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Laboratory Maintenance of the Lower Dipteran Fly *Bradysia (Sciara) coprophila*: A New/Old Emerging Model Organism

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Citation

Gerbi, S.A. Laboratory Maintenance of the Lower Dipteran Fly *Bradysia (Sciara) coprophila*: A New/Old Emerging Model Organism. *J. Vis. Exp.* (206), e66751, doi:10.3791/66751 (2024).

Date Published

April 19, 2024

DOI

10.3791/66751

URL

jove.com/video/66751

Abstract

Laboratory stocks of the lower dipteran fly, *Bradysia (Sciara) coprophila,* have been maintained for over a century. Protocols for laboratory upkeep of *B. coprophila* are presented here. These protocols will be useful for the rapidly increasing number of laboratories studying *B. coprophila* to take advantage of its unique biological features, which include (1) a monopolar spindle in male meiosis I; (2) non-disjunction of the X dyad in male meiosis II; (3) chromosome imprinting to distinguish maternal from paternal homologs; (4) germ line-limited (L) chromosomes; (5) chromosome elimination (paternal chromosomes in male meiosis I; one to two X chromosomes in early embryos; L chromosomes from the soma in early embryos); (6) sex determination by the mother (there is no Y chromosome); and (7) developmentally regulated DNA amplification at the DNA puff loci in larval salivary gland polytene chromosomes.

It is now possible to explore these many unique features of chromosome mechanics by using the recent advances in sequencing and assembly of the *B. coprophila* genome and the development of transformation methodology for genomic engineering. The growing scientific community that uses *B. coprophila* for research will benefit from the protocols described here for mating the flies (phenotypic markers for mothers that will have only sons or only daughters; details of mass mating for biochemical experiments), checking embryo hatch, feeding larvae, and other comments on its rearing.

Introduction

A complete understanding of biological principles requires the study of many diverse organisms spanning the Tree of Life. Although a broad range of organisms were described through the end of the 19th century, by the middle of the 20th century experimental studies became restricted to a handful of less than a dozen model organisms. With the advent of the genomic era and the goal to sequence genomes from all species in the Tree of Life[1], we are now in a position

to expand the types of organisms being used for laboratory experiments and to reap the advantage of their diversity. Such expansion of emerging model organisms for experiments has a prerequisite of being able to maintain them in the laboratory. Here, protocols are described for rearing one such emerging new/old model organism.

The majority of animal life on Earth is accounted for by four super-radiations of Insects[2]. Within the Insects, there are about 158,000 species of Diptera (true flies)[3], with about 3000 species in the family Sciaridae (black fungus gnats)[4]. The fruitfly *Drosophila* is the most thoroughly studied of the Dipteran flies. The lower Dipteran fly (Nematocera), *Bradysia (*previously called *Sciara) coprophila*, diverged 200 million years ago from *Drosophila*, which is a "higher Dipteran" fly (Brachycera). Therefore, *B. coprophila* is in a favorable taxonomic position for comparative studies with *D. melanogaster* (**Figure 1**). Moreover, *B. coprophila* has many unique biological features that are worthy of study in their own right[5,6,7]. Many of these features disobey the rule of DNA constancy in which all cells of an organism have the same DNA content. In *B. coprophila,* (i) the paternal genome is eliminated on a monopolar spindle in male meiosis I; (ii) there is non-disjunction of the X dyad in male meiosis II; (iii) germ line-limited (L) chromosomes are eliminated from the soma; and (iv) one or two X chromosomes are eliminated in the early embryo depending on the sex of the individual. Chromosome imprinting to distinguish maternal from paternal homologs was first discovered in *B. coprophila* and is at play for many of these chromosome elimination events. In addition to chromosome elimination, another bypass of DNA constancy occurs via developmentally regulated, locus-specific DNA amplification at the DNA puff loci in larval salivary gland polytene chromosomes. Studies of these unique features require laboratory maintenance of *B.coprophila*; details of its husbandry are presented here to facilitate such studies.

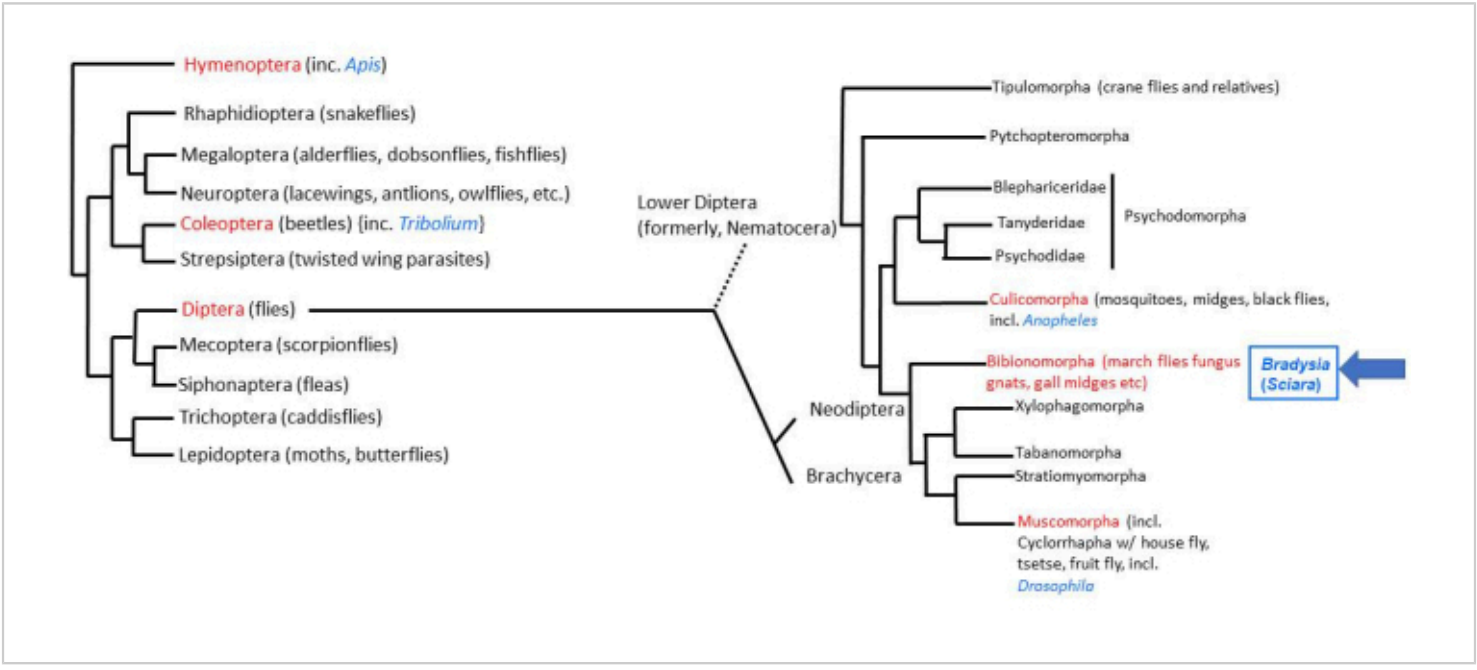


Figure 1: Phylogeny of *Bradysia (Sciara) coprophila*. Popular model organisms are indicated in blue font and their taxonomic order in red font. *Bradysia* and other Sciarid fungus gnats as well as mosquitoes are lower dipteran flies (formerly, suborder Nematocera), whereas *Drosophila* species are higher dipteran flies (suborder: Brachysera). Information on the left side of the figure is from Misof et al.[33]; information on the right side is from Bertone et al.[34] and Wiegmann et al.[2]. Please click here to view a larger version of this figure.

