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THE DEVELOPMENT OF A WATER-JACKETED MEMBRANE FEEDER REPELLENT TESTING SYSTEM FