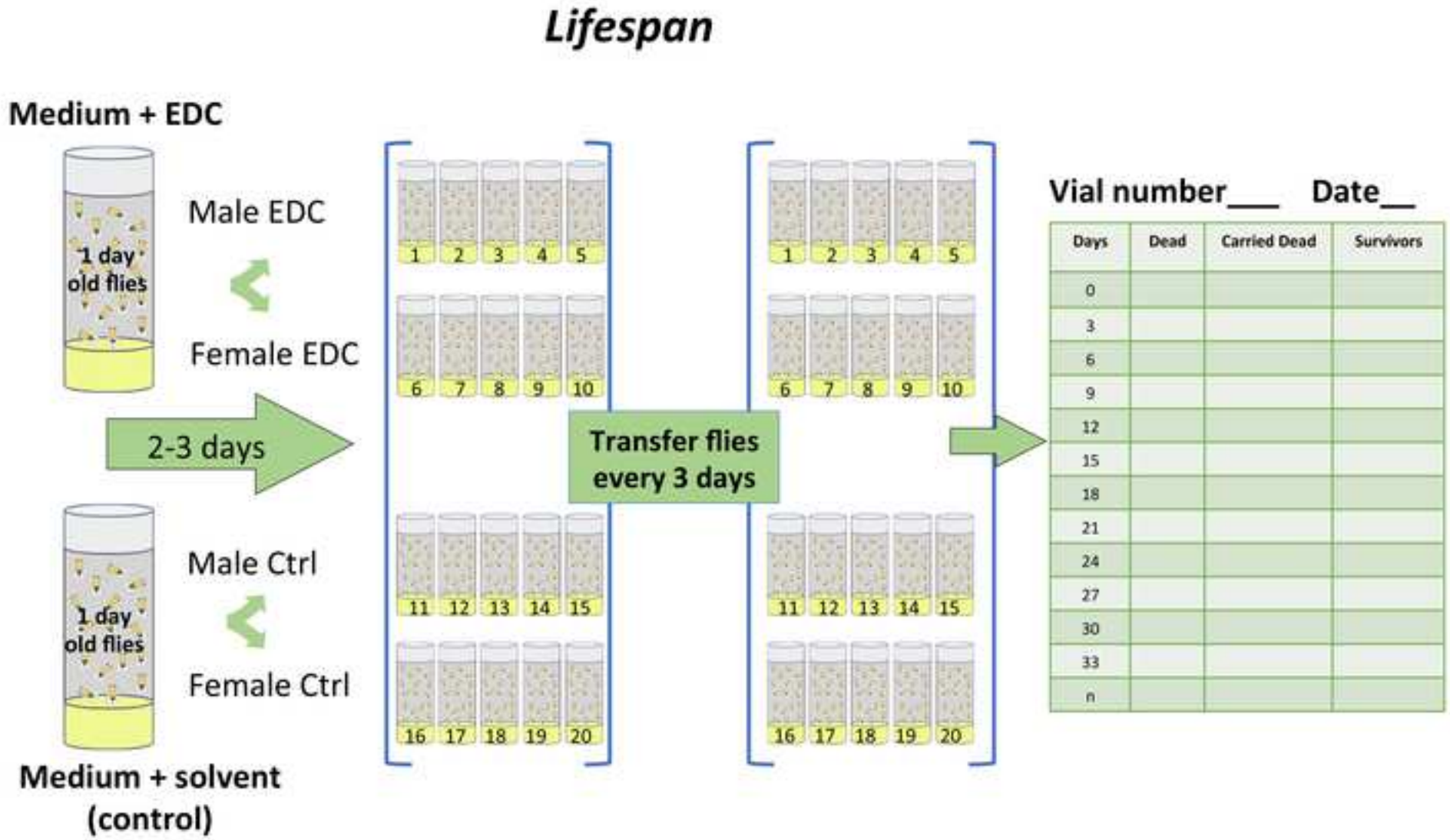


Plate number_____ Date _____

Days	Total Eggs.	Unfertilized + Dead embryos	Pupae	Adults
1				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
n				

