Topic Introduction

Points to Consider When Establishing and Rearing *Culex* Mosquitoes in the Laboratory

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Culex mosquitoes transmit several pathogens to humans and animals, including viruses that cause West Nile fever and St. Louis encephalitis and filarial nematodes that cause canine heartworm and elephantiasis. Additionally, these mosquitoes have a cosmopolitan distribution and provide interesting models for understanding population genetics, overwintering dormancy, disease transmission, and other important and ecological questions. However, unlike *Aedes* mosquitoes that produce eggs that can be stored for weeks at a time, no obvious "stopping" point exists in the development of *Culex* mosquitoes. Therefore, these mosquitoes require nearly continuous care and attention. Here, we describe some general considerations when rearing laboratory colonies of *Culex* mosquitoes. We highlight different methods so that readers may choose what works best for their experimental needs and laboratory infrastructure. We hope that this information will enable additional scientists to conduct laboratory research on these important disease vectors.

INTRODUCTION

Culex mosquitoes transmit viruses that cause West Nile fever (Goldberg et al. 2009; Andreadis 2012), St. Louis encephalitis (Bailey et al. 1978), and Japanese encephalitis (Wispelaere et al. 2017), as well as filarial nematodes that cause canine heartworm (Vezzani et al. 2011) and elephantiasis (Harb et al. 1993). Moreover, *Culex* mosquitoes are an important vector of Ross River virus in inland Australia (Russel 2002) and Sindbis virus in Egypt (Turell et al. 2002), and *Culex tarsalis* can transmit Rift Valley fever to humans and cattle in West Africa (Bergren et al. 2021). Admittedly, the global burden on human health by *Culex*-vectored diseases is far less than those transmitted by *Aedes* mosquitoes (e.g., dengue, Zika, yellow fever, and chikungunya) and *Anopheles* mosquitoes (e.g., human, avian, and murine malaria). Therefore, most mosquito research has focused on *Aedes* and *Anopheles*. However, members of the *Culex pipiens* complex that are highly competent vectors of West Nile virus extend further into temperate environments in North America, South America, Africa, Europe, Asia, and Australia (Ciota and Kramer 2013) than *Ae. aegypti* and *Ae. albopictus* (Kraemer et al. 2015). Therefore, a high proportion of the human population is at risk of contracting West Nile virus, as it is endemic or potentially endemic in at least 114 countries (Chancey et al. 2015).

THE CHALLENGES OF WORKING WITH *CULEX*

Apart from the differences in disease burden, another reason why researchers have gravitated to working with *Aedes* mosquitoes is likely because of the ease with which they are reared and maintained

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in the laboratory. The eggs of nearly all *Aedes* species can be stored under humid conditions for weeks, if not months, allowing researchers to hatch an appropriate number of larvae whenever they are ready to begin an experiment (Beckel 1958; Kalpage and Brust 1974; Shroyer and Craig 1983; Parker 1985). Unfortunately, *Culex* eggs are not as hardy and cannot be stored for any length of time. This is likely due to accelerated rates of embryogenesis relative to *Aedes* and *Anopheles* species, as well far lower levels of embryonic desiccation resistance (Vargas et al. 2014). Notably, *Culex* larvae will hatch in 24–72 h depending on temperature (Kiarie-Makara et al. 2015). Therefore, researchers who work with *Culex* mosquitoes must continuously maintain the mosquitoes in the laboratory. Additionally, because most existing laboratory strains of *Culex* have been established relatively recently, they are not as well adapted to artificial laboratory conditions. Finally, most *Culex* species are zoophilic, preferring to feed on live, nonhuman hosts (Schultz 1992; Tuno et al. 2017), and are not nearly as aggressive biters as *Aedes* or *Anopheles* mosquitoes (Hawley 1988; Wanji et al. 2003). These characteristics make rearing *Culex* in the laboratory more challenging, as it is often difficult to achieve high levels of blood feeding and egg production in laboratory colonies.