Previously, the genus *Sciara* had the largest number (700) of species for any eukaryote, prompting Steffan to subdivide them[8]. Subsequently, Shin proposed that the Sciaridae family be subdivided into the subfamily Sciarinae (with six genera including *Sciara, Trichosia,* and *Leptosciarella*), the subfamiliy Megalosphyinae (including the genus *Bradysia*) and three other groups (including *Pseudolycoriella*)[9]. The phylogeny of the Sciaridae has been studied further by several groups in recent years[9,10,11]. Over the past several decades, the names of many organisms in the Sciaridae family have changed[12]. Although most of the literature spanning more than a century refers to the organism that we study as *Sciara coprophila*, its current taxonomic name is now *Bradysia coprophila (syn. Bradysia tilicola and other synonyms)*[10]. They are found worldwide and are commonly known as fungus gnats since they eat mushrooms and other fungi. They were first described in 1804 by Meigen[13] in Europe and subsequently by Johannsen[14,15] in North America. *B. coprophila* was collected at Cold Spring Harbor Laboratory and laboratory stocks were established by Charles Metz in the early 1900s when he was a graduate student at Columbia University with Thomas Hunt Morgan. Thus, the current stocks reflect a century of inbreeding. Similarly, the biology of *B. coprophila* was further elucidated by decades of cytogenetic studies by Helen Crouse (who did her Ph.D. work with Barbara McClintock).

In the 1930s, *Bradysia* (*Sciara*) competed with *Drosophila melanogaster* as a model system for genetic studies. Despite its many unique biological features, *B. coprophila* was eclipsed by *D. melanogaster* as a popular model organism since radiation-induced phenotypic mutations were needed for genetic studies and were easier to achieve in the latter, even though *B. coprophila* is only slightly more resistant to gamma irradiation than *D. melanogaster*[16]. In the modern era of genomics, this is no longer a concern. Since the genome sequence[17,18,19] (Urban, Gerbi, and Spradling, data not shown) and methods for transformation[20,21] (Yamamoto and Gerbi, data not shown) for *B. coprophila* have recently become available, the time is now ripe to utilize it as a new/old emerging model system, as seen by the growing community of scientists that have adopted it for their research. This article describes procedures for its laboratory maintenance.

B. coprophila lacks a Y chromosome, and the sex of the offspring is determined by the mother. Females that have the X' ("X-prime") chromosome with a long paracentric inversion will have only daughters, whereas females that are homozygous for the standard (non-inverted) X chromosome will have only sons[5] (**Figure 2**). Sequence information is available for the X' chromosome[19], but the molecular mechanism remains to be elucidated on how the X' chromosome determines that offspring will be females. Males never have the X' chromosome, and after fertilization, females are X'X (heterozygous for the X') or XX. Adult X'X females can be distinguished from XX females by phenotypic markers on the wing (**Figure 3**). X'X females (who will have only daughters) can be recognized by the dominant Wavy (W)

wing marker on the X' (as in the HoLo2 stock)[22]. Alternatively, XX females (who will have only sons) can be recognized by the recessive petite (p) wing marker on the X as in the 91S stock[23]. In this case, X'Xp females will have full-length (not petite) wings and will have only daughters. The stock 6980 carries a recessive marker on the X chromosome for swollen (sw) veins[24], as well as the dominant marker Wavy on the X', allowing two markers for selection for crosses. The degree of expression of Wavy can vary and seems weaker in overcrowded vials where food is limiting or if the temperature becomes too warm. The Wavy wing phenotype is exceptionally strong if larvae are kept in the cold room (4°-8 °C) instead of the usual 21 °C. Although the recessive petite wing marker is not variable and is very easy to identify, 91S stocks are used less frequently since they are less healthy than the HoLo2 stock. The *B. coprophila* mating schemes are presented here (**Figure 2**), and described in detail for the HoLo2, 7298, and W14 stocks (**Supplemental File 1**), the 91S stock **(Supplemental File 1**), the 6980 stock **(Supplemental File 1**), and the translocation stocks **(Supplemental File 1**). The translocation stocks are no longer extant; they were reciprocal translocations of heterochromomeres (H1, H2, and H3) on the short arm of the X that contains the ribosomal RNA genes[25,26,27].

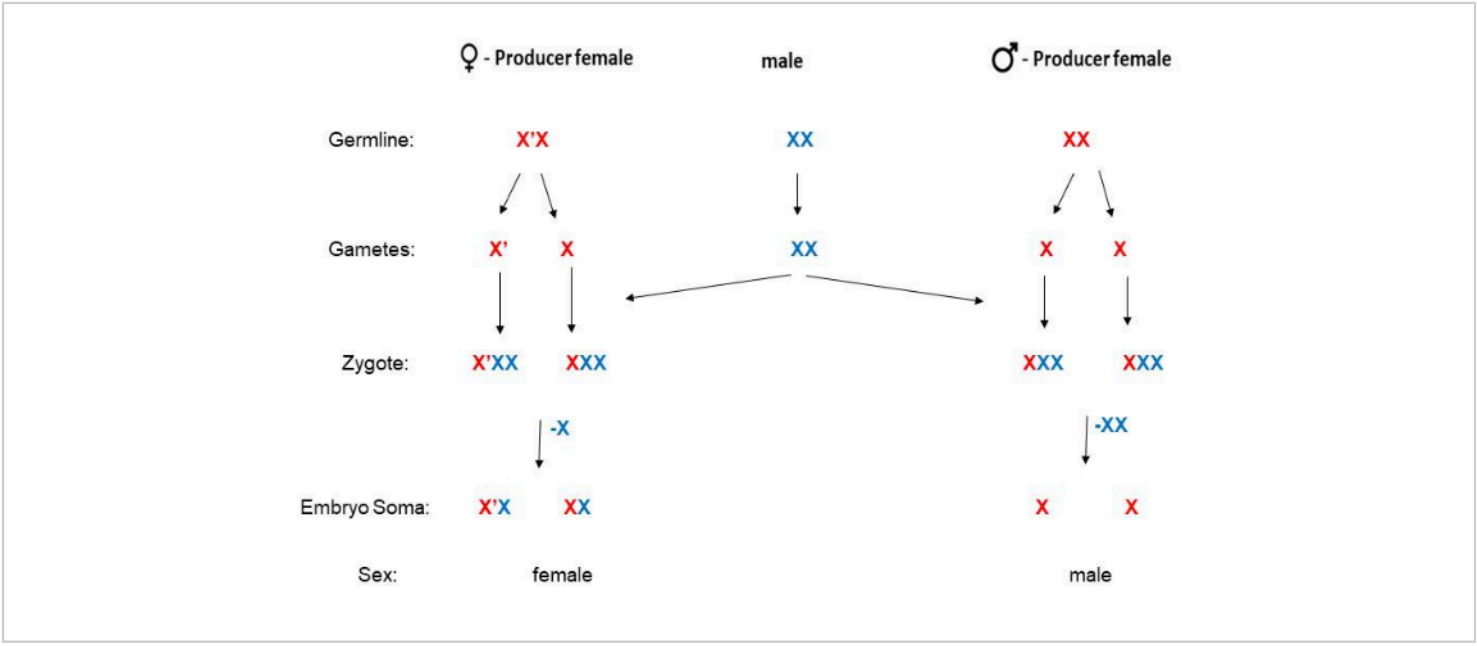


Figure 2: Mating scheme for *B. coprophila*. This organism has no Y chromosome (the male soma has a single X); mothers determine the sex of their offspring. XX mothers have only sons and X'X females have only daughters. The X' chromosome has a long paracentric inversion when compared to the X chromosome. The paternal or maternal lineage of the X (or X') chromosome is denoted by blue or red, respectively, in this figure. Sperm are haploid for the autosomes but have two copies of the X chromosome due to non-disjunction in meiosis II. The somatic lineage of early embryos eliminates one or two copies of the paternally derived X if they will be female or male, respectively. Please click here to view a larger version of this figure.