Figure



Figure

	Medium + solvent							Medium +EDC						
Day	1	2	3	4	n	dead flies	living flies	11	12	13	14	n	dead flies	living flies
0	0	0	0	0	0	0	100	0	0	0	0	0	0	100
3	0	0	0	0	0	0	100	0	0	0	0	0	0	100
6	0	0	0	0	0	0	100	0	0	0	0	0	0	100
9	0	0	0	0	0	0	100	0	0	0	0	0	0	100
12	0	0	0	0	0	0	100	0	0	0	0	1	1	99
15	0	0	0	0	0	0	100	0	0	0	0	0	0	99
18	0	0	0	0	0	0	100	1	0	0	0	0	1	98
21	0	0	0	1	0	1	99	0	0	0	0	1	1	97
24	0	0	1	0	0	1	99	0	0	1	0	0	1	96
27	1	1	0	0	0	2	98	0	1	0	0	0	1	95
30	0	0	0	0	0	0	98	0	1	0	0	0	1	94
33	0	0	1	1	1	3	97	0	0	1	0	0	1	93
36	0	0	0	0	0	0	97	0	0	1	2	0	3	90
39	0	0	0	1	0	1	96	0	1	1	1	1	4	86
42	1	0	0	0	0	1	95	2	1	0	0	0	3	83
45	0	0	0	1	0	1	94	1	2	1	1	3	8	75
48	0	0	0	0	0	0	94	1	1	1	1	1	5	70
51	0	1	1	0	0	2	92	0	1	1	2	2	6	64
54	1	1	0	0	0	2	90	2	2	2	2	0	8	56
57	0	0	0	1	1	2	88	0	0	1	2	3	6	50
60	1	1	0	0	0	2	86	0	1	2	1	0	4	46
63	2	1	0	0	0	3	83	0	0	3	1	2	6	40
66	0	1	2	0	0	3	80	2	2	0	0	0	4	36
69	1	1	2	1	1	6	74	2	0	2	2	2	8	28
72	1	1	2	0	0	4	70	2	1	1	1	1	6	22
75	2	2	1	0	1	6	64	1	1	2	2	2	8	14
78	2	3	2	1	1	9	55	2	3	2	2	3	12	2
81	2	2	2	2	2	10	45	1	1	0	0	0	2	0
84	2	1	3	2	2	10	35	0	0	0	0	0	0	0
87	3	3	6	3	4	19	15	0	0	0	0	0		
90	5	3	2	3	2	15	0	0	0	0	0	0		
						Mean lifespan	74,38834951							57,99

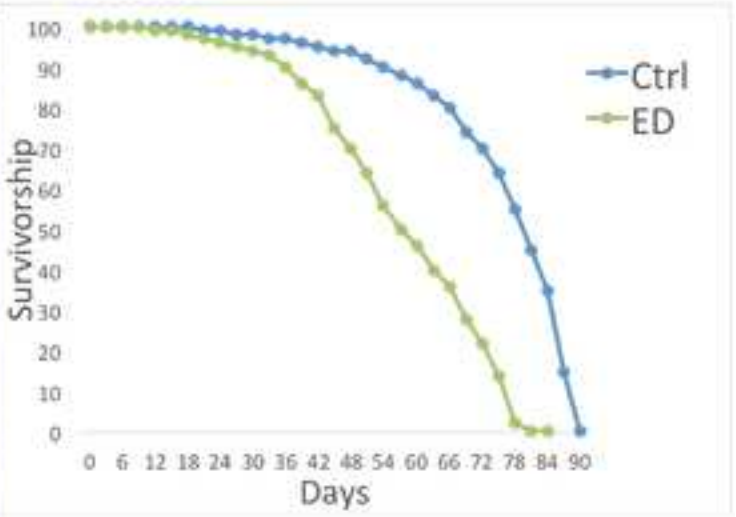


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_RE_Bovier 2018.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
17α-Ethinylestradiol	Sigma	E4876-1G	
Agar for Drosophila medium	BIOSIGMA	789148	
Bisphenol A	Sigma	239658-50G	
Bisphenol AF	Sigma	90477-100MG	
Cornmeal	CA' BIANCA		
Diethyl ether	Sigma		
Drosophila Vials	BIOSIGMA	789008	25x95 mm
Drosophila Vials	BIOSIGMA	789009	29x95 mm
Drosophila Vials	Kaltek	187	22X63
Embryo collection cage	Crafts		Plexiglass cylinder (12,5 x7 cm) with an open end and the other end
Ethanol	FLUKA	2860	
Etherizer	Crafts		cylindrical glass container with a cotton plug
Glass Bottle			250mL Bottles
Glass Vials	Microtech	ST 10024	FLAT BOTTOM TUBE 100X24
Hand blender Pimmy	Ariete		food processor
Instant Success yeast	ESKA		Powdered yeast
Laying tray	Crafts		plexiglass trays (11 x 2,6 cm) in wich to pour medium for laying
Methyl4-hydroxybenzoate	SIGMA	H5501	
Petri Dish	Falcon	351016	60x5
Red dye no. 40	SIGMA	16035	
Stereomicroscope with LED lights	Leica		S4E
Sucrose	HIMEDIA	MB025	
Tomato sauce	Cirio		

closed by a rectangular base in which a slot allows the insertion of special trays for laying

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Title of Article: METHODS TO TEST THE EFFECTS OF THE ENDOCRINE DISRUPTORS IN DROSOPHILA MELANOGASTER

Author(s): BOVIER T.F., CAVALIERE D., COLOMBO M., PELUSO G., GIORDANO E., DIGILIO F. A.

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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