CURRENT *CULEX*-REARING PROTOCOLS

Despite the challenges, we were able to successfully establish a colony of Northern house mosquitoes, *Cx. pipiens*, from central Ohio in 2013 (Buckeye strain) (see Protocol: **Establishing a *Culex* Colony from Field-Collected Eggs** [Meuti et al. 2023a]) that we have continuously maintained (see Protocol: **Rearing and Maintaining a *Culex* Colony in the Laboratory** [Meuti et al. 2023b]). Several other researchers have also established colonies of *Cx. pipiens*, including the autogenous form *Cx. pipiens molestus* that can generate their first batch of eggs without a blood meal (Roubaud 1930; Dobrotworsky 1955; Spielman 1957; Mutebi and Savage 2009; Gao et al. 2019), the anautogenous form, *Cx. pipiens pipiens* (Paul et al. 2005; Yurchenko et al. 2020), and the Southern house mosquito, *Culex quinquefasciatus* (Sundararaman 1949; de Melo Chalegre et al. 2015). Additionally, Kauffman et al. (2017) have provided an excellent and detailed protocol for rearing *Aedes* and *Culex* mosquitoes and have used their protocol to successfully rear *Cx. pipiens*, *Cx. quinquefasciatus*, and *Cx. tarsalis*. Although this is more difficult, other researchers have established colonies of *Culex salinarius* (Wallis and Spielman 1953; Wallis and Whitman 1968; Eldridge et al. 1972), *Culex territans* (Desser et al. 1973), and *Culex erraticus* (Coon 2006), whereas limited numbers of *Culex restuans* have been reared in a laboratory but only after forcing males and females to copulate (Eldridge et al. 1972). The research that has been done on these species and strains has greatly enhanced our understanding of the physiology, ecology, and behavior of *Culex* mosquitoes. However, several important research questions remain, particularly about physiological and behavioral differences among different populations of *Culex* mosquitoes. Therefore, we need other researchers to rise to the challenge of working with *Culex* mosquitoes.

CONSIDERATIONS WHEN COLLECTING THE DESIRED *CULEX* SPECIES FROM THE FIELD

Different species of *Culex* mosquitoes vary in their geographic and temporal distributions, as well as their microhabitat preferences and which vertebrate hosts they prefer to bite. These factors should be kept in mind when trying to collect and/or establish a desired *Culex* species from the field. Here, we provide recommendations on how to collect the most common and medically important species of *Culex* in North America.

First, to collect *Cx. quinquefasciatus*, *Cx. pipiens*, *Cx. restuans,* and *Culex erraticus*, we would recommend using the gravid H_2O recipe included in our protocol (Protocol: **Establishing a *Culex* Colony from Field-Collected Eggs** [Meuti et al. 2023a]) without modification. *Cx. quinquefasciatus* occupies a southern range, whereas *Cx. pipiens* occupies a more northern range. However, these two species have been known to hybridize between 34°N and 46°N (Yurchenko et al. 2020). Therefore, researchers living within the hybrid zone should be aware that they may not collect pure strains of either species and should check for hybridization by using previously described protocols (Smith and Fonseca 2004). Additionally, the time of year can greatly influence which species is collected. For example, east of the Mississippi River, *Cx. restuans* is far more abundant early in the year than *Cx. pipiens*, making mid-May through mid-July ideal for collecting *Cx. restuans* and mid-July through August ideal for collecting mostly *Cx. pipiens* (Jackson and Paulson 2006; Helbing et al. 2015; Meuti laboratory, unpubl. data). Similarly, *Cx. erraticus* is more abundant later in the year (August until mid-October; Meuti laboratory, unpubl. data). Therefore, if researchers wish to establish populations of *Cx. erraticus*, they should time their collections appropriately.

Within the southeastern United States, *Culex nigripalpus* is commonly collected from gravid traps across the urban-to-rural gradient (Hancock and Camp 2022). Notably, the range of this species has been expanding northward into the Midwest (Akaratovic et al. 2021). Therefore, researchers living within the current range can likely collect these mosquitoes using our accompanying protocol (Protocol: **Establishing a *Culex* Colony from Field-Collected Eggs** [Meuti et al. 2023a]) without modification.

To collect *Cx. tarsalis*, which is distributed throughout the western half of North America, we recommend creating more dilute gravid H_2O, using one-half to one-quarter of the ingredients included in Protocol: **Establishing a *Culex* Colony from Field-Collected Eggs** (Meuti et al. 2023a). As *Cx. tarsalis* is more halotolerant than other *Culex* species (Bohart and Washino 1978; Du and Millar 1999), researchers can add up to 0.5% saline (NaCl) to the gravid H_2O to increase the proportion of *Cx. tarsalis* collected relative to other species.

Cx. salinarius can be collected from almost any H_2O source, although they are most commonly found in high concentrations in salt marshes (Murphey 1961). Therefore, researchers who want to establish laboratory colonies of *Cx. salinarius* can easily collect them by placing gravid H_2O traps at these locales. If researchers are trying to collect *Cx. salinarius* outside salt marshes, it is important to note that females that have been raised in fresh H_2O prefer to oviposit in fresh H_2O. However, females of *Cx. salinarius* that have been reared in freshwater will still lay their eggs in H_2O with high salinity (Murphey 1961). Therefore, researchers can collect *Cx. salinarius* in areas where they are less abundant by filling tubs with gravid H_2O containing a ∼3% saline solution (NaCl). Although this procedure may reduce the total number of *Culex* egg rafts that are collected, it will increase the proportion, and likely the total number, of *Cx. salinarius* egg rafts.

Cx. territans prefers to feed on amphibians (Burkett-Cadena et al. 2008). Adults of *Cx. territans* are therefore more abundant near creeks that have high frog populations and are easiest to find in late April through mid-May in Columbus, Ohio. During this time, gravid adult females of *Cx. territans* can be collected by aspirating them from culverts (A. Siperstein, pers. observations). Adult females can be placed directly into a cage and transported back to the laboratory where they will likely lay eggs in any nutrient-rich H_2O source that is provided to them.

CONSIDERATIONS WHEN ESTABLISHING REARING PROTOCOLS

Larval Growth and Development

Although *Culex* larvae are container breeders that are adapted to developing in nutrient-rich H_2O, they are highly susceptible to bacterial overgrowth, and entire pans of larvae can die within 24–48 h. To prevent this mortality, it is imperative that the larvae are not "overfed" (too much food is given to the larvae at any point in time). Underfeeding, although not ideal, will cause the mosquitoes to develop more slowly. Therefore, it is almost always better to underfeed (add slightly less food to larval containers) than to overfeed. The exception to this idea is when generating autogenously reproducing mosquitoes, which have higher nutritional requirements during their larval phase and where underfeeding reduces body size and overall fecundity and can increase mortality (Kassim et al.

2012). Please also note that larval growth and development are directly dependent on temperature. In the accompanying protocols (Protocol: **Establishing a *Culex* Colony from Field-Collected Eggs** [Meuti et al. 2023a]; and Protocol: **Rearing and Maintaining a *Culex* Colony in the Laboratory** [Meuti et al. 2023b]), we rear *Culex* mosquitoes under high temperatures (24°C–27°C). We also routinely rear *Cx. pipiens* at lower temperatures (18°C) that, in combination with short photoperiods (light:dark, 8 h:16 h), induce overwintering dormancy or diapause in the mosquitoes (Eldridge 1968; Sanburg and Larsen 1973; Spielman and Wong 1973). *Culex* mosquitoes reared at lower temperatures should be fed less frequently, ideally once every 2 to 4 d.

When feeding the larvae, one should check the color and scent of the mosquito-rearing H_2O. If it is dark brown/cloudy or highly pungent and/or if there are any dead larvae (bloated and white in appearance) floating near the bottom, it is best to immediately filter the mosquito larvae out of their old and dirty container and then place them into a new container with clean H_2O and fresh food. Additionally, if you see excessive biofilm accumulation on the sides of the pan or top of the H_2O, it is also a good idea to filter the H_2O. Please see Protocol: **Rearing and Maintaining a *Culex* Colony in the Laboratory** [Meuti et al. 2023b] for detailed instructions on how to filter mosquito larval water.

Pupal Development

Like most mosquitoes, *Culex* species generally only spend ~36 h at 25°C in the pupal phase (Birley 1979). Therefore, when *Culex* mosquitoes are reared at high temperatures, it is necessary to remove pupae from the larval contains and place them into an adult cage at least once a day, which will ensure that no adult mosquitoes escape. In our experience, the pupal phase is the most sensitive period in mosquito development. Therefore, it is also important to ensure that cups containing pupae are not overcrowded; we recommend placing no more than 150 pupae in a plastic cup with a diameter of 11.4 cm and height of 4.4 cm. Placing small pieces of Styrofoam (~3 cm in diameter) in the pupal cup may also facilitate adult emergence by providing a platform for newly emerged adult mosquitoes, and placing a paper cone on the top of the deli cup may prevent adult mosquitoes from falling in and drowning in the pupal H_2O. Last, the pupal cup should be filled halfway using H_2O from the larval pan, as the use of fresh H_2O can result in high mortality, possibly because of osmotic shock.

Caring for Adult Mosquitoes

Adult mosquitoes require constant access to moisture, and they perform best in humid environments. If you are rearing *Culex* mosquitoes in an environmental chamber that does not have humidity control, the relative humidity within the cage can be increased by placing a wet sponge on top of the cage and covering the cage with a plastic bag. Moreover, both male and female mosquitoes need to have access to both H_2O and sugar sources for reproduction and fertility (Kassim et al. 2012). We have reared *Culex* mosquitoes on 10% sucrose solutions only, and they do quite well (Meuti et al. 2015a,b). However, to promote reproduction, we have found that it is better to offer them additional and more natural sugar sources, such as organic (pesticide-free) raisins and honey. The sucrose solution and H_2O source should be covered to prevent mosquitoes from drowning, and the liquid should be made available to the mosquitoes via a cotton dental wick, piece of cotton roll, or filter paper. This setup will permit adult mosquitoes to feed and hydrate freely, without any risk of drowning. Although adult mosquitoes require substantially less careful monitoring than the larval and pupal phases of mosquitoes, it is necessary to frequently check cages that contain adult mosquitoes to ensure that they have access to food and H_2O. Replace food and H_2O at least once per week to prevent mold growth or fermentation of the sugar solution. After blood feeding, it is best to check the cages every day, as females must have access to a sugar and H_2O source to produce a high number of egg rafts that will hopefully yield abundant viable larvae.

Blood Feeding Newly Established and Laboratory Strains of *Culex*

Blood Source

The best way to encourage mosquitoes to blood feed is to offer a live vertebrate host, as it will provide the mosquitoes with the necessary visual, olfactory, and temperature cues. Moreover, *Culex* species are well adapted to specific hosts and generally produce greater numbers of eggs when they are allowed to feed on avian hosts (Shroyer and Siverly 1972; Downe and Archer 1975; Nayar and Sauerman 1975; Lyimo and Ferguson 2009; Richards et al. 2012; Alto et al. 2014). Therefore, if animal use protocols are in place, it would be best to offer an appropriately restrained avian host, as any host movements will further deter biting (Edman et al. 1974; Darbro and Harrington 2007).

Another option is to use live humans. If the need to establish a colony outweighs the risks and annoyances of using human volunteers, we recommend that the volunteer offer an unwashed foot or arm to the mosquitoes. Additionally, we recommend that the volunteer avoid consuming foods that contain garlic or citrus, as they are repellents to mosquitoes (Bhuyan et al. 1974; Giatropoulos et al. 2012) and can be emitted in the sweat/pores of the host (Sato et al. 2020). However, field-collected females may be infected with viral pathogens that were vertically transmitted from their mothers. Generally, the rate of vertical transmission is quite low (0.3%–0.6% for West Nile virus; Goddard et al. 2003), but the potential for infection should be reflected in any biosafety protocols that are in place to protect human volunteers and should be clearly communicated to the person who is planning to put their foot, hand, or arm into the mosquito cage.

If *Culex* species can be induced to take a blood meal from an artificial source, we strongly recommend this option. Avian blood that is heated in an artificial membrane feeding system (e.g., Hemotek) can be offered. Artificial feeding systems are convenient and allow researchers to maintain mosquito colonies without creating extensive biosafety or animal use protocols. Sodium citrate–treated blood from a variety of sources, including chickens, can be easily purchased (e.g., Lampire Biological Laboratories and Pel-Freeze Biologicals). Additionally, Griffith and Turner (1996) reported that it is possible to induce females of *Cx. quinquefasciatus* to consume a blood substitute and continue to lay viable eggs. Note that other laboratories have had success using circulating H_2O baths to warm blood (Deng et al. 2012; Witmer et al. 2018), whereas Kauffman et al. (2017) described how to use sausage casings to warm and offer blood. Although circulating H_2O baths and sausage casings warmed in H_2O are less expensive, they are also slightly more challenging to use. These artificial feeding systems lack many of the necessary cues that mosquitoes need to orient to the blood source. Moreover, the artificial membranes (e.g., Parafilm, cellulose paper, and sausage casings) are quite different from the skin of vertebrate hosts. Therefore, it is not surprising that many if not most field-collected *Culex* females may fail to feed on blood offered through an artificial membrane. To help encourage newly collected *Culex* females to feed on artificial blood feeders, we recommend that the membrane for the system be rubbed against the skin of a bird or human (i.e., the foot and ankle) to transfer some of the host volatiles. We also recommend placing a source of carbon dioxide (e.g., dry ice) near the cage. Finally, we recommend that the blood be supplemented with adenosine triphosphate (ATP; Sigma-Aldrich; ~200 µL of 0.1 M ATP per 15 mL of blood), as it encourages female mosquitoes that initially penetrate the membrane to feed to repletion (Galun 1967; Galun et al. 1985; Griffith and Turner 1996).

Timing of the Blood Meal

Mosquito species vary in the age at which they become competent to take a blood meal. Females of *Aedes aegypti* are ready to bite and lay eggs as early as 24 h after adult emergence (Howard 1923; Armstrong and West 1966) and most will bite 24–72 h after emergence (Christophers 1960). The age at which *Culex* mosquitoes first bite/blood feed has not extensively been investigated, although high rates of biting are achieved 5 d after adult emergence, especially when the mosquitoes have been reared under long photoperiods and high temperatures (Eldridge 1968). In our experience, females of *Cx. pipiens* blood feed well when they are 5–10 d old, and therefore we recommend this time frame when offering the first blood meal to adult mosquitoes.

Most *Culex* mosquitoes are crepuscular, and therefore offering a blood meal, whether avian, human, or artificial, at dawn or dusk will likely stimulate blood feeding. As field-collected *Culex* females will likely need several opportunities to blood feed before they bite, we recommend offering the first blood meal 3 d after adult emergence and every day or two thereafter. We also recommend leaving the blood source in the cage for as long as possible. If artificial blood feeders are being used, we recommend that they remain in the cage for at least 1–2 h to stimulate blood feeding; we have had the most success feeding mosquitoes with an artificial blood source overnight.

Offering Extra Blood Meals

Female *Culex* mosquitoes can take multiple blood meals and produce several batches of eggs (up to four batches according to Awahmukalah and Brooks 1985). Therefore, if researchers need to rapidly build up their populations of *Culex*, they can offer the same cage of *Culex* females multiple blood meals. We recommend waiting at least 1 wk between blood feedings for mosquitoes reared at 24°C–27°C to ensure that every female that initially took a blood meal has enough time to oviposit and reinitiate a subsequent gonotrophic cycle. Moreover, as the sex ratio becomes more male-biased in older females (Qutubuddin 1953; pers. observations) and because most female mosquitoes will only survive ∼6 wk after adult emergence at 22°C–26°C (Oda et al. 1999; Alto et al. 2012), we recommend blood feeding each cage a maximum of four times. Additionally, whenever possible, we suggest rearing the offspring of 5- to 10-d-old females that have been blood fed for the first time, as it will yield a high number of larvae with a sex ratio of nearly 1:1 males to females (Gómez et al. 1977).

Working with Multiple Strains, Species, or Populations of *Culex* Mosquitoes

If you are rearing multiple strains or species of *Culex* mosquitoes, it is also very important to ensure that everything is properly labeled so that you do not unintentionally cross laboratory strains. Several members of the *Cx. pipiens* complex (e.g., *Cx. pipiens molestus*, *Cx. pipiens pipiens*, and *Cx. quinquefasciatus*) can produce fertile hybrids (Smith and Fonseca 2004; Bahnck and Fonseca 2006). To avoid such a problem, we recommend using a set of separate and clearly labeled containers to collect egg rafts, rear larvae, and allow pupal emergence within adult cages. We also suggest that separate labeled disposable pipettes are used for different strains and species. Whenever possible, it is best to work with different mosquito strains in separate rearing spaces and/or work with one species/strain at a time.

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