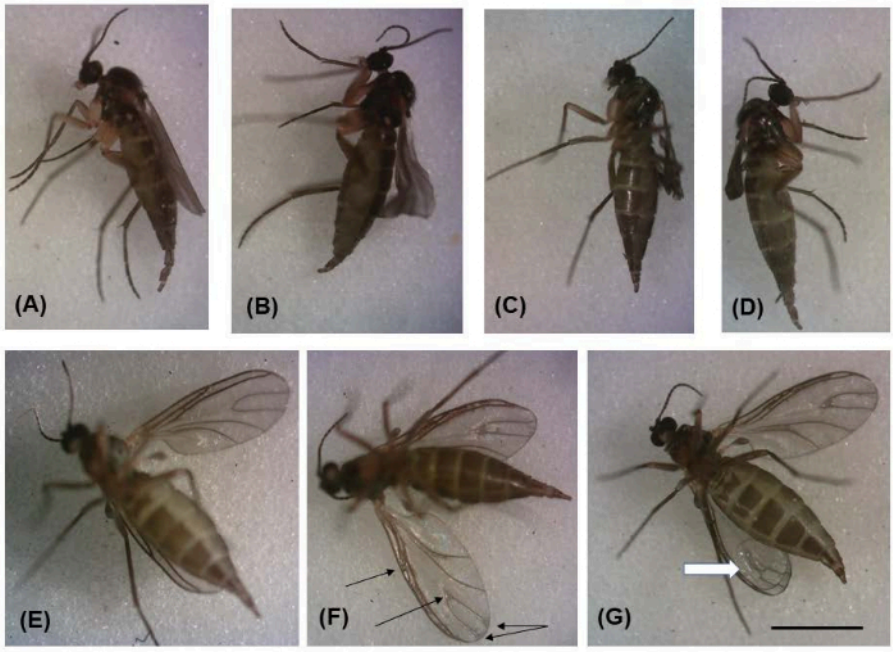


Figure 3: Wing phenotypes of *B. coprophila*. Adult female flies are shown with various wing phenotypes: (**A**) straight-wing (XX), (**B**) Wavy wing (X'_WX), (**C**) extreme Wavy wing (X'_WX) phenotype that has a shriveled appearance after storing larvae in the cold roo**m,** (**D**) petite wing (XpXp) that is vestigial-like, (**E**) straight wing with wildtype (XX) and not swollen veins**,** (**F**) straight wing with swollen veins (XswXsw) where small bubbles (black arrows) appear on the upper edge of the wing and/or near the tip of both wings, (**G**) extreme example of swollen where a blister (white arrow) occurs on one or both wings. Males lack the X' chromosome and therefore will never have Wavy wings, but they have petite or swollen wings in the 91S stock or 6980 stock, respectively. Scale bar = 1 mm. Please click here to view a larger version of this figure.

The goal in stock maintenance is to perform crosses where half the crosses are from female-producing mothers and half the crosses from male-producing mothers to have equal numbers of female and male adults in the next generation for subsequent crosses. However, this also involves planning since the life cycle for males is shorter than for females and adult males emerge up to a week before adult females. Nature accommodates for this asynchrony between the sexes by having the male embryos emerge as larvae 1-2 days after the female larvae from a cross on the same date. However, to ensure that male and female adults are available at the same time for laboratory crosses, female development can be somewhat speeded up by leaving vials with female larvae at room temperature rather than at 21 °C or placing vials with male larvae at slightly cooler temperatures (e.g., 16 °C). Another route that is more foolproof is to do crosses with female-producer mothers on Monday and crosses with male-producer mothers on Friday of the same week. The easiest route, which is what we employ, is to perform crosses with female-producer and male-producer mothers on the same day each week and perform crosses on that day in each consecutive week. In that approach, adult females from a

cross on week 1 can be mated with adult males that emerged from a cross on week 2.

The life cycle for female *B. coprophila* is 5 weeks when raised at 21 °C (**Table 1**). The length of their life cycle is somewhat longer at cooler temperatures or if they are underfed. The life cycle of male B. *coprophila* is ~4-4.5 weeks since they pupate 0.5-1 week before females. The end of each larval instar is marked by the shedding of the cuticle, which is triggered by a burst in the level of the steroid hormone ecdysone. Unlike *D. melanogaster,* which has three larval instars, *B. coprophila* has four larval instars.

Developmental Stage	Days post mating (dpm)	Length of stage (days)
Egg laid	1-2	
Embryo	1-2 to 7-8	~7 days
Larva		
Larval instars 1, 2, and 3	7-8 to 16-19	~10
4th larval instar pre-eyespot	16-19 to 21-24	5
4th larval instar eye-spot stage	21-24 to 25-28	4
Pupa	25-28 to 30-33	5
Adult	lives 1-2 days at 21 °C if mated or lives 2-3 weeks at 16 °C if not mated.	

Table 1: Life cycle of female *B. coprophila* at 21 °C.

B. coprophila can be kept anywhere in the range of 15 °C-25 °C, with development progressing more slowly at cooler temperatures. This insect prefers a humid environment (being found in the soil of houseplants or mushroom beds), so we keep a beaker with deionized water in the incubator. *B. coprophila* can be kept at room temperature in a metal bread box with a loosely fitting lid and containing a beaker of water, but they go into heat shock at 37 °C[28], which is a danger in hot climates. Michael Ashburner and others have tried with little success to store *D. melanogaster* in the cold to reduce the time needed for stockkeeping. In contrast, a major advantage of *B. coprophila* is that vials with mid-stage larvae can be stored for up to 3 months on an open shelf in the cold room (4-8 °C) with minimal care of only feeding once a month. They develop extremely slowly in the cold up to the pupal stage and will emerge as fertile adults when the vials are brought back to 21 °C. Presumably, this mimics their overwintering in the wild. This cold-induced developmental stalling might be comparable to that seen after gamma irradiation of mid-stage *B. coprophila* larvae[16], but developmental stalling is not seen in late-stage larvae that have passed the point of no return for their normal developmental progression.

Protocol

The protocols described here represent a century of experience from the *Bradysia* (*Sciara*) stock centers overseen

sequentially by Charles Metz, Helen Crouse, and Susan Gerbi as well as input from others.

1. Mating crosses

1. Use one adult female and two adult males per 28 mm diameter glass vial. Once 100% success is achieved in recognizing wing phenotypes for mothers who will have just daughters or sons, use two females and two males per vial to increase the number of larvae/vial. Add females to each vial before adding the males who wake up faster from the anesthesia than the females.

   NOTE: The description below presumes that you have CO_2; alternatively, ether can be used to anesthetize the adult flies.

2. If using ether to anesthetize the adult flies, tape a pad of folded laboratory wipes (e.g., kimwipes) to the inside of a round glass lid of a Coplin jar and use a dropper to transfer some ether from the bottle to moisten the laboratory wipe pad (but not saturated and so wet that ether liquid will fall off it and risk drowning the flies). For step 1.6 below, place the moistened ether pad on top of the opened vial for ~1 min until the adults stop moving. Once the adults have been transferred to a white ceramic tile plate (used instead of the white fly-pad), periodically (when the flies' legs begin to twitch) hold the pad that has been freshly ether-moistened over (not touching) the flies on the plate for ~1 min.

   CAUTION: Ether is flammable and should be stored in a ventilation hood and not in a refrigerator.

3. Arrange within arm's reach a tray with vials of adult females, a tray with adult males, and a tray of empty vials containing 2.2% (wt/vol) agar. On the lab bench, place placard notes stating "female" or "male" and the wing phenotype of the mother so that the vial with adults selected for the cross will be placed into the correct group, where all vials in one group will have just male progeny and all vials in the other group will have just female progeny.

   NOTE: Be sure that no condensation droplets are within the vial as the adults will stick to the droplets. If the vials were stored in a plastic box, put the vials on a lab bench at room temperature for at least 1 h before use to let the condensation evaporate.

4. Turn on the CO_2 gas and the lamp for the dissecting microscope.

   NOTE: A light source with fiber optics is preferred as it emits less heat, which causes the anesthetized flies to wake up faster.

5. Vigorously tap the vial with adults on a rubber pad so that the adults fall to the bottom of the vial and remove the plug; insert the CO_2 gun nozzle and add back the plug.

6. Depress the nozzle trigger so that CO_2 flows into the vial for ~1 min to anesthetize the adults.

7. Put a foot on the foot pedal so that CO_2 will flow onto the white fly-pad rather than the gun nozzle. Keep the foot pedal depressed the entire time that flies are on the white fly-pad (or else intermittently depress the foot pedal whenever the flies' legs begin to twitch).

