AliveSCENT: Evaluating the potential use of limonene as a mosquito repellent

Mohammed Aldulaimy, Emma Angelo, Yesenia Collins, Ginelle Cote, Emily Fugazzotto, Sophia Lundberg, Meshwa Patel, Sam Puma, and Colin Riley

BioBuilderClub, Tyngsborough High School, Tyngsborough, MA, USA

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As vectors for disease transmission, mosquitoes are responsible for over 700,000 deaths worldwide each year. There are many methods to prevent mosquito bites, including the use of repellents like DEET or citronella candles, and the use of "bug zappers" to reduce mosquito populations. A bacterially-produced insect repellent could be a safer alternative to repellent sprays and flames, and cleaner than the mess created by bug zappers. AliveSCENT, a biologically-inspired mosquito deterrent that utilizes the native methylerythritol 4-phosphate (MEP) pathway of *Escherichia coli* for the production of limonene, has been proposed as a novel strategy for preventing mosquito bites that could have public health applications. Limonene is a naturally occurring fragrant compound, and a major component of most citrus scents, including lemon. Although the smell of the isolated compound has been compared to known insect repellents, such as citrus and turpentine, it has not been established whether limonene repels mosquitoes on its own. Before this compound is engineered into recombinant bacteria, its effectiveness as a repellent must be evaluated. This work tested *Aedes albopictus* mosquitoes for limonene sensitivity. Female mosquitoes between one and two weeks old were placed into bug dorms containing a cotton ball treated with one of four concentrations of limonene. The number of mosquitoes that landed on the cotton ball during each trial was recorded and analyzed. The results of this investigation were inconclusive, and further testing is required. This will help guide final plasmid assembly, and the overall direction of this project.

Keywords: Mosquito, Aedes albopictus, distillation, limonene, repellent

Mentors: *Rebekah Ravgiala, and Michael Sheets*
Direct correspondence to rebekah.ravgiala@tyngsboroughps.org; michael.sheets4@gmail.com

Watch a video introduction by the authors at https://youtu.be/6M1ziVSs6Is

Background

As one of the deadliest animals in the world, the mosquito causes the deaths of over half a million people each year (Eflein, 2020). Out of over 3,500 species of mosquitoes worldwide, not all bite humans or animals, but those that do are considered vectors, or carriers, of disease. They can spread many diseases, including dengue fever, yellow fever, West Nile virus, and malaria (Hartman, 2011). To become infected with a disease, a mosquito must pick up a pathogen when biting an infected host. The pathogen will then develop inside the mosquito over a period of days, and can then be passed on to another organism when the mosquito bites it. Only female mosquitoes bite, because they require the protein from blood to produce and nourish eggs. Mosquitoes bite humans and animals because of their attraction to carbon dioxide emissions, which are found in body heat, sweat, and smell (Centers for Disease Control and Prevention, 2020a). The female mosquito's proboscis is the part of the animal that pierces the skin and draws blood. In males, it is solely used to drink flower nectar (Centers for Disease Control and Prevention, 2020a). For the purposes of this experiment, the mosquito species *Aedes albopictus*, or Asian tiger, was used. This species was chosen because it is very easy to raise in comparison to others, because it inhabits the geographic location in which the proposed experiments would take place, and because it carries the diseases that AliveSCENT aims to prevent. The species originates from East Asia, but has since spread and adapted to many parts of the world, including the United States.

Figure 1. *This figure depicts the anatomical differences between male and female Aedes albopictus. Females are larger than males, whereas the antennae of males are more bushy, and usually shorter (Hartman, 2011).*

Currently, there are many methods to repel mosquitoes, including *N,N*-diethyl-*meta*-toluamide (DEET), the active ingredient found in many insect repellents, citronella candles, and bug zappers. DEET is not always the best option, as it can cause rashes and irritation, and has not been approved for children under two months old. In addition, studies have not been conducted to observe its effects on pregnant women (Is DEET Bad for You?, 2019). Most bug sprays containing DEET have a very strong and unpleasant smell. Citronella candles can pose a fire hazard, and bug zappers are mostly ineffective against mosquitoes, killing mainly harmless and beneficial insects (Lewis, 1996).

Last year, AliveSCENT, a novel insect deterrent that utilizes the native methylerythritol 4-phosphate (MEP) pathway of *Escherichia coli* for the production of limonene, was conceived as a possible solution to this public health problem. Limonene is a monoterpene with a citrus scent that has the potential to repel insects. It smells like lemons or turpentine, and turpentine has been shown to have some success in repelling mosquitoes (Barton et al., 2020). Orange peel extract typically contains 90–95% limonene. For the purposes of this study, limonene was extracted from orange peel using steam distillation.

A plasmid has been designed with two devices to promote the production of limonene in *E. coli* (see Figure 2). Device A is designed with a constitutive promoter, and a geranyl pyrophosphate synthase coding sequence that will be cloned into a red fluorescent protein (RFP)-encoding vector. This was introduced into the plasmid as a biological marker used to detect successful operon function. Device B is designed with a stationary phase promoter and a limonene synthase translational unit that will also be inserted into an RFP-encoding vector as a biological marker. Prior to building the plasmid to code for the production of limonene in *E. coli*, the effectiveness of limonene as a mosquito repellent needed to be tested. Thus, limonene was extracted from orange peel to test on *A. albopictus* mosquitoes that were raised in a laboratory environment.

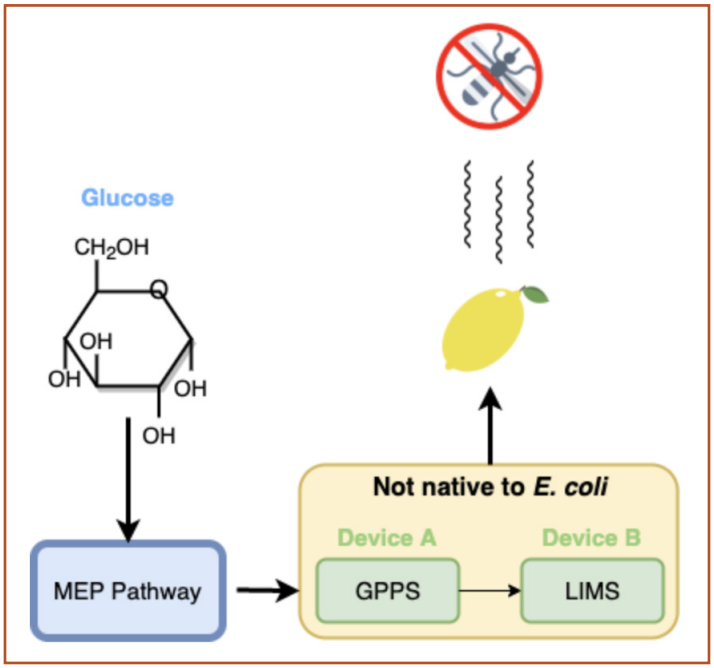

Figure 2. *General description of the biosynthesis of D-limonene. This schematic illustrates the coupling of the native MEP pathway of E. coli with synthetic devices A and B. These two devices will be constructed on a single plasmid.*

Materials and methods

Limonene extraction

In order to test the effects of limonene on mosquito behavior, the compound must first be extracted from orange peel (Barton et al., 2020). A distillation apparatus was used to extract the limonene (Figure 3), using modifications of a previously published method (Lesson 4, 2013).

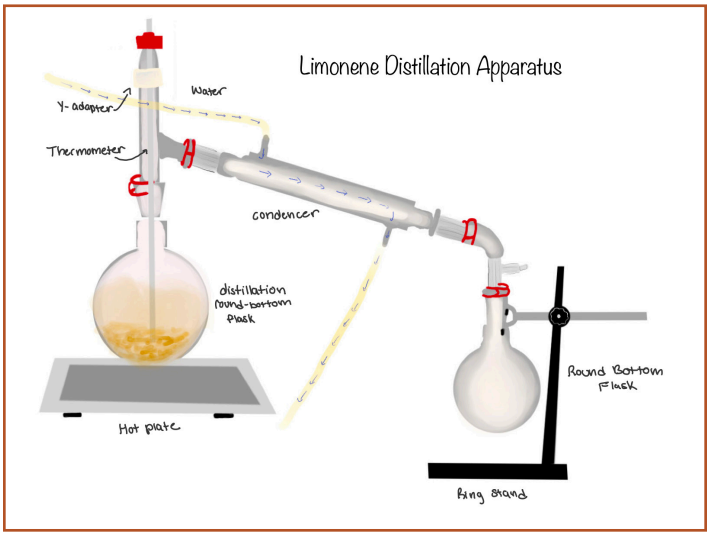

Figure 3. *Schematic of steam distillation apparatus used for limonene extraction. Arrows indicate flow of water from the injector spout of the faucet. Oil-water mixture was collected in the round bottom flask and subsequently transferred to a separating funnel.*

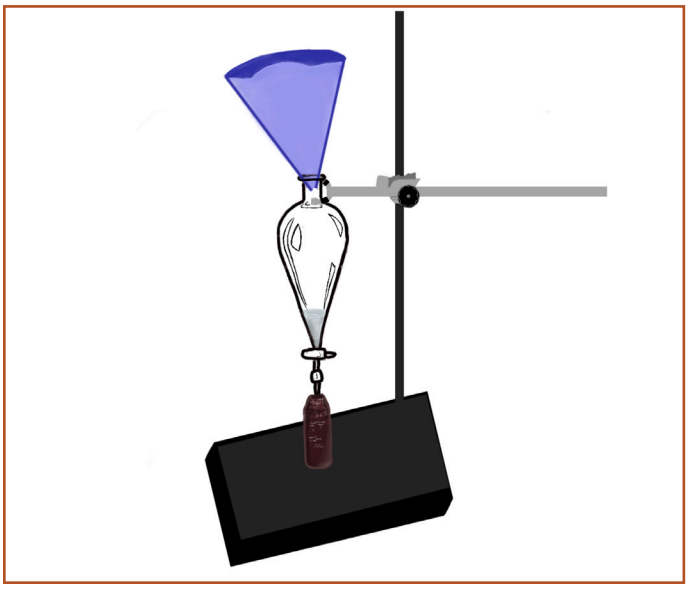

Figure 4. *Schematic of the separating funnel step used to separate the unpurified limonene essential oil from the rest of the distillate. The denser water layer was removed and the floating, unpurified limonene oil layer was captured in an amber vial.*

First, the peel of six navel oranges was zested. Only the zest from the orange part of the peels was collected; all other parts of the oranges were discarded. The orange zest was then placed into the boiling flask of a distillation apparatus (Carolina Biological Supply Company, Cat. No. 725635), followed by 250 mL of distilled water. The apparatus was placed over a hotplate. The hotplate was heated to 200 °C, and the temperature of the internal thermometer was kept below 100°C to make sure the water and orange zest mixture did not reach boiling point. The oil–water mixture collected in the distillation apparatus was placed into a separating funnel for oil layer capture (Figure 4). The bottom layer (the water layer) was poured into a waste container while the top 5 mL layer (oil layer) containing an unknown concentration of limonene was collected in a 10 mL amber vial for later use.

Limonene solution preparation

Four solutions containing increasing amounts of extracted limonene were prepared. Each solution consisted of a 1:1 ratio of a 10% sucrose solution, and one of four different concentrations of oil containing limonene: 0% (negative control), 25%, 50%, and 100%. An analytical test to determine the purity of the collected oil was not performed, but Jongedijk et al. (2016) reported that citrus oil can contain between 70–98% limonene. In place of formal analysis, a comparative smell test was performed using commercially available limonene (Floraplex Terpenes, LLC) as the standard. The distillation product and the reference sample smelled nearly identical when compared by the authors. Micropipettes were used to transfer the sucrose solution, limonene extract, and distilled water into each of the properly-labeled vials (see Table 1).

Limonene Concentration	Volume of %10 Sucrose Solution (mL)	Volume of Limonene Extract (mL)	Volume of Distilled Water (mL)
0%	1	1	1
25%	1	0.25	0.75
50%	1	0.5	0.5
1000%	1	1	1

Table 1. *Amounts of each substance in the different limonene test samples. Final volume of test solutions is 2 mL, with a constant volume of 10% sucrose in water, sucrose and varying amounts of limonene distillation product.*

Mosquito growth and development

Bug dorm and aspirator assembly

The growth and development of mosquitoes began with the assembly of cages (Figure 5) and aspirators (Figure 6) in order to move and sex mosquitoes. Aspirators were made using 10 mL serological pipets, 8” of latex tubing (0.4” O.D.) for flexibility, 2 mL Eppendorf tubes as mouthpieces, and mosquito netting (Walmart) to act as a barrier for mosquitoes, cut into 1.5” × 1.5” squares.

Figure 5. *An 8″ × 8″ × 8″ bug dorm was constructed and used to keep the adult A. albopictus mosquitoes in a contained area. These bug dorms also played the dual role of testing chambers in this investigation.*

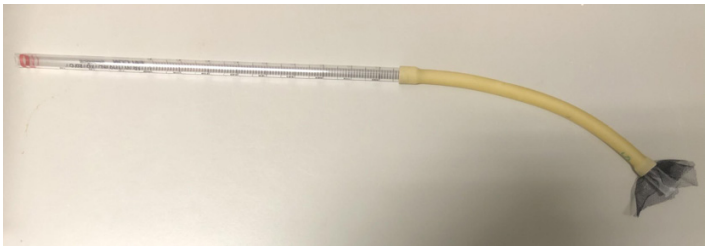

Figure 6. *A homemade mosquito aspirator was assembled using a 10 mL serological pipet (left), 8″ rubber tubing (middle), mosquito netting and an Eppendorf tube with the lid and bottom removed (right). Using this aspirator, adult mosquitoes could be carefully transferred to the bug dorm without the risk of inhaling the mosquito, due to the mosquito netting partition.*

Mosquito conditions for care and feeding

Mosquito eggs were first hatched in a 14″ × 10.5″ × 3″ plastic tray containing approximately one inch of spring water. This tray was also covered in mosquito netting to prevent any matured mosquitoes from escaping. Mosquito larvae were fed using a pinch of crushed pellet fish food (Hikari Micro Pellets) every day. When larvae reached the pupal stage of the mosquito life cycle, they were transferred into either a 32 oz or 40 oz yogurt container with approximately one inch of distilled water using a disposable graduated Pasteur pipette (0.5 mL capacity, 150 mm length). Once adult mosquitoes emerged, they were aspirated and transferred to a bug dorm. All containers and cages were kept in a small 12′ × 8′ room with LED lighting operating on a 12-hour light/dark cycle. Each day, a cotton ball saturated with 10% sucrose solution was placed in the chambers along with a 100 mm diameter Petri dish filled with distilled water to provide additional moisture. This process was continued until there were over 50 female mosquitoes in the population (Figure 7).

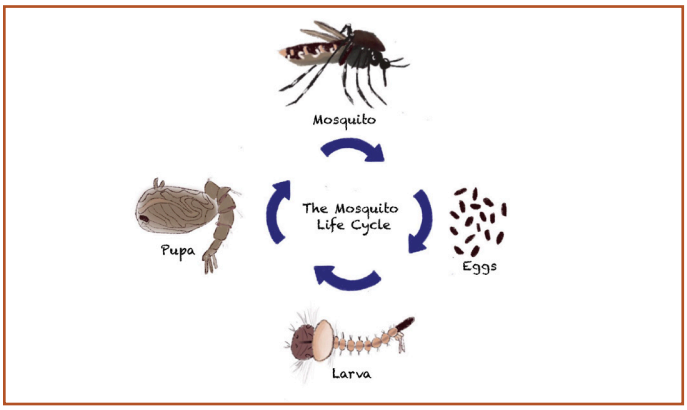

Figure 7. *A. albopictus life cycle. After a day or two when submerged in water, worm-like larvae, also called "wigglers," will hatch from eggs. In less than a week, larvae will develop into pupae with distinctly dark, densely colored anterior. The pupae can then be transferred into another container with water. Within a few days, mature, adult mosquitoes will emerge from pupae (Center for Disease Control and Prevention, 2020b), where they can then be transferred to the bug dorm using aspirators.*

The effects of limonene concentration on mosquito behavior

Sample population

Before data collection, female mosquitoes were separated from the population by aspiration and isolated in a testing bug dorm. Mosquitoes were sorted by gross examination, as females tend to be larger and have lightly feathered antennae, while males are smaller and have bushy antennae (Figure 1). To ensure that mosquitoes would be hungry and attracted to the sucrose solution to be used as the control, mosquitoes isolated for testing were starved for a 24 h period prior to experimentation.

Testing protocol

Two bug dorm chambers containing 25 starved female mosquitoes each were isolated in separate classrooms to prevent scent diffusion between bug dorms. Testing was conducted simultaneously to efficiently utilize the time available. This decision was made prior to experimentation to ensure that there would be a large enough sample mosquito population to carry out the investigation in the

event that mosquitoes did not survive overnight. Classrooms used as testing sites were separated by a hallway, and the two rooms had closed doors. There were also no windows open during the experiment, to prevent scent diffusion. Classroom one tested the 0% and 50% limonene samples and classroom two tested the 25% and 100% limonene samples. Each mosquito sample was given a 10 min adjustment period before the trials to account for the change in temperature and humidity. The designated prepared solutions were then inverted to mix the sucrose and limonene solutions and soaked into the cotton ball directly before the trial began. This was to ensure attractive sucrose was sufficiently mixed with limonene. The cotton ball was then placed into an empty petri dish bottom and inserted into the testing chamber. The number of mosquitoes landing on the cotton ball was recorded in 30 s intervals for 10 min. The 10 min testing duration was chosen after referring to a similar experiment conducted with mosquitoes (Grieco, 2007).

Time (min)	Limonene Concentration			
	%0	%25	%50	%100
0	0	0	0	0
0.5	0	0	0	0
1	0	0	0	0
1.5	0	0	0	0
2	0	0	0	0
2.5	0	0	0	0
3	0	0	0	0
3.5	0	0	0	0
4	0	0	0	0
4.5	0	0	0	0
5	0	0	0	0
5.5	0	0	0	0
6	0	0	0	0
6.5	0	0	0	0
7	0	0	0	0
7.5	0	0	0	0
8	0	0	0	0
8.5	0	0	0	0
9	0	0	0	0
9.5	0	0	0	0
10	0	0	0	0

Table 2. *Data table reporting the number of A. albopictus at different concentrations of limonene every 30 s for 10 min. Each limonene solution consists of a 10% sucrose solution (1 mL; the standard food sustaining the mosquito population) and a limonene and water mixture (1 mL), as specified in Table 1.*

When testing was complete, the cotton ball was removed from the chamber and the mosquitoes received a 30 min recovery period between trials. This amount of time was chosen after discussions with Dr. Joseph Wagman, the team's mosquito consultant. After the recovery period, the trials for the remaining limonene concentrations were conducted under otherwise identical conditions.

Laboratory safety

Throughout the experiment, proper personal protective equipment was worn by researchers including lab aprons, non-latex gloves, and safety goggles. Non-latex gloves were used for hand protection and as a measure to prevent cross-contamination between limonene concentrations during testing. Lab materials, including used pipette tips and petri dishes, were disposed of properly. The extracted limonene was stored in an airtight, lightproof container and kept within its optimum temperature range. Limonene is a category 3 flammable liquid according to the Globally Harmonized System (GHS), so it was stored away from any open flames. Mosquitoes were closely monitored daily and during experimentation to ensure humane handling of live specimens. The mosquito nursery where the specimens were raised and housed was selected because it is a closet within a classroom. There were two doorways, to reduce the likelihood of possible escapees, as well as reducing the chance of exposing mosquitoes to environments outside the controlled conditions. Aspirators were used to transfer mature mosquitoes. Since the mosquitoes were hatched from eggs, there was no chance that any disease could be passed to the researchers. Researchers' hands spent minimal time in the bug dorms, only going in and out quickly to replace food and water, while the bug dorm entrance was held securely so no mosquitoes could escape. Dead specimens were disposed of in the regular garbage, because they are not considered a biohazard.

Results

This experiment was conducted to test the effectiveness of limonene as a potential mosquito repellent. Preliminary results are inconclusive. The protocol called for the observation of the number of mosquitoes making contact with limonene extract-treated cotton ball samples every 30 s for 10 min. Mosquitoes did not exhibit any preference for the control, which consisted of equal parts 10% sucrose solution and distilled water, over the different concentrations of limonene that replaced the distilled water in the test samples (Table 1).

The results, reported in Table 2, show that no mosquitoes landed on the 0% limonene concentration cotton at any time during testing, which was unexpected. Theoretically, the sucrose solution should have appealed to the mosquitoes since they had been regularly fed on this solution and starved for 24 h. In addition, the mosquitoes did not land on the cotton balls soaked in 25%, 50%, or 100% limonene solutions. Over the course of the experiment, mosquitoes displayed little to no move-

ment and remained on the sides of the chamber during testing with the exception of slight leg movement.

Potential sources of error can be identified that might have contributed to these results. Firstly, the cotton balls may not have been completely soaked in the test solutions. Both the method and the volume used to treat the cotton ball with the designated limonene solution may have interfered with mosquito olfaction. It is possible that not enough of the solution was absorbed by the cotton ball and any solution that was absorbed was located on the interior of the cotton ball as opposed to the cotton ball exterior. Secondly, the mosquitoes that were tested may not have been in the best physical condition, since they were starved for 24 h before the testing in order to amplify their feeding behavior. This strategy may have had unanticipated, adverse effects. A third source of error could be that the experiment took place in the middle of the day in a bright environment, as opposed to their dimly-lit nursery. Mosquitoes tend to be less active during the day due to the hotter temperatures. Instead, they become active during dawn and dusk when there is less sun exposure and the temperature begins to fall (When Are Mosquitoes Most Active, 2020). Even though a 10 min window was used between moving the mosquitoes to another area for testing and actually running the experiment, this might not have had the desired effect because the environment in the testing area was different from the mosquito nursery.

Discussions

The purpose of this experiment was to determine if limonene, an aromatic hydrocarbon found in the oil of citrus fruit peels, is an effective insect repellent. If limonene is proven to act as such, this would prompt the construction of AliveSCENT. As a non-toxic mosquito repellent, AliveSCENT has the potential to deter the presence of mosquitoes and have public health applications. The scent produced by limonene has been likened to turpentine, which has in the past been shown to deter certain insects (Barton et al., 2020). However, there was still a need to determine if limonene would deter mosquitoes at all.

This marked the beginning of a nearly year-long endeavor. To meet this goal, the team needed to accomplish three things: Extract limonene, raise mosquitoes, and run a final experiment. The project was not without its challenges. One of the most difficult parts of this project was to extract limonene from orange peel. The team used steam distillation to obtain the essential oil from orange zest, but it cannot be determined how much of the oil reclaimed is limonene. This makes it difficult to determine if the distillation was a success. However, limonene makes up 70–98% of orange peel, so it is very likely that limonene was extracted (Barton, et al. 2020). In addition, it is the opinion of the authors that the scent was noticeably similar to that of commercially available limonene.

Raising the *A. albopictus* mosquitoes proved to be more challenging than expected. The first batch of eggs never hatched, most likely due to the cold weather they were shipped in. However, the next batch was kept in more ideal conditions, and hatched relatively quickly. There was success in raising the mosquitoes from larvae to the adult stage. The procedures for mosquito care were adapted from published mosquito rearing protocols (Foggie & Achee, 2009). Unfortunately, there was a high mortality rate among the mosquitoes, with many dying each day. This could have been due to the room's conditions such as temperature or humidity, but they were fed and otherwise cared for regularly.

For the final experiment, the results were inconclusive, so it can be neither confirmed nor refuted that limonene is a possible candidate as a natural insect repellent. It was expected that the mosquitoes would land on the cotton ball soaked in the control (sucrose only) solution. When limonene was added, this was expected to deter the mosquitoes from landing on the cotton ball. However, within our observation window, mosquitoes did not land on either treated or untreated cotton balls. Therefore, no claim can be made about the effectiveness of limonene as a mosquito repellent. Additional testing must be performed.

This project is far from over, as there are many error sources that need to be corrected in future testing. This includes the use of higher amounts of limonene and sucrose solution on the cotton balls, and increasing the sample population size of mosquitoes used in the experiment. The results of subsequent investigation will determine the next steps the team will take.

Next steps

The purpose of this experiment was to provide proof-of-concept evidence to support the claim that limonene is an effective insect repellent. As previously stated, the reported results can neither confirm nor reject this claim since the control conditions did not produce the chemotactic behavior expected of starved mosquitoes. The unresponsiveness of the mosquitoes in this preliminary investigation suggests several next steps.

First, the protocol will be improved to include a limonene extract and 10% sucrose solution mixture with a total volume of 10 mL rather than 2 mL. This five-fold increase will mimic the volume of sucrose water that is routinely applied to cotton balls during daily feeding. Second, given the likelihood that a change in setting from the dimly-lit mosquito nursery to a brightly-lit classroom for testing may have caused the mosquitoes' reported behavior, future testing will take place in the original nursery. This will eliminate testing location changes as a source of error. Lastly, a commercially-produced limonene (Floraplex Terpenes, LLC) that is claimed

to be "very concentrated" will replace the steam distilled limonene extract. By repeating the experiment described under the Materials and Methods section using this substitute, and with the change of experiment location, baseline results can be obtained and will be compared to experimental results. Analysis of experimental results will determine whether limonene can truly be used as a mosquito repellent.

If limonene is determined to be an effective mosquito deterrent, future experimentation will involve the construction and subsequent transformation of *E. coli* with a second plasmid containing geranyl diphosphate synthase (GPPS), required to close the MEP pathway for maximum limonene production, but the team will consider *P. putida* as an alternative chassis due to its reported high tolerance of limonene (Ramos et al. 2015). Several techniques for limonene capture from engineered microorganisms have been described by Jongedijk et al. (2016) including culture extraction and solvent overlay, among others, and have been considered as likely candidates in this experimental design. Solvent overlay has been shown to reduce cytotoxicity in cultures and is most suited for large-scale recovery of limonene. Solvent overlay requires the use of industrial-grade chemicals that may not be accessible in this setting, so culture extraction by lysis will be the preferred method for limonene recovery in this project. In addition, culture extraction is also preferable to solvent overlay when the desired end-product is limonene as an essential oil (Jongedijk et al. 2016). Evaluation of limonene's effectiveness as a functional mosquito repellent will be carried out with the use of a choice chamber. The plasmid design has been changed compared to the initial concept, to now include two devices (Figure 2). The team is looking at plasmid pGL403 from the Addgene plasmid library for inspiration because it contains both GPPS and limonene synthase (LIMS), which are necessary for limonene production.

If limonene is proven ineffective, other essential oils could be tested using the same experimental procedures, such as lavender or peppermint. New plasmid design and construction would follow subsequent determination of *E. coli* as a suitable chassis candidate for the expression of these scents.

Author contributions

M.A., E.A., Y.C., G.C., E.F., A.J., S.L., M.P., S.P., and C.R. conducted background research. Y.C., G.C., and C.R. designed the experimental protocols. Y.C., E.F., S.L., M.P., and C.R. performed the distillation and collection of limonene. M.A., E.A., Y.C., E.F., S.L., M.P., S.P., and C.R. conducted nursery assembly and mosquito care. M.A., Y.C., E.F., M.P., S.P., and C.R. performed the final experiment. Y.C. wrote the abstract, which was edited by E.F., M.P., G.C. Y.C. and S.L. developed the video and created the manuscript graphics. Y.C., G.C., E.F., S.L., M.P., and C.R. wrote the manuscript. Y.C. and E.F. compiled references and edited the manuscript.

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