Protocol

Rearing and Maintaining a *Culex* Colony in the Laboratory

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After overcoming the significant obstacle of getting adult *Culex* mosquitoes to reproduce and blood feed in the laboratory, maintaining a laboratory colony is much more achievable. However, great care and attention to detail are still required to ensure that the larvae have adequate food without being overwhelmed by bacterial growth. Additionally, achieving the appropriate densities of larvae and pupae is essential, as overcrowding delays development, prevents pupae from successfully emerging as adults, and/or reduces adult fecundity and alters sex ratios. Finally, adult mosquitoes should have constant access to H_2O and nearly constant access to sugar sources to ensure that both males and females have adequate nutrition and can produce the maximum number of offspring. Here, we describe our methods for maintaining the Buckeye strain of *Culex pipiens* and how other researchers might modify them to suit their specific needs.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Adenosine Triphosphate (ATP) Solution for Mosquito Feeding (0.1 M) <R>

The ATP helps make the blood seem "fresh" to the mosquitoes and encourages them to feed to repletion (Galun 1967).

Bleach

Blood source

The preferred source is commercially purchased chicken blood treated with an anticoagulant (e.g., sodium citrate–treated chicken blood from Pel Freeze or Lampire Biologicals). Other options are human volunteers with appropriate institutional review board and biosafety protocols in place and live chicken or other avian host if proper Institutional Animal Care and Use Committee and biosafety protocols are in place.

Dish soap (optional, see Step 8; e.g., Dawn or Palmolive dish soap)

Grass (e.g., Kentucky bluegrass, crabgrass, or other local grass)

H_2O (distilled and hot tap)

Reverse osmosis H_2O can also be used.

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Larval food (Tetramin tropical fish flakes that are ground with a coffee grinder)

Rodent or puppy chow can also be used. Additionally, if you need to promote autogenous egg development (e.g., laying the first batch without a blood meal), such as for Cx. pipiens molestus*, protein-rich food sources like a slurry of beef liver powder (2.53% wt/vol solution) or ground brine shrimp should be used to feed mosquito larvae (Gao et al. 2019).*

Mosquitoes (plastic deli-cup container where egg rafts have been placed and allowed to hatch into first-instar larvae)
Sugar source (e.g., raisins, honey, and/or 10% sucrose)

Equipment

Cotton, braided dental rolls (e.g., Pearson, ~16.2-cm) or filter paper (e.g., Whatman Filter Paper, 42.5-mm circle); see step 19
Covered plastic or glass container (e.g., GladWare home mini round food storage containers, 4-oz or 118-mL; see Step 19)

A hole, ~1-cm-diameter, can be drilled through the lid to allow filter paper or a cotton dental wick to fit through.

Environmental chamber

A room where lights are on a photoperiod timer and that is temperature- and/or humidity-controlled can also be used. To stimulate mosquito growth and reproduction, we recommend setting the chamber/room to long-day conditions (>14 h light/d) and temperatures of 24°C–27°C. Culex *mosquitoes will develop at temperatures as low as 15°C, but development will be slower. If you want to induce diapause/overwintering dormancy,* Culex *mosquitoes should be exposed to photoperiods of <12 h light/d and temperatures of <21°C. Both reproductively active and diapausing* Culex *mosquitoes prefer humid conditions (relative humidity of >70%). This can be achieved by using a humidifier and/or placing moist sponges on top of the mosquito cages.*

Graduated plastic cylinder (1-L)
Hemotek artificial blood feeding system

See Introduction: ***Points to Consider When Establishing and Rearing*** **Culex** ***Mosquitoes in the Laboratory*** *(Meuti et al. 2023) for options if the strain you are working with will not feed on a Hemotek system.*

Kitchen scrubbing brush (e.g., Vikan Dish Brush)
Labeling tape
Large black trash bag
Manual counter
Marker
Mesh cages (31-cm^3 or larger recommended)
Metal spatula or white plastic spoon (see Step 30)
Microplate sieve with openings <300 µm (e.g., Endecotts)
Paper towels
Parafilm
Petri dishes
Plastic bag (optional, see Step 18)
Plastic deli cups (e.g., diameter of 11.4 cm; height of 4.4 cm; holding volume of 250–500 mL)
Plastic, disposable transfer pipette (e.g., 3-mL; Fisher Scientific; labeled with strain name)
Plastic shoe-box-sized containers (e.g., Sterlite, 11 in. × 6.6 in. × 2.7 in)
Scissors
Sponges
Styrofoam (optional, see Step 17)
White plastic container or a clear plastic container with a white paper towel or piece of paper underneath it

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METHOD

Rearing *Culex* Larvae to Adulthood

1. Use an appropriately labeled plastic transfer pipette to draw up newly hatched, first-instar larvae from the containers (day 1 of larval life; see Table 1).
2. Pipette the larvae out in small droplets onto a white plastic container, wrapping the droplets around the pan. Ideally, place fewer than 10 larvae per droplet to facilitate easy counting.
3. Use the manual counter to count every mosquito larva in each droplet and continue to count until approximately 220 larvae are in the white plastic container.
4. Using the plastic 1-L graduated cylinder, measure out 450 mL of distilled H_2O.
5. Label a plastic, shoe-box sized container (hereafter "larval rearing pan") with label tape.

   We recommend that labels include the initials of the researcher, the species, strain, cohort/lab generation, rearing conditions, purpose of the mosquitoes (e.g., general colony maintenance; title of the relevant experiment), and the date.
6. Tilt the white plastic container with the mosquito larvae over a larval rearing pan and use the distilled H_2O to transfer all the larvae into the rearing pan.
7. Place ~50 mg of larval food into the rearing pan with the larvae.
8. Repeat until the desired number of pans of larvae have been made. If any larvae remain, kill the larvae by adding bleach (~10% of the total volume) or dish soap.

   Typically, we recommend creating 12 pans of Culex *mosquitoes for each strain and rearing condition (approximately 2640 larvae in total).*
9. Place the containers of mosquito larvae into an environmental chamber that is set to the appropriate conditions (see Introduction: **Points to Consider When Establishing and Rearing *Culex* Mosquitoes in the Laboratory** [Meuti et al. 2023]).
10. On day 2, add 100 mg of larval food to each of the larval pans.
11. On day 3, monitor the larvae for growth and development. Remove any dead larvae from the pan with a disposable transfer pipette and discard.
12. On day 4, add 250 mg of larval food to each of the larval pans.
13. On day 5, monitor the pans for bacterial growth and filter if necessary.

    Pans should be filtered if there is excessive bacterial growth (e.g., the mats or clouds of bacteria are evident and the H_2O becomes opaque), if the H_2O is highly pungent, and/or if more than 20 dead, white larvae are present on the bottom of the pan.

    i. To filter the larvae, first use the plastic transfer pipette to remove and discard any dead larvae.

    ii. Then, prepare a clean larval pan, measure 400 mL of distilled H_2O, and place the microplate sieve in the sink.

    iii. Carefully pour the pan containing the larvae through the sieve.

    iv. Quickly and gently rinse the larvae with distilled tap H_2O. Then, transfer the larvae into the clean container using the 400 mL of clean distilled H_2O.

    v. If the pan was filtered, add 300 mg of larval food after transferring larvae to their new pan, move the label to the new plan, and add a note of when the pan was filtered.
14. On day 6, monitor the pans for bacterial growth and filter if necessary, using the instructions detailed in Step 13.
15. On day 7, add 350 mg of larval food to each of the larval pans.
16. On day 8, monitor the pans for bacterial growth and filter if necessary, using the instructions mentioned in Step 13.

TABLE 1. Feeding schedule for rearing *Culex* larvae at 24°C–27°C

Pan (50 mg food)					100 mg food		No feed	250 mg food		No feed	No feed	350 mg food		No feed	Pick 1		Pick 2		Cage Info
Date	Number of pans	Initial	Purpose (main colony/apt ID)	Cohort	Date	Initial	Date	Date	Initial	Date	Date	Date	Initial	Date	Date	Initial	Date	Initial	Emergence date/notes
7.25.22	12	AS	Main colony	F42	7.26	MW	7.27	7.28	AS	7.29	7.30	7.31	MEM	8.01	8.02	MEM	8.03	MW	Emerged 08.02
8.04.22	6	MW	FlyBox	F43	8.05	MW	8.06	8.07	MEM	8.08	8.09	8.10	MW	8.11	8.12	AS	8.13	MEM	Placed into small cages

The top row indicates how much food should be given or when mosquito pupae should be picked (Pick 1 and 2). Labels for each column represent other important information that should be recorded starting when the pans of larvae are established and each subsequent day thereafter. Researchers use their initials to indicate who fed the mosquito larvae and when, allowing multiple people to easily and accurately care for laboratory colonies.

17. On day 9, monitor the pans to see if any pupae have developed. If so, collect the pupae.
    i. With the label tape and marker, label plastic deli cups with the species, strain, your initials, and date. Fill with ~1″ of the larval rearing H_2O to avoid osmotic shock.
    ii. Use an appropriately labeled plastic transfer pipette to suck up the pupae and place them into the prepared container.
    iii. Once the cup has 150 pupae, start placing them into a new labeled plastic deli-cup container to avoid overcrowding. If desired, add a small piece of Styrofoam (~2.5-cm-diameter) to the cup to provide newly emerged adults a place to climb off the H_2O surface.
18. Place the container into an appropriately labeled cage (e.g., your initials, species, strain, cohort, and the next day's date through the end of adult emergence).

    If the cage is in a non-humidity-controlled environment, place a wet sponge on top of the cage and cover the cage with a plastic bag. This retains high humidity in the cage, which enhances adult mosquito survival.
19. Provision the cage with a H_2O source and food (e.g., 10% sucrose solution, raisins, and/or honey-soaked sponges).

    H_2O and sucrose solutions can be provided to mosquitoes by placing them in covered plastic or glass containers and allowing either filter paper or cotton to wick the solution.
20. On day 10, transfer any pupae from the larval rearing containers into a new labeled, plastic deli cup. Place the deli cup with the pupae into the adult mosquito cage.
21. On day 11, transfer any remaining pupae into a new open container. Place the pupal cup into a previously established adult mosquito cage.

    At this point, fewer than 10% of the pupae should remain. We recommend killing any remaining larvae to synchronize mosquito development, which is useful for subsequent experiments. To kill remaining mosquito larvae, first consolidate them by filtering all the H_2O in the larval pans through the microplate sieve. Then use a small amount of hot tap H_2O to transfer the larvae into a deli cup container and then add an equal amount of bleach to create a 50% final solution.

Blood Feeding Adult *Culex* Females

Anautogenous female Culex *mosquitoes need to be blood-fed so that they can produce eggs and allow the laboratory strain to persist. Only long-day-reared, nondiapausing females will blood feed. For established colonies, we recommend that cages containing adult females that were reared at 24°C–27°C be fed 5–10 d after adult emergence, whereas long-day mosquitoes that are reared at 18°C–21°C should blood feed 14–20 d after adult emergence.*

22. One to two days before blood feeding, remove the sugar sources from the cages. Ensure that the mosquitoes have access to regular H_2O and wet sponges if they are not in a humidity-controlled environment. Remove all plastic deli cups from the cages to prevent mosquitoes from drowning and/or laying eggs prematurely.

    Removing the sugar sources makes it more likely that female mosquitoes will be hungry and ready to take a blood meal.
23. Prepare the Hemotek artificial membrane system to blood feed females.
    i. If blood was previously frozen, thaw it overnight at 4°C or for ~4 h at room temperature.
    ii. With scissors, cut squares of Parafilm for every heating unit of the Hemotek that you plan to use. Rub the squares of Parafilm against human skin (stinky feet work best) so that they pick up human odors, allowing female mosquitoes to find the blood source.
    iii. Gently stretch the Parafilm so that it forms an ~6″ thin square without tearing it.

        If any holes or tears are present, start with a new piece of Parafilm.
    iv. Place one piece of stretched Parafilm over a metal blood feeding cylinder such that the side of the Parafilm that has been rubbed against human skin is facing upward. Secure the Parafilm in place by wrapping it tightly around the metal cylinder and then sliding a black O-ring into the groove. Repeat these steps for each feeding cylinders. Place the cylinders in a Petri dish

such that the Parafilm side is down and roll up any excess Parafilm that has contacted the bottom of the metal blood feeding cylinder.

v. Add 200 µL of a 0.1 M ATP solution to 15 mL of chicken blood using an adjustable pipette or plastic transfer pipette, and gently mix the blood to distribute the ATP.

vi. Using a disposable plastic pipette, transfer the chicken blood to the Hemotek blood-feeding cylinder. Slowly pipette the blood in, being careful to avoid bubbles. Lift the cylinder up to make sure that a layer of blood completely or almost completely covers the bottom of the Parafilm.

vii. Without setting the cylinder on the bench (still hold it with one hand in the air), place plastic plugs in the holes, and screw the blood feeding cylinder into the heating units. Place the heating unit on a Petri dish.

viii. Repeat this process with all blood-feeding cylinders until they are filled with blood and turn on the blood feeding unit.

ix. Place the heaters with the blood on top of each cage containing *Culex* females that are ready to blood feed. Cover the cage and heaters with a large black trash bag to simulate evening. Then exhale into the large mosquito cage to raise the CO_2 levels and stimulate blood feeding.

As Culex *mosquitoes are crepuscular, it may be best to feed at dawn or dusk. If possible, it is likely best to feed the cages within the incubator in which they are reared. If mosquitoes do not feed well, it might be necessary to move the heating units inside the cages, ensuring that the cloth sleeves of the mosquito cage are secured around the electrical cord of the Hemotek so that mosquitoes do not escape. It might also be necessary to try moving the cages to warmer or cooler rooms, and/or removing H_2O sources. If available, dry ice can also be placed near the cages to increase CO_2 levels, and/or dirty and stinky socks can be placed near the feeding units to further stimulate feeding.*

24. Leave the Hemotek heating units on each cage for at least 2 h and up to 14 h.

The heating units can be transferred to other cages as well.

25. Disassemble the Hemotek blood feeder.

i. After the cages have had access to the blood for an appropriate amount of time, turn off the artificial blood feeder. Transfer the metal blood feeding cylinders to Petri dishes.

ii. Disassemble the blood feeding units. Use the gray-handled screw tool (provided with the Hemotek artificial blood feeding system) to remove the plastic plugs. Pipette the old blood back into the chicken blood container or use paper towels to manually remove as much congealed blood from the feeding cylinder as possible and dispose of the old blood and used Parafilm according to approved biosafety protocols.

iii. Place the plugs, cylinders, and the gray screw tool in a container with a 10% bleach solution for 5–10 min. Scrub them with a brush until they are clean, rinse them with hot tap H_2O, and allow them to air dry.

Leaving the blood feeding cylinders or other materials in bleach for long periods of time can damage them. Therefore, they should be soaked in bleach for no more than 15 min and rinsed thoroughly.

iv. Wipe down the bench, Petri dishes, and heating units of the blood feeder with paper towels and a 10% bleach solution.

26. Provision each cage with a sugar solution.

At this stage, we provision our cages with 10% sucrose, raisins, and honey-soaked sponges to help promote egg laying.

27. Monitor the cages daily to ensure that mosquitoes have access to both sugar and H_2O and that mosquitoes are in a humid environment.

If females do not have these resources, they will fail to produce egg rafts or only produce very small ones (e.g., containing only approximately 50 eggs instead of approximately 200 eggs).

28. Two days after blood feeding, make oviposition H_2O.

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i. Use scissors to cut off a handful of grass tips.

ii. Place the freshly cut grass into a 1-L plastic graduated cylinder and fill the container with 500 mL of distilled H_2O.

Freshly cut grass is best. Avoid placing soil in the oviposition H_2O, as it can contaminate the H_2O with microbes. Also, avoid using grass that may have been treated with fertilizers or pesticides.

iii. Allow the grass and H_2O mixture to rest for ~48 h at room temperature.

29. Four days after blood feeding, place the oviposition H_2O into the cages that have been blood-fed.

i. Label a deli container with the strain, species, date, and cohort.

ii. Pour the oviposition H_2O into the labeled container such that the oviposition H_2O is 5- to 8-cm-deep (~200-mL) and some grass clippings are present.

The grass clippings may give female mosquitoes a surface to land and stand on while ovipositing.

iii. Place the container inside the appropriate cage.

30. Five days after blood feeding, collect *Culex* egg rafts.

i. Label a new clean deli cup and fill it with 2–4 cm of distilled H_2O.

ii. Using a metal spatula or white plastic spoon, carefully scoop out egg rafts and transfer them to the prepared container.

iii. Move the oviposition H_2O back into each cage as it is likely that more females will lay egg rafts the following day.

31. Once the larvae hatch, begin at Step 1 to establish new pans of mosquito larvae.

DISCUSSION

Using this protocol, researchers can continuously maintain *Culex* species in the laboratory. Depending on the needs and interests of the researcher as well as the source population of *Culex* mosquitoes, the photoperiodic and temperature conditions can be adjusted. For example, populations collected from higher latitudes will likely fare better in the laboratory if they are exposed to longer photoperiods (>14 h/light day) and lower temperatures (24°C) compared with mosquitoes that are collected from tropical and subtropical locations.

Researchers can induce diapause in temperate *Culex* mosquitoes by exposing mosquitoes to short photoperiods and lower temperatures (Sanburg and Larsen 1973; Spielman and Wong 1973). If researchers are interested in the differences between diapausing and nondiapausing mosquitoes, we strongly recommend rearing mosquitoes under the same low temperature (18°C or 20°C) and using different photoperiods (Meuti et al. 2015a,b; Fyie et al. 2021). This is because diapause induction is primarily driven by differences in photoperiod, and therefore a high proportion of nondiapausing and reproductively active mosquitoes can be obtained even when mosquitoes are reared at low temperatures (Meuti et al. 2015a,b). Moreover, as mosquitoes are ectothermic organisms, holding temperature constant allows researchers to accurately characterize physiological differences between diapausing and nondiapausing mosquitoes.

Rearing mosquitoes at lower temperatures and/or allowing mosquitoes to enter diapause may be favorable, as this allows researchers to maintain mosquitoes in the laboratory for 2–5 mo with little active care; after adult mosquitoes enter diapause, their food sources can be removed, and they will survive for several months if they are held under humid conditions and have access to H_2O (Meuti et al. 2015a). Diapause can be terminated in the laboratory by exposing mosquitoes to higher temperatures (24°C–27°C) and longer photoperiods (>13 h light/d) for 1–2 wk. Postdiapause females can then take a blood meal and produce the next generation of mosquito offspring. If researchers plan to study diapause long term, regularly allowing mosquitoes to complete diapause and reproduce after diapause termination will ensure that the diapause incidence of the laboratory colony remains high.

RECIPE

Adenosine Triphosphate (ATP) Solution for Mosquito Feeding (0.1 M)

Dissolve 551 mg of adenosine 5′-triphosphate disodium salt hydrate (Sigma-Aldrich) in 8 mL of distilled H_2O. Adjust the pH to 7 using 1 M potassium hydroxide (VWR) and raise the final volume to 10 mL. Store 200 µL aliquots of the solution for up to 1 yr at −20°C.

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