8. Remove the nozzle and plug from the vial and invert the vial over a white fly-pad under a dissecting microscope.

9. Tap the bottom of the inverted vial against the microscope so that the adults fall from the vial onto the white fly-pad.

10. Select the fattest adults (recently eclosed with white abdomens) and use fine-tipped forceps to gently pick

up the adult by its middle or hind leg. Do not injure the front legs that are used for the mating dance. Do not use adults that have just eclosed and whose bodies are fully white and not yet turned black because their wings will be short and not fully developed so that the wing phenotype cannot be scored. Adults with slimmer abdomens can still be used, though they have reduced fertility. Do not use skinny adults whose wings are raised vertically away from their body, since they are dead.

11. With the other hand, remove the plug from the vial with 2.2% (wt/vol) agar. With the hand holding the forceps with the adult, tap the forceps vigorously against the top inside wall of the vial so that the adult falls to the bottom of the vial. Replace the plug into the vial.

12. Repeat steps 1.5-1.11 above to set up each vial containing female adults. Use a paintbrush to sweep the unused flies from the white fly-pad back into their parent vial. Put a checkmark on the label of the parent vial to indicate that it has been used for mating (though it can be used again if needed).

13. For routine stock maintenance, set up 6-8 vials with female-producer mothers and 6-8 vials of male-producer mothers (**Figure 4**). Set up half of the vials with mothers (or fathers) from one adult vial and the other half of the new vials using a different adult vial to minimize genetic bottlenecks.

    1. Occasionally, a mis-segregation event will produce an exceptional male in female-producing vials. If a vial with adult females has an exceptional male, remove and squish to kill that male. If possible, discard all females from that vial and wait a few days until more adult females eclose and can be safely used for crosses.

       NOTE: It is preferable to not use the females in that vial for crosses because they may have mated with the exceptional male and not produce a fertile cross.

14. Once the adult females have been added to all the vials, repeat steps 1.5-1.11 to add two adult males (**Figure 4**) to each vial already containing female flies. Tap the vial with females on a rubber pad so that they do not escape when the two males are added sequentially.

    NOTE: Work quickly and do not overanesthetize the adult flies as that will kill them.

15. Add a label to each vial stating the stock, the cross (for female or male progeny), whether the mother came from adult vial #1 or #2, and the date of mating. Also enter the information above into a notebook and state the number of vials set up for each cross.

    NOTE: It is convenient to add a paper towel to separate the male-producing vials from the female-producing vials in the tray.

16. Leave the vials undisturbed on the countertop for ~15 min to be sure that the adults wake up and fly around. Observe if they are mating (very soon after they wake up) where the female and male are oriented posterior to posterior (the male clasper will grab the female's pointed ovipositor) (**Figure 4, bottom**).

    NOTE: An adult female will accept an adult male just once, so do not jostle the vials after mating as this could separate the male from the female while in the process of mating and that female will not re-mate.

17. Place the tray with mated vials in the incubator (e.g., 21 °C). Label the tray with the name of the stock (e.g., HoLo2) and the week of the 5 week cycle (weeks 1, 2, 3, 4, or 5).

NOTE: Keep the tray with just-mated flies in a separate part of the incubator or another incubator as a reminder to not feed the vials until the larvae have emerged (described below).

2. Mass mating

NOTE: Typically, a single *B. coprophila* mother will have 60 offspring in her brood. When a larger number of offspring is required for experiments, a mass mating can be done instead of the single-pair mating described above. The mass mating can be done in the standard 28 cm diameter glass vials when the mothers will be collected a day later for induced egg-laying and embryo collection. However, if a larger number of larvae are needed, the mass mating is done in a jar with a larger surface to prevent overcrowding. Pierce several small holes in the lid of the jar so that the larvae can get some air to breathe.

1. Follow the steps above in section 1 but use fine-tipped forceps to move all female-producer mothers (or all male-producer mothers) to a front corner of the white fly-pad. Use 10-15 anesthetized fat adult females and sweep this group with a paintbrush into the vial or jar with small holes pierced in the lid.
   NOTE: Perform steps 2.1 and 2.2. quickly for mass mating to avoid over-anesthesia that would prevent fertile crosses.
2. Select 20-25 anesthetized fat adult males and move them with fine-tipped forceps to the front corner of the white fly-pad.
3. Vigorously tap the vial or jar with the females on a rubber pad so that they do not escape when the plug or lid is removed to sweep in the anesthetized cluster of adult males from the white fly-pad using a paintbrush.

3. Embryo collection after mass mating

1. One day (24 h) after mating, perform steps 1.5-1.11 to anesthetize the adult flies (mixture of females and males) and transfer them to a white fly-pad.
   NOTE: Oogenesis is not yet completed when the adult flies mate, which triggers finishing meiosis; the mature eggs are fertilized by sperm stored in the spermatheca when the eggs are discharged [29]. Completion of oogenesis can take 1-2 days, which is why 1 day is allowed after mating before egg-laying is induced.
2. With fine-tipped forceps, pick up an adult female by her wings and place her on a 100 mm diameter Petri dish containing 2.2% (wt/vol) agar, with her wings inserted into the agar. Repeat this step sequentially for each adult female on the white fly-pad. Discard the adult males on the fly-pad.
3. Once all the female flies are impaled on the agar, induce egg-laying by gently squeezing the head with forceps until the female fly has seizure-like movements. Alternatively, gently squeeze her thorax. She will then lay a cluster of fertilized eggs within 30-60 min. Cover the Petri dish with its lid that is moistened with a water-dampened laboratory wipe to prevent electrostatic attraction of the eggs to the lid.

4. Checking "hatch" of larvae

1. Check for "hatch" of larvae 1 week after mating; remove the plug from the vial and use a dissecting microscope to score for larvae. Their black jaw will open and close repeatedly and they will slowly move forward. If there are only a few larvae, write "few" on the vial label, as a reminder to feed that vial less. Examine each vial in the

tray with that cross and enter the number of vials with larvae in the notebook in a column next to the number of vials that were set up in that mating.

NOTE: Deep yellow eggs will not develop. White eggs are likely to develop and 1 day before the larvae emerge, black pigment (the future jaw) will develop at the anterior end of the egg. Do not mistake mold with a white filament ending in a black sphere at its end for larvae-the mold will not move, unlike the larvae that crawl forward on the agar.

2. Add a small bit of straw (just once) to each vial with larvae to control excess humidity and provide a hiding place of cover for the larvae.

3. Move the tray to the incubator (e.g., 21 °C) with trays of larvae from previous weeks' mating crosses and begin to feed the vials with newly emerged larvae (see section below on feeding).

NOTE: Generally, the larvae will be in a cluster near their dead mother and they will begin to eat her; presumably this transfers the yeast from the mother's gut to the gut of the larvae. You can begin feeding on the day that the larvae emerge or within 2 days thereafter.CAUTION: If feeding is delayed, the larvae will eat each other, with only one fat larva remaining per vial!

4. Continue to check vials that did not yet have larvae for 3 days per week. If no larvae have emerged after 7-10 days, discard the vial or store it for vial washing.

5. Making the food

NOTE: All the food ingredients should be pesticide-free!

1. Use a tablespoon to measure the following ingredients by volume and deposit them into a metal or glass pan (e.g., an 8 inch x 8 inch metal baking pan): 4 parts oat straw (8 Tbsp), 2 parts Shitake mushroom powder (4 Tbsp), 1 part spinach powder (2 Tbsp), 1 part nettle powder (2 Tbsp). Use the tablespoon to mix the ingredients well in the pan.

NOTE: Alternatively, 2 parts of just spinach powder or just nettle powder can be used instead of 1 part of each.

2. Cover the pan with aluminum foil and autoclave on a dry cycle for 20-30 min. Let it cool to room temperature overnight or longer.

3. Remove the foil from the pan and break up the caked sterilized food mixture, using a grinding motion with the tablespoon to create a powdery mixture.

4. Add 1 part (2 heaping Tbsp) of Brewer's yeast and mix well into the autoclaved food mixture.

NOTE: The Brewer's yeast is not autoclaved as that would kill the yeast.

5. Transfer the food mixture to a sterile capped jar.

NOTE: The same type of 240 mL jar as used for mass mating can be used, and the recipe above will fill the jar. Similarly, the same type of sterilized capped jar should be filled with just straw that was autoclaved in a foil-covered pan.

6. Feeding

NOTE: Adjust the amount of food given according to the age and amount of larvae. Give a lot of food to jars with many larvae from a mass mating. Give just a light sprinkle of food to Petri plates with larvae that are being stored for a few days for developmental staging. The feeding method described below was developed in the lab of Charles Metz [29], and it has been used by his lab and by Helen Crouse, Susan Gerbi, and others successfully for a century.

1. Wash hands and rinse well to remove soap.

NOTE: Gloves are not recommended as they diminish the feel of your fingers for regulating the amount of food given to each vial.

2. Store a capped sterile jar with ground straw and a sterile jar with food in the incubator (e.g., 21 °C) where the larvae are kept. Remove the lid from the jar and pour some food into a clean bowl (e.g., a candy dish) for easier access; replace the lid on the jar containing the remaining food. Keep the open bowl with food in a metal or glass small tray; this will inhibit mites or other insects from crawling over the wall of the tray to enter the bowl with food.

3. Pick up some food between the second and third fingers (or between the thumb and second finger). With the other hand, remove a vial from the tray and remove the plug, holding it with fingers while feeding. Examine the vial for the age and number of larvae and deposit the appropriate amount of food into the vial by rotating the two fingers holding the food against one another. Vials with newly emerged larvae just need a few grains of food. Vials with older larvae should have a thin layer of food covering the top of the agar.

4. If there is white mold in the vial, use 70% ethanol sprayed on a laboratory wipe to wipe clean a long metal probe (e.g., with a wooden handle), remove the plug, and insert the clean probe into the vial to tap down the mold on the surface of the agar. If there is a lot of mold, swirl the probe to wrap the mold around the probe to remove it from the vial. Do not disturb the top of the agar as larvae are living there; add just a small amount of food and replace the plug in the vial. Wipe the probe clean with a laboratory wipe dampened with 70% ethanol before storing the probe or using it to clean another vial.

5. Once all vials in a tray have been fed, replace the tray in the incubator and remove the next tray for feeding as in step 6.3.

6. After feeding is completed, pour the remaining food from the bowl into the previously sterilized jar, cap the jar, and store it in the incubator.

7. Collection of larvae or pupae on Petri plates

1. For small numbers of larvae, use a metal probe or spatula that has been wiped with 70% ethanol to dig into the top layer of agar in a vial to transfer the adhering agar with some larvae to a 100 mm diameter sterile Petri dish half-filled with 2.2% (wt/vol) agar.

2. For larger numbers of larvae, insert a spatula along the wall at the bottom of the vial to ease the agar plug out of the vial and deposit it face-side up in an empty Petri dish. Use a dissecting microscope to find the larvae at the top of the agar plug and transfer them with fine-tipped forceps to a 100 mm diameter sterile Petri dish half-filled with 2.2% (wt/vol) agar.

3. Use a dissecting microscope to sort the larvae with fine-tipped forceps into groups of the same developmental stage.

   NOTE: Due to slight developmental asynchrony, there will be several different clusters of sorted larvae on the Petri plate with 2.2% (wt/vol) agar.

4. Add a tiny sprinkle of food and place the Petri dish with larvae in the incubator. Remove it daily for observation with the dissecting microscope for selection of the desired developmental stage.

   NOTE: For DNA puff studies, early eyespot larvae proceed through 10x5, 12x6, 14x7, and edge eye/drop-jaw stages with ~1 day at each of these stages [30,31].

Choose pupae whose eyes are ¼ to ½ filled with pigment to have meiosis I and II stages in the pupal testes.

8. Cold room storage of larvae

1. As a backup, keep in the cold room about four vials of female larvae and four vials of male larvae from 2 consecutive weeks of crosses.
2. For the backup storage, feed larvae that are early 4th instar (pre-eyespot stage) and put them in an open tray on a shelf in the cold room; feed these vials just once a month.
3. Remove the 16 vials from the 2 consecutive weeks of mating crosses from the cold after 2-3 months (mark this on a calendar as a reminder); place them in an incubator (e.g., 21 °C) to feed them normally and let them develop into adults to use for crosses.
4. When the vials are removed from the cold room, put a fresh set of vials with early 4th instar larvae in the cold room so that these vials are always available as a backup for stocks.

   NOTE: Viability after cold room storage has not been tested systematically for different developmental stages, but our experience reveals that cold storage of early 4th instar larvae works well.

9. Vial washing

1. After the adults have died (~2-3 weeks after eclosion), remove the vials from the incubator and place them at 60-70 °C for 1 hour to kill any remaining organisms.
2. Remove the cotton plugs and store them in a plastic box.
3. Use a spatula to scrape the agar plug from the vial into a waste can.
4. Soak the vials overnight or longer submerged in water in a dishpan.
5. Use a test tube brush saved just for this purpose (never used for vessels with chemicals nor with soap) and scrub up and down in the vial under running tap water. Place the cleaned vial open side down in a metal basket. Fill the basket with cleaned vials.

   NOTE: Never use soap in the vials as any residual soap could kill *B. coprophila*.
6. Add a wire mesh lid to the basket, and while holding the lid in place, invert the basket to fill all the vials with deionized water. While still holding the lid in place, invert the basket to empty the deionized water from the vials. Repeat the deionized water rinses 4x.
7. Place the baskets with cleaned vials on paper towels or an open mesh platform (e.g., lab cart) to dry overnight or longer. Store the dry vials in a drawer.

   NOTE: Do not let vials with dead adults remain too long before washing them as it could invite an infestation of mites.

10. Pouring agar

1. Fill a large container (metal basket or metal tray) with clean vials and add a cotton plug to each vial. Reuse the plugs taken from old vials when the vials were washed, and use the plugs repeatedly until they degrade and fall apart and should be discarded. Sterilize the container with the plugged vials on a dry cycle for ~30 min and let them cool to room temperature to store them in the autoclave container. Always keep two containers (one actively in use and one spare) with autoclaved plugged vials on hand.

2. Place 11.0 g of agar powder in a 1 L Erlenmeyer flask and add 500 mL of distilled water to make a 2.2% (wt/vol) agar solution sufficient to pour ~24 vials (~21 mL/vial). Swirl the flask and place it in a microwave oven.
   NOTE: From here on, use a heat-resistant autoclave glove to handle the flask. The same 2.2% (wt/vol) agar solution can be poured into Petri plates (for picked larvae) or jars (for mass matings) if these are needed.
3. Microwave for 1 min, remove the flask to swirl it, replace it in the microwave oven, and microwave it again for 1 min. Repeat this several times.
4. Watch the flask through the glass door of the microwave oven. When the agar solution begins to foam and boil up, immediately use the manual stop button. Do not swirl the flask at this point (it could boil over), and carefully remove it from the oven to the counter and let it rest for a few minutes.
5. Remove the plug from a sterilized vial and hold it with one hand; use the other hand to pour ~2.5 cm high 2.2% agar into the sterilized vial. Then, replace the plug into the vial. Repeat this until all the agar has been poured.
   NOTE: If too little agar is poured into the vial, it will dry up more quickly during use and shrink away from the walls of the vial. If too much agar is poured into the vial, it is hard to focus on the top layer when scoring the "baby-hatch" of larvae with a dissecting microscope.
6. Let the poured vials remain on the counter at room temperature for 1-2 days so that the agar hardens and the moisture evaporates completely. Use the vials for mating crosses or store them lying on their side in a plastic box with a tight-fitting lid; place a paper towel dampened with water on top of the vials before placing the lid on the box (this will prevent the agar from drying out and shrinking away from the wall of the vial). Store the box with poured vials at room temperature for a few days or in a 4 °C refrigerator or cold room for up to 2 weeks. Before using the stored vials, remove them from the box and leave them on the counter at room temperature for an hour or two to let the condensation evaporate from inside the vials (the adult flies would stick to the condensate and drown).

11. Making plugs for vials

1. Place a clean vial in a styrofoam 50 mL test-tube holder (force the tube in so it stands up straight). Cut a square of cheesecloth (2-4 layers thick) and place it on top of the vial.
2. Place scraps of cheesecloth from previously made plugs on top of the cheesecloth to create a thicker layer. Place cotton on top of the cheesecloth and press down into the vial, making sure to tightly fill the top inch or so of the vial.
3. Hold the top of the ends of the cheesecloth together and tie it with string. Cut the excess string and excess cheesecloth (leave enough tails of the string to keep it tied and enough cheesecloth to comfortably grasp the plug).
   NOTE: Fit plugs so that they are snug. You should be able to pull them out with your index and forefinger while you hold them in one hand. If you pick up the vial by the plug and it falls out, it is too loose. If it makes a loud popping sound when pulled out, it may be too tight. If too tight or if the plug has a layer of hard, dry agar on it, the flies may suffocate. If the plug is a little too loose or slipping down the sides of the vial, it can be tapped on the countertop to squash it into a slightly wider size.

12. Typical weekly schedule (for greatest efficiency, perform tasks in the order listed)

1. Monday (~30 min)
    1. Feed vials with larvae.
    2. Pour agar into sterile clean vials with plugs.
2. Wednesday (~2 h)
    1. Place vials with dead adults in an oven for 1 h and store for washing.
    2. Check for larvae "baby-hatch".
    3. Feed vials with larvae.
    4. Perform weekly mating crosses.
    5. Other duties as needed: autoclave clean vials with plugs to have a reserve supply as needed; make new food and autoclave straw as needed; autoclave jars as needed for food and straw.
3. Friday (~30 min)
    1. Check for larvae "baby-hatch".
    2. Feed vials with larvae.

Representative Results

The protocols described here have led to proven success in raising *B. coprophila*. When recently eclosed fat adults are chosen for mating (**Figure 4**), over 90% of the crosses can be fertile and yield offspring. The fertility success does vary with different stocks (**Table 2**). Stock 7298 (X' chromosome with Wavy marker) was the healthiest of the stocks but went through a period of decline, apparently due to activation of DNA mobile elements creating genome rearrangements[32]. The HoLo2 stock represents a healthy strain derived from 7298, where apparently the genome rearrangements have stabilized, and it has replaced the parental 7298 stock in the stock center. The HoLo2 stock is the one that was used to sequence the *B. coprophila* genome and is the one used most widely by various laboratory groups. Recently, CRISPR mutagenesis of HoLo2 flies has been used to create the W14 stock with a white eye phenotype to be used for transformation with fluorescent eye markers (Yamamoto and Gerbi, data not shown). The W14 strain is exceptionally robust. The 6980 stock (Wavy wing and swollen vein markers) is somewhat less robust and the 91S stock (petite wing marker) is even less robust.

Successful crosses result in embryos (**Figure 5**). Embryos undergo elimination of the imprinted paternal X chromosomes at the 7^{th} to 9^{th} cleavage division. In addition, germ-line limited L chromosomes are eliminated from the somatic lineage in embryos at the 5^{th} to 6^{th} cleavage division. The embryos emerge as larvae, which should not be confused with mold that also may be present (**Figure 6**). Eyespots (anlage to the adult eyes) appear at the second half of the 4^{th} larval instar (**Figure 7**). The size of the eyespots provides a convenient phenotypic marker for the onset and progression of DNA puff amplification, which is one of only two known examples of naturally occurring developmentally regulated site-specific intrachromosomal DNA (gene) amplification. Subsequently, pupae develop, and the amount of pigment filling their eyes can serve as a developmental marker for meiosis I and II in spermatogenesis (**Figure 8**) with its unique chromosome behaviors in these divisions.

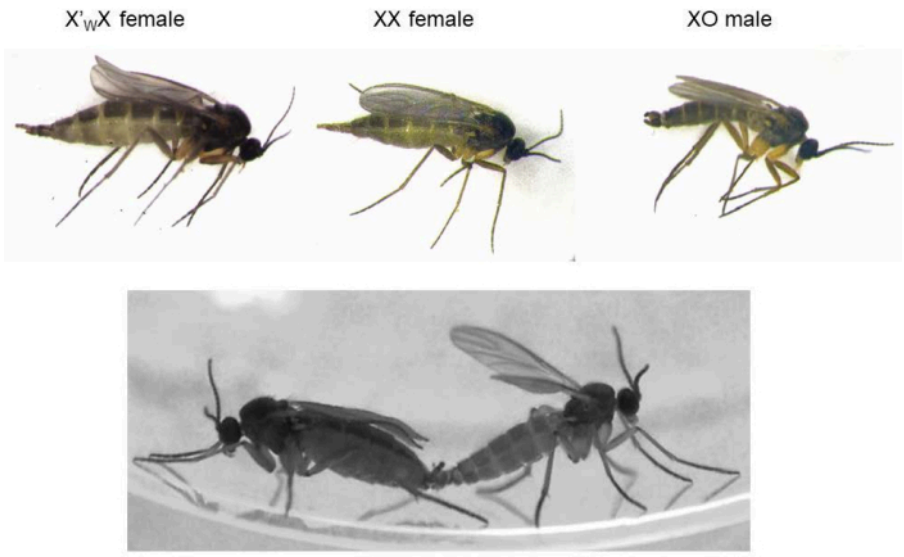
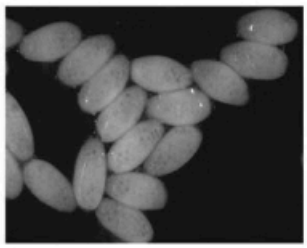


Figure 4: Mating of adult *B. coprophila* flies. The upper panel shows the three types of adult flies in the HoLo2 stock: female-producing mothers with Wavy wings (X'_WX adult females), male-producing mothers with straight wings (XX adult females), and males with straight wings (X0 adult males). Note the pointed ovipositor at the posterior end of the female flies and the hook-shaped clasper at the posterior end of the male flies. The lower panel shows a male and female mating, where the male clasper has grabbed the female's ovipositor. The sperm will be stored in the spermatheca of the female and will fertilize the eggs as they are discharged to the outside. The length of adults is 2.0 mm (males), 2.5 mm (females). Please click here to view a larger version of this figure.



Cycle	Hours after egg deposition
Syngamy	1.4 ± 0.5
1	1.6 ± 0.5
2	2.0 ± 0.4
3	2.3 ± 0.4
4	2.9 ± 0.5
5	3.3 ± 0.5
6	3.7 ± 0.3
7	4.2 ± 0.5
8	4.9 ± 1.4
9	5.6 ± 0.9
10	7.0 ± 1.3
11	9.3 ± 1.1

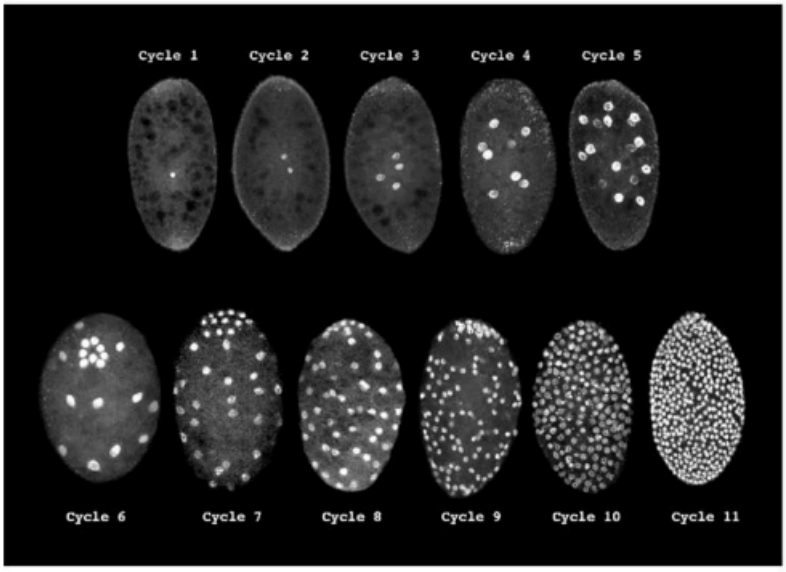


Figure 5: *B. coprophila* embryos. The upper left panel is a view of embryos using standard light in a dissecting microscope; the nuclei in the syncytial cytoplasm appear as black dots. The panel on the right uses fluorescence microscopy to visualize the propidium iodide-stained nuclei of embryos. The embryos have an average length of 200 microns and an average width of 150 microns. The nuclei for the germ cells cluster at the posterior pole of the embryo as seen for cycles 6 (embryo tilted forward), and 7-9, after which they are interspersed with somatic nuclei. L chromosome elimination in the somatic lineage occurs in cleavage division 5 or 6; X chromosome elimination in the somatic lineage occurs in the 7^{th}, 8^{th}, or 9^{th} cleavage division. Cellularization occurs during interphase of cycle 11. The table at the left shows the average length of time for each division cycle at 22 °C. The table on the left and panel on the right are adapted with permission from de Saint Phalle and Sullivan[35]. Please click here to view a larger version of this figure.

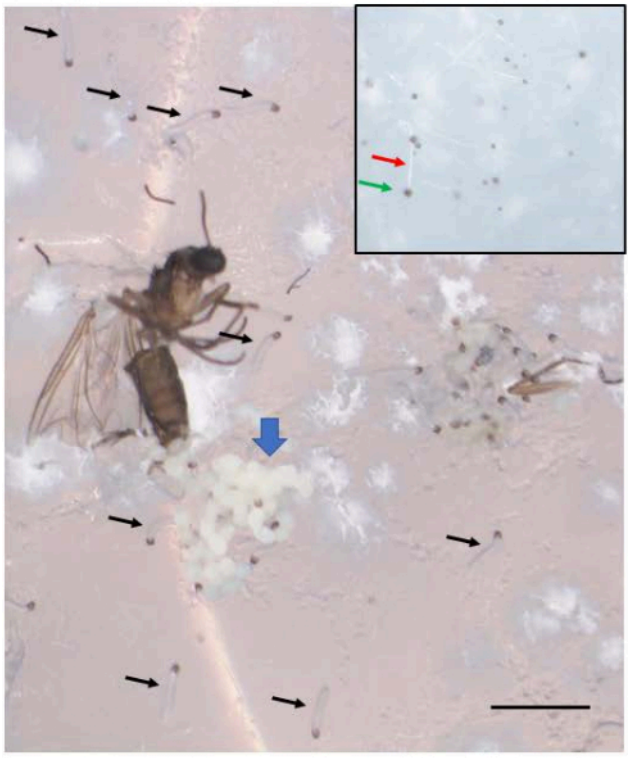


Figure 6: *B. coprophila* embryos emerge as larvae. A cluster of embryos (blue thick arrow) is seen near the ovipositor of the adult female who died after egg-laying. A week after egg-laying, the embryos become young larvae, several of whom are indicated by the black arrows. The newly emerged larvae have a black jaw at the anterior end and a translucent body. They move on top of the agar surface and should not be confused with mold that does not move. The inset shows some mold, with a white filament (red arrow) and a black spore at its tip (green arrow), and is very slightly smaller than the newly emerged larvae. Scale bar = 1 mm. Please click here to view a larger version of this figure.

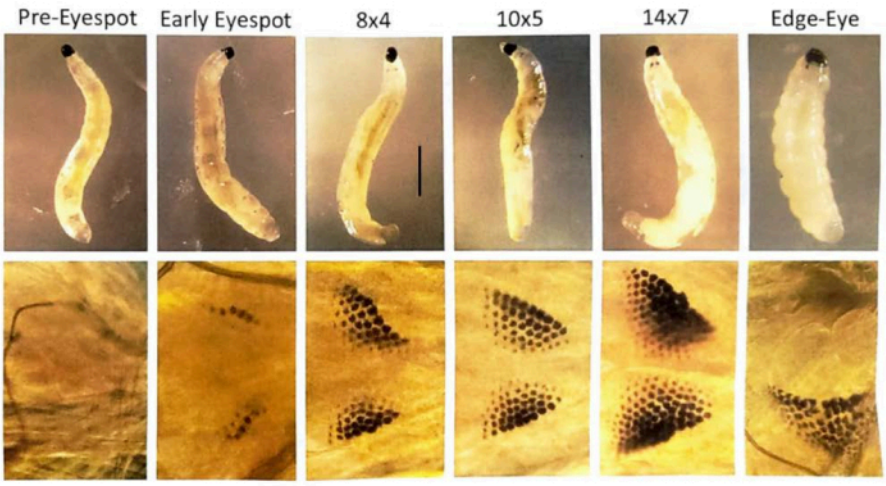


Figure 7: Eyespot stages of *B. coprophila* larvae. Eyespots form at the anterior of the larva, just behind the jaw, and are composed of pigment granules that increase in number. The eyespots are the anlage to the adult eye. The upper panel shows larvae that were visualized with a dissecting microscope; the lower panel is a magnified view of the eyespots using a phase contrast microscope to visualize a larva on a microscope slide with a drop of distilled water and a coverslip lightly floating on top. The nomenclature of the eyespot stages is according to Gabrusewycz-Garcia[30] where the number of granules is counted in the longest row (e.g., 12) and the number of additional rows excluding the longest row is noted (e.g., 6 for eyespot stage 12x6). Initiation of site-specific DNA amplification in the salivary gland polytene chromosomes begins at eyespot stage 10x5 and is completed at 14x7 when there is a burst of transcription at the locus and expansion of the DNA puffs[31]. At the subsequent edge-eye/dropped jaw stage, the eyespot granules begin to merge, and they move laterally away from the midline; the length of the larval body shortens. Further, the DNA puffs condense at this stage. It takes about one day at 21 °C to traverse each eyespot stage. Scale bar = 1 mm. Please click here to view a larger version of this figure.

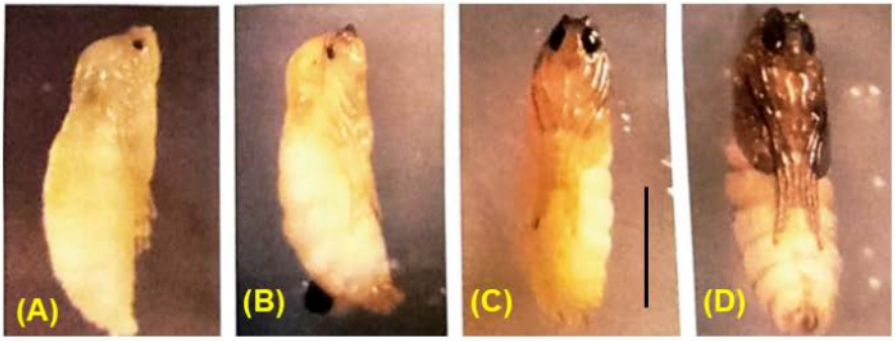


Figure 8: Development of *B. coprophila* pupae. During pupation, all larval tissues histolyze except for the nervous system and are replaced by adult tissues that arise by cell divisions of the imaginal discs. The body color changes from white to tan to brown to black. Pigment gradually fills in the pupal eye; meiosis I and II occur in male pupae with eyes that are 1/4 to 1/2 filled with pigment[36]. Scale bar = 1 mm. Please click here to view a larger version of this figure.

Stock name	Markers	Fertility rate	Comments
7298	Wavy (W) wing	~75%	
HoLo2	Wavy (W) wing	~90%	derived from 7298
W14	Wavy (W) wing; White eyes	~95%	derived from HoLo2
6980	Wavy (W) wing; swollen (sw) veins	~65%	
91S	weak Wavy (W) wing; petite (p) wings	~50%	the Wavy marker was introduced in a cross to rescue 91S

Table 2: Stocks of *Bradysia (Sciara) coprophila*. Table 1 of Gerbi[6] lists these markers and others that are no longer extant. Five translocations (T1, T23, T29, T32, T70) at the centromere end of the X[27] are summarized in **Figure 8** of Gerbi[6] but are no longer extant.

Supplemental File 1: HoLo2 (and 7298 and W14) crosses, 91S cross and rescue, 6980 cross, translocation crosses. Please click here to download this File.

Discussion

The protocols presented here for the husbandry of *B. coprophila* will be useful for scientists who wish to raise this organism in their laboratories for experiments to delve into

its unique biological features. The initial description of the feeding method using yeast and mushroom powder sprinkled onto an agar base to maintain *B. coprophila*[29] was used in the Metz laboratory to rear 14 different species of Sciarid flies[5]. Subsequently, it was observed that the addition of nettle and/or spinach powder further increased the vitality of *B. coprophila* (Gabrusewycz-Garcia, personal communication). These methods have been successful for the maintenance of related species within the Sciaridae family, including *Bradysia impatiens* and *Lycoriella ingenua* that are currently in culture (Robert Baird, personal communication).

Other methods (such as the alternative feeding methods described below) have been tried to raise *B. coprophila*, but the protocols described here have been optimized to have the most favorable ratio of larvae per surface area of agar to obtain the most fertile fat adults and minimize the growth of mold. To scale up, mass mating can be done in glass vials as described in protocol 2 above. Alternatively, a few (2-4) adult females can be placed together with twice as many adult males in a flask such as that used to raise *Drosophila* (disposable 6 oz. = 177.4 mL square bottom *Drosophila* polypropylene bottle). In both cases, the researcher must be fully confident that the flask contains only all female-producer or all male-producer mothers.

Only feed the larvae since the pupae and adults do not eat. Do not feed the vial if larvae have turned into pupae (a sign of this is when the first early-to-emerge adult flies appear). Once the adults eclose, put the vials into a cooler incubator (e.g., 16 °C) if available as this will allow the adults to live longer. Feed three times per week (e.g., Monday, Wednesday, Friday), increasing the amount of food given per vial as the larvae grow older. Feed generously, and you will be rewarded with fat fertile adults. However, if you feed too much, white mold will appear and that is a sign to reduce the amount of food you are depositing in a vial. Further, if you feed too much, a thick pad of food will develop on top of the agar and make it harder for the adults to emerge (you can remove the pad with a forceps, but be careful to not take larvae out with the pad-it is best to not have to do this at all). Vials with few larvae (marked "few") need less food. If you feed too little, the larvae will climb the walls of the vial in search of food. Underfed larvae result in small adults that are less fertile.

Alternative feeding methods

A variety of methods have been attempted to feed larvae just once during the larval stage rather than 3 times per week. *B. coprophila* will not grow on *Drosophila* style food. John Urban (personal communication) tried mixing *B. coprophila* food in with the agar, but too much mold grew. He found that adding two mold inhibitors (tegosept and propionic acid) in combination and separately, trying several different concentrations, were all toxic to *B. coprophila* at levels that inhibit mold. The agar should be pH 6-7 (neutral) as *B. coprophila* gets sick at an acidic pH (as with propianic acid). Alternatively, to obviate thrice weekly feeding, he tried using a spatula or syringe without a needle to dispense a thick yeast paste (Red Star active dry yeast mixed with a little distilled water to moisten it) as a dollop on top of the agar in each vial one week after mating (i.e., about the time the larvae will begin to emerge).

Another method to avoid thrice weekly feeding is to add a living culture of fungi to each vial. Bath and Sponsler[37] reported that a slanted agar surface with Sabouraud's medium should be streaked with a fungal culture of the genera *Chaetoconidia* (best) or else *Baplosporangia* or *Xllescheria*. The fungus was grown several days to one week before *B. coprophila* was introduced. No feeding was needed after this.

A variant of this method was also employed by Ellen Rasch (personal communication). In our hands, the vials were too wet with this method and the larvae drowned, but it could be tried again to optimize the number of larvae relative to the vials with live fungi.

Arthur Forer (personal communication) has had some success in rearing *B. coprophila* the same way as crane flies[38]. With this approach, pupae were reared on moist *papier mâché*. Subsequently, the adults were mated and the eggs were deposited on fresh moist *papier mâché*. The resulting larvae were kept on *papier mâché* in petri dishes and fed with powdered nettle leaves twice a week. Pupae were put into a cage to repeat the cycle.

Yukiko Yamashita (personal communication) has tried without success to rear *B. coprophila* on soil, mimicking the conditions where they are found in nature in potted plants and greenhouses with high humidity. However, mold can become a problem when the humidity level is raised. Nonetheless, moist soil has been used with success to raise *Pseudolycoriella* (previously *Bradysia*) *hygida* larvae in plastic boxes with moist soil; they are fed decomposed Ilex paraguariensis leaves, supplemented in late larval life with 1.2% yeast extract, 1.4% cornstarch, 0.8% oatmeal flour, 1.2% agar[12]. Similarly, the moist soil can be replaced by moist peat moss with crushed kidney beans to raise Sciarid flies[39,40].

Still other methods have been employed to maintain laboratory cultures of *Bradysia*: (i) autoclaved potato to which yeast and dried blood fertilizer are added[41]; (ii) manure[42,43,44] to which dried blood can be added[45]; (iii) plastic containers with cotton pads and dampened paper towels with ground soybeans[46].

Mites

Mites can be transferred from *Drosophila* to *B. coprophila*. To minimize this, it is best to keep *B. coprophila* in a separate incubator or room, not close to *Drosophila* stocks. Moreover, do any *B. coprophila* maintenance work early in the day before handling *Drosophila*. Mites can also be transferred to *B. coprophila* from houseplants, so do not keep plants in the same room as *B. coprophila*. If mites invade the vials, they can be seen as small white spherical organisms crawling on the body of *B. coprophila*. Chemical treatments that work to destroy mites in *Drosophila* cannot be used for *B. coprophila* as the chemicals kill *B. coprophila* (*B. coprophila* is also sensitive to organic fumes such as phenol). The only treatment to rid *B. coprophila* stocks of mites is to manually collect the embryos on an agar plate, examine each for the absence of mites, and then transfer them to fresh agar vials using a fine paintbrush. Cotton-filled gauze plugs and cellulose acetate foam flugs (as used for *Drosophila* polypropylene vials) both help to prevent the entry of mites into the vials.

Usefulness of the husbandry protocols

The protocols described here will enable the growing community of scientists to rear *B. coprophila* as a new/old emerging model organism to study its unique biological features. New laboratory groups are encouraged to join the growing community to maintain and investigate the unique biological features of *Bradysia* (*Sciara*).

Disclosures

The author has no conflicts of interest to declare.

Acknowledgments

Special thanks to previous *B. coprophila* stock keepers (Jacob E. Bliss, Paula Bonazinga, Anne W. Kerrebrock, Ingrid M. Mercer, Heidi S. Smith) and research personnel (especially Robert Baird, Michael S. Foulk, Donna Kubai, John M. Urban, Yutaka Yamamoto) for fine-tuning the husbandry protocols. Initial instructions on care of *B. coprophila* were provided by Helen V. Crouse, Natalia Gabrusewycz-Garcia, Reba M. Goodman, Charles W. Metz, and Ellen Rasch. With gratitude to Yukiko Yamashita and Anne W. Kerrebrock for taking on the *Bradysia* (*Sciara*) stock center. Much appreciation to the following people for their helpful preparation of figures: Brian Wiegmann (**Figure 1**), John M. Urban (**Figure 4** upper panel), Laura Ross (**Figure 4** lower panel), Yutaka Yamamoto (**Figure 5** left panel), Leo Kadota (**Figure 7** and **Figure 8**). Many thanks to Ava Filiss and the Brown University Multidisciplinary Laboratory for their help with photography and filming. Thanks to Robert Baird for comments on this manuscript. Our research and maintenance of *B. coprophila* has been supported by NIH and NSF, including the most recent support from NIH GM121455 to S.A.G. Further details about *B. coprophila* are available on the *Bradysia* (*Sciara*) Stock Center websites (https://sites.brown.edu/sciara/ and https://sciara.wi.mit.edu) that are currently being constructed.

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