

# Testing Mosquitoes for Student Inquiry: Husbandry Lessons in the Lab

ERICA KOSAL, BEVERLY I. ANAELE



## ABSTRACT

Because mosquitoes are a public health concern, several chemical insect repellents have been created and used for many years. While some of these products, such as DEET and permethrin, are effective at controlling mosquito populations, their excessive use may lead to animal, human, and environmental harm if applied improperly. Understanding the life cycles of mosquitoes, their feeding preferences, and their responses to natural plant extracts could enable scientists to develop more environmentally safe but still effective insect repellents. Various types of plant extracts (e.g., American beautyberry, *Callicarpa americana*) hold promise. In order to study such plant–mosquito interactions, we had to establish basic husbandry practices for successfully rearing and maintaining mosquito populations in the lab. We discuss the protocols we have used for housing mosquitoes and creating plant extracts and offer suggestions for how students can use both for inquiry.

**Key Words:** mosquito; blood feeding; behavior; beautyberry; repellent.

## ○ Introduction

Mosquitoes carry human pathogens, and their populations may increase in number and distribution with climate change (Reiter, 2001). The evolutionary ties between mosquitoes and the diseases they transmit are interesting. For example, the parasitic protozoan species in genus *Plasmodium* have coevolved with their mosquito hosts. Scientists have concluded – based on current strains containing chloroplasts from its photosynthetic ancestors – that *Plasmodium malariae*, the species that causes malaria, evolved from a far less harmful aquatic species (Nair & Striepen, 2011). After emerging from water as adults, female mosquitoes can transfer parasitic protists when they bite humans (Wellems et al., 2009). Humans suffer from several mosquito-borne illnesses besides malaria, including those caused by the Zika virus, which spread to the United States in 2016 (Grennell, 2018), and the West Nile virus, which was reported to have killed one North Carolinian in 2018 (NCDHHS, 2018a).

Historically, in response to public health concerns, scientists have developed synthetic chemicals to control mosquito

populations. For example, DEET (N, N-Diethyl-meta-toluamide) and permethrin are used to safeguard against mosquito bites; however, these repellents may cause additional harm (Jackson et al., 2008; Toynton et al., 2009). For example, pesticide residues can find their way into the human urinary system; one 2007 study detected levels of organophosphate pesticide metabolites in farmworkers' children in eastern North Carolina (Arcury et al., 2007). Excessive levels of organophosphate exposure can lead to a number of adverse health effects, such as acute cholinergic syndrome, which increases internal levels of acetylcholine and potentially results in tachycardia (severely increased heart rate) and muscle rigidity (Suratman et al., 2015). Natural alternatives, predicted to be safer to the environment, exist in various types of plant extracts. Studying how these plant extracts can influence mosquito behavior and population size is a worthwhile research approach for high school or undergraduate students and can easily be achieved in the academic environment.

In the laboratory, conditions for studying mosquitoes need to support larvae and adults as they complete their life cycles. Once a male and female mosquito have copulated (on land or in the air), the female requires a blood meal to nourish and lay her eggs. She finds her host through cues of CO<sub>2</sub>, lactic acid in sweat, and body heat (McMeniman et al., 2014). About two days later, she lays the eggs on the surface of stagnant water. Larvae emerge from the eggs four to five days later, at which point oxygen and food intake become critical (Das et al., 2007; Mega-Catch, 2019). In response to the need for oxygen, tracheal trunks located at the larva's eighth abdominal segment open. Microorganisms found in the water serve as food sources when needed, and as the larvae eat they molt four times, growing larger heads until they ultimately pupate. The pupal stage is a somewhat stagnant period in which food is not a necessity and the animals are relatively motionless; however, the pupae will dive suddenly if the water is disturbed (Becker et al., 2003). After three days, the pupae morph into adults by building up pressure with air and pushing their maturing bodies out of their exoskeletons.

The life cycle of a mosquito from egg to adult is quite short, so populations can increase quickly. With multiple generations, it is easy to study the changes in a mosquito population over time.

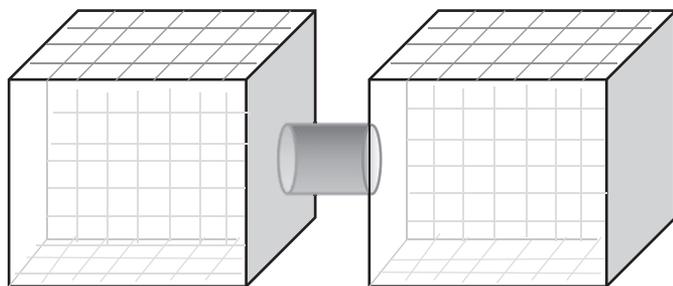
Studying mosquitoes in the laboratory is a cost-effective and safe means for cultivating student inquiry. Acquiring mosquitoes is inexpensive: you can order them from a biological supply company for a few dollars, or they can be collected from the field in warmer months when the larvae are easily found in stagnant water. The only issue with the latter approach is that the exact species of mosquito collected may be unknown. Indeed, even the biological supply company from which mosquitoes were purchased for this study did not identify the species. Nevertheless, the advantages of using mosquitoes as a study specimen include minimal workspace for research cages, low overall cost, and safety. The use of bovine blood to feed the females – instead of blood from human veins – means there is no risk of disease transmission or inflammatory responses.

Overall, there is much potential for student engagement and research in a high school or college undergraduate classroom at minimal cost. For this study, we spent about \$10 for 28 mosquitoes, \$215.24 for four rearing cages (for adults), \$119 for the bovine blood, \$29 for two breeding chambers (with contained water for eggs, larvae, and pupae), and \$16.76 for incidentals (cat food, sucrose, etc.). Thus, for several hundred dollars, students can conduct a yearlong study of mosquitoes. The cost is even lower after the start-up year, as the cages and some of the other incidentals can be reused year after year. Cheaper alternatives to these materials could also be used, such as beakers instead of breeding chambers and handmade cages instead of purchased ones.

## ○ Methods

### Mosquito Husbandry

Rearing cages (21 × 21 cm; BioQuip, <https://www.bioquip.com>) were positioned in an incubator set at 28°C. These cages were used to house the adults. The top, bottom, and back faces were netted and any small openings were taped closed. A tunnel constructed of white copy paper was put in place between two cages (Figure 1) so that mosquitoes could fly to different cages during behavioral treatment observations. A lightbulb (150 W, 130 V) was clamped to the top rack of the incubator to evenly distribute light, set for a day: night cycle of 12:12 hours. To regulate humidity levels, a beaker of 500 mL water was placed in the incubator and replenished twice a week. Damp paper towels (checked daily) were also taped to the back netting of each cage. A humidity gauge (bought at Walmart) was used to monitor humidity levels in the incubator, kept at ~26% with the aid of beakers of water and the moist paper towels. Please note that the incubator, while ideal for controlling temperature and humidity, is not mandatory. If such equipment is unavailable, cages

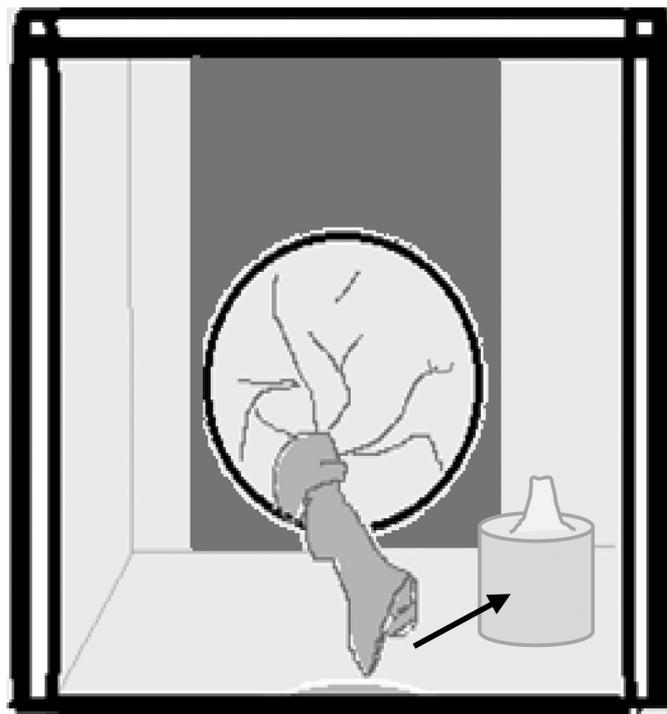


**Figure 1.** Makeshift tunnel formed between two rearing cages to give mosquitoes the option to move freely from one side (with treatments) to the other (no treatments).

could be placed at room temperature. The duration of the life cycle of the mosquitoes would increase and fewer generations of mosquitoes would be found within the study frame, but a large data set could still be collected. Additionally, insect rearing cages could be made from scratch. Several ideas are readily available through Internet searches (e.g., Goodwin, 1984).

Upon shipment arrival (from Niles Biological, <https://www.nilesbio.com/>; species unidentified), adult mosquitoes were carefully transferred from their plastic transport bag by making a hole in the bag. Once all adults had flown out into the cage, they were fed daily by pipetting 15 mL of a 10% sucrose solution onto a fresh expanse of cotton (unrolled cotton balls), which was placed on the top netting of the cage and left for 24 hours. All eggs, larvae, and pupae in the transport bag were decanted into a cylindrical breeding chamber (BioQuip) filled with 600 mL tap water. The breeding chambers served as a reservoir for eggs and for the development of larvae and pupae. To support these animals, one-fourth of a solid cat-food tablet (Purina brand) was dissolved in water and added for feeding. The lower half of the breeding chamber was placed in the prepared cage (Figure 2, arrow) to allow emerging adults to freely fly out into the cage. If the breeding chamber became cloudy, the water was pipetted out (eggs, larvae, and pupae were visible to the eye) and replenished with 10 mL fresh tap water.

After adults emerged and were three to five days old, they were starved of sucrose solution for 24 hours. Following this starvation



**Figure 2.** Prepared cage setup for purchased adult mosquitoes and for emerging adults from the smaller breeding chamber (indicated by arrow; purchased from BioQuip). Notice that this breeding chamber was inserted within the larger rearing cage. This allowed metamorphosing individuals in the breeding chamber to escape into the outer environment and feed on sucrose solution applied to a cotton expanse placed on the top netting. The back of the cage was covered with damp paper towel (darker area) to maintain humidity.

period, bovine blood was provided during the adults' active evening hours (NCDHHS, 2018b). To prepare this blood meal, 6 mL bovine blood with citrate phosphate dextrose anticoagulant (already mixed; Lampire Biological Products, <http://www.lampire.com/>) was centrifuged in a 15 mL tube at 22°C at 2500 rpm for 10 minutes. Both the supernatant and buffy coat were removed and filled with an equal amount of RPMI 1640 cell culture medium (Sigma-Aldrich, <https://www.sigmaaldrich.com>) that was then extracted and replaced for a total of three wash cycles. At the third wash, the RPMI medium was left with the blood and stored at 4°C overnight for a maximum of 8–10 days (the blood can be maintained for longer periods by storing in a freezer). When the stored red blood cells were to be used for feeding, the blood was once again centrifuged at 22°C at 2500 rpm for 10 minutes and the supernatant removed (Das et al., 2007; Llinás, 2013).

To feed the adults, 30.5 mL blood was pipetted onto an expanse of cotton 23 × 23 cm wide and 0.32 cm thick (similar to the sugar delivery). This process was repeated on another sheet of cotton and they were placed on the tops of two mosquito cages. Two heating pads (23 × 23 cm) set at 37°C (to mimic the range of human body temperature) were placed on top of the blood-soaked cotton sheets, with a 23 × 23 cm wide sheet of parafilm positioned between the blood and heating pad to protect from staining (Figure 3). Mosquitoes were allowed to feed for ~30 minutes. If they were not actively feeding, the cage was tapped or air was blown into the netting to release CO<sub>2</sub> and stimulate the mosquitoes. After this blood-feeding period, the netting on the tops of the cages was wiped down with damp paper towels. Sugar was administered on two fresh cotton surfaces (as described above) and left on top of the cages overnight. Additionally, a 50 mL beaker of water was placed inside each cage for egg deposition, and the female mosquitoes were given a period

of three days to lay their eggs. The water was subsequently poured into a new breeding chamber.

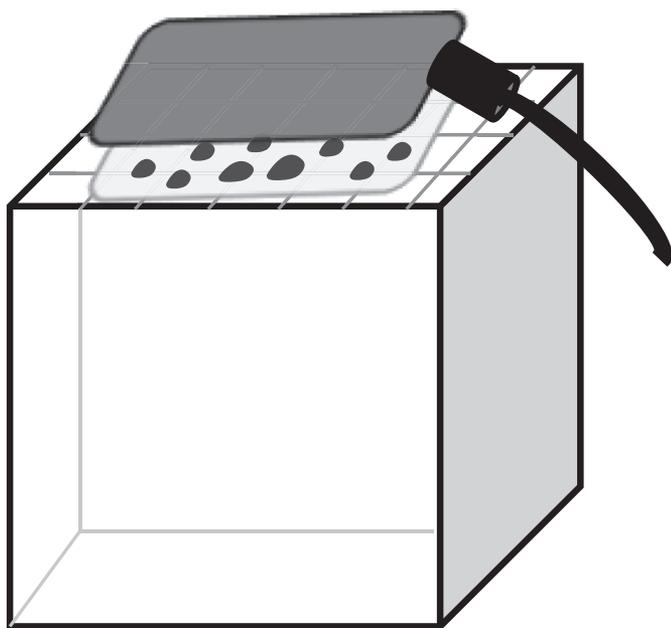
## Studying Mosquito Behavior: Treatment Application

Students can easily test how mosquitoes respond to natural plant extracts or chemicals. In the study described here, American beautyberry (*Callicarpa americana*) was chosen for investigation. The protocol outlined below can be modified as necessary for other plant extracts that students may be interested in investigating, such as citronella, lemongrass, thyme, or combinations of these extracts. Students could also examine mosquito behavior toward a repellent on different types of fabrics to see if absorbance of the repellent makes a difference. Of further interest may be male mosquito behavior and blood feeding, as some males were observed feeding on bovine blood despite the fact that only female mosquitoes are known to take blood for egg deposition. This may have occurred because male mosquitoes were also starved the night before blood feeding and the blood solution contained some glucose. Males can be distinguished from females by the visible hairs on male antennae; females lack hair on their antennae (Marois et al., 2012).

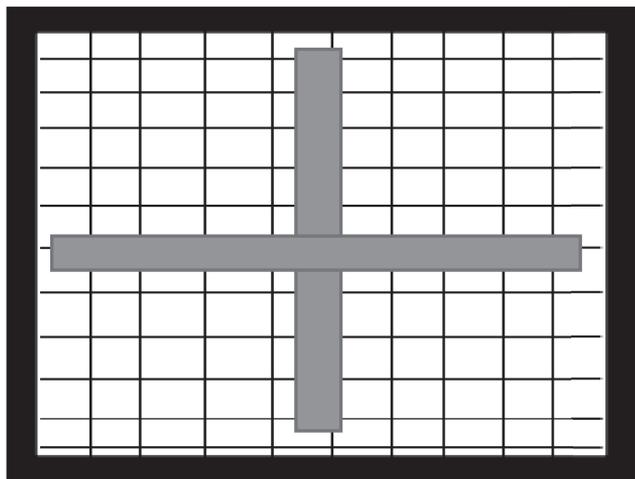
In this study, four treatment groups were selected for investigation: 70% beautyberry (BB) extract, 50% BB extract, 25% BB extract, and 5% BB extract. In addition, there were two control groups: 90% ethanol and 0% ethanol. To make the extracts, beautyberry leaves were first ground as fine as possible with a mortar and pestle. The leaves were weighed before and after grinding. The ground leaves were placed in solvent to soak overnight (Azwanida, 2015). The 70% BB extract was produced by dissolving 7.04 g of ground leaves in 10 mL of 90% ethanol. Each of the other extracts was prepared in similar fashion by dissolving ground leaves in 10 mL of 90% ethanol, using 5.01 g of leaves for the 50% BB, 2.57 g for the 25% BB, and 0.50 g for the 5% BB. If desired, students could instead create a 100% BB solution and use dilution methods to produce the same results.

When testing mosquito response to the extracts, cheesecloth was used to create four delineated sections 11.5 × 11.5 cm wide (this material is thin enough to imitate application of a repellent on skin or clothing), and 3 mL of each of the four treatments was applied drop-wise to their respective quadrants (Figure 4). Positioning one layer of cheesecloth on top of a paper towel during treatment application allowed for optimal absorption of the BB extract. This cheesecloth was then laid on the top netting of one cage. Blood pipetted onto cotton (as described above) was laid over the cloth containing the plant extract, and another sheet of cotton without a plant extract or 90% ethanol was laid on the second cage as a control (0% ethanol). They were covered with a thin parafilm sheet to avoid staining the heating pads, set to 37°C to warm the blood. Mosquito behavior was not significantly different when exposed to ethanol compared to the no-ethanol treatment. This validated that using ethanol to make the plant extracts was not altering mosquito behavior and that any differences noted with different concentrations of BB extract were due to the concentration of the plant itself.

Mosquito behavior was video recorded and documented for ~30 minutes as mosquitoes explored their environment with their mouthparts and legs, walking and visibly feeding on blood. Subsequent analysis of the recording was used to construct an ethogram (Table 1). At two-minute intervals, behaviors that might indicate



**Figure 3.** Apparatus setup during feeding procedure. A modification of Rutledge et al.'s (1976) model, this system incorporates a heating pad to warm blood to the appropriate temperature of 37°C. Blood was placed on top of a thin piece of cotton and the cotton was placed on top of the cage.



**Figure 4.** Bird's-eye view of cheesecloth on top of a cage. Use of four delineated sections separated by tape allowed for simultaneous testing of all treatment groups in one trial and alternating testing of controls in another. Mosquitoes thus had a choice between treated squares, which is similar to natural conditions.

mosquito interest or attraction at particular treatment sites were noted. Ethogram categories included (1) searching with proboscis (mouthparts), (2) rotating legs, (3) walking around cotton expanse, and (4) visibly feeding on blood (indicated when stomach turned red).

As we will report in another paper (in review), mosquitoes showed some differences in blood-feeding behavior across BB extract concentrations compared to the ethanol control. In this case, we were interested only in how a particular plant extract affects mosquitoes. Further research to determine potential negative environmental effects of ethanol or BB extract alone would be worthwhile.

## ○ Further Considerations

We encountered and resolved a few challenges while implementing this study. First of all, flexibility in investigators' schedules was required, since mosquito life cycles and shipment deliveries were restricted to warmer times of the year. Additionally, it was not known how many males and females would arrive. On rare

occasions, one or more mosquitoes escaped from their cages. Tape was used to secure openings, taking care to ensure that mosquitoes were not exposed to the sticky part of the tape.

When considering the larvae and pupae in the breeding chamber, water level turned out to be critical; we determined that 600 mL was needed to enable emerging adults to exit successfully. This volume also reduced the number of drowning mosquitoes. When feeding larvae, using excess cat food led to increased pollution in their environment. One-fourth of a cat-food pellet was enough to provide nutrients and was replenished only if it was significantly diminished. Moreover, after trials in several locations, blood feeding and sucrose feeding were found to be better performed at the top of the cage rather than at the bottom because it simplified the heating method by use of heating pads. Initially, a sugar-filled beaker inside the rearing cages was used; however, it disrupted the animals too much. Keeping the feeding consistent at the top of the cages proved more ideal.

Lightbulbs that give off heat should not be used, as this heat influences the temperature of the incubator. Even if an incubator is not used, consideration of the type of lightbulb used will become important to help the animals survive optimally. With the incubator set at 28°C, learning to monitor accompanying moisture levels was of paramount importance, as the drying out of mosquitoes led to their rapid mortality. To prevent this, damp towels were taped to the back of each cage and a beaker of water (with volume depending on the size of the incubator) was placed on a rack. If an incubator is not used, it is suggested that students experiment with damp towels and placement before adding mosquitoes to the setup, to optimize mosquito survival.

Once the husbandry techniques are established, the mosquito-related questions that students can raise and investigate are endless. In addition to the plant preference behavior explored here, students could study mosquito mating behavior, habitat preference or choice, responses to toxins, selection of food types, responses to changing rearing conditions (such as temperature and humidity), or the influence of other selective pressures on populations.

Additional supplemental work could function well with the mosquito study. For example, students could partner with local high school or middle school educators or students in a collaborative fashion to collect data from different areas or to create mosquito biology questions and pool data for larger sample sizes. College students could partner with younger students to help with community service and learning. Finally, related content could be coupled with the mosquito studies. For example, if mosquitoes are collected from local water sources, ecological topics and investigations could easily

**Table 1. Sample of a student ethogram of mosquito behaviors. Male (M) and female (F) behaviors were recorded every two minutes over a 30-minute period. Each behavior was also labeled with the corresponding ethanol control or BB concentration. Chi-square analyses were performed later.**

Time	Searching with Proboscis	Rotating Leg(s)	Walking around Cotton Expanse	Visibly Feeding on Blood
0:00	2 (F) 90% EtOH	2 (F) 90% EtOH	1 (F) 0% EtOH	1 (F) 90% EtOH
2:00	1 (M) 90% EtOH 1 (M) 0% EtOH 1 (F) 90% EtOH	2 (F) 90% EtOH	1 (F) 0% EtOH	4 (F) 0% EtOH 1 (F) 90% EtOH 2 (F) 90% EtOH
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Total	---	---	---	---

be paired with the laboratory experiments conducted on the mosquitoes directly.

## ○ Acknowledgments

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## References

- Arcury, T.A., Grzywacz, J.G., Barr, D.B., Tapia, J., Chen, H. & Quandt, S.A. (2007). Pesticide urinary metabolite levels of children in eastern North Carolina farmworker households. *Environmental Health Perspectives*, 115, 1254–1260.
- Azwanida, N.N. (2015). A review on the extraction methods use in medicinal plants, principle, strength and limitation [sic]. *Medicinal & Aromatic Plants*, 4, 196.
- Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M.B., Dahl, C. & Kaiser, A. (2003). *Mosquitoes and Their Control*, 2nd ed. Dordrecht, The Netherlands: Springer.
- Das, S., Garver, L. & Dimopoulos, G. (2007). Protocol for mosquito rearing (*A. gambiae*). *Journal of Visualized Experiments*, 2007(5), 221.
- Goodwin, M. (1984). Make a simple science project for kids: a live insect cage. *Mother Earth News*, May–June. <https://www.motheearthnews.com/diy/science-project-for-kids-zmaz84mjzloeck>.
- Grennell, A. (2018). What happened to Zika? *PBS News Hour*, July 6. <https://www.pbs.org/newshour/science/what-happened-to-zika>.
- Jackson, D., Luukinen, B., Buhl, K. & Stone, D. (2008). DEET general fact sheet. National Pesticide Information Center, Oregon State University Extension Services. <http://npic.orst.edu/factsheets/DEETgen.html>.
- Llinás, M. (2013). Blood washing technique for fresh blood. Penn State University. [http://llinaslab.psu.edu/wp-content/uploads/2013/12/Washing\\_Blood.pdf](http://llinaslab.psu.edu/wp-content/uploads/2013/12/Washing_Blood.pdf).
- Marois, E., Scali, C., Soichot, J., Kappler, C., Levashina, E.A. & Catteruccia, F. (2012). High-throughput sorting of mosquito larvae for laboratory studies and for future vector control interventions. *Malaria Journal*, 11, 302.
- McMeniman, C.J., Corfas, R.A., Matthews, B.J., Ritchie, S.A. & Vosshall, L.B. (2014). Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. *Cell*, 156, 1060–1071.
- Mega-Catch (2019). Mosquito life cycle. <https://www.megacatch.com/mosquito-faqs/mosquito-life-cycle.html>.
- Nair, S.C. & Striepen, B. (2011). What do human parasites do with a chloroplast anyway? *PLoS*, 9, e100137.
- NCDHHS (2018a). DHHS reports state's first death this year related to West Nile Virus. Press release, North Carolina Department of Health and Human Services. <https://www.ncdhhs.gov/news/press-releases/dhhs-reports-state%E2%80%99s-first-death-year-related-west-nile-virus>.
- NCDHHS (2018b). Diseases & topics: mosquito-borne illnesses (arboviruses). North Carolina Department of Health and Human Services. <https://epi.publichealth.nc.gov/cd/diseases/arbo.html>.
- Reiter, P. (2001). Climate change and mosquito-borne disease. *Environmental Health Perspectives*, 109(Supplement 1), 141–161.
- Rutledge, L.C., Moussa, M.A. & Belletti, C.J. (1976). An *in vitro* blood-feeding system for quantitative testing of mosquito repellents. *Mosquito News*, 36, 3.
- Suratman, S., Edwards, J.W. & Babina, K. (2015). Organophosphate pesticides exposure among farmworkers: pathways and risk of adverse health effects. *Reviews on Environmental Health*, 30, 65–79.
- Toynton, K., Luukinen, B., Buhl, K. & Stone, D. (2009). Permethrin general fact sheet. National Pesticide Information Center, Oregon State University Extension Services. <http://npic.orst.edu/factsheets/PermGen.html>.
- Wellems, T.E., Hayton, K. & Fairhurst, R.M. (2009). The impact of malaria parasitism: from corpuscles to communities. *Journal of Clinical Investigation*, 119, 2496–2505.

ERICA KOSAL is Director of the Life Sciences First Year Program and a Teaching Professor in the Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695; e-mail: [efkosal@ncsu.edu](mailto:efkosal@ncsu.edu). BEVERLY ANAELE is a graduate of the Public Health Department at Thomas Jefferson University, Philadelphia, PA 19107; e-mail: [beverly.anaele@gmail.com](mailto:beverly.anaele@gmail.com). When the research was conducted, both authors were in the Biology Department at North Carolina Wesleyan College, Rocky Mount, NC 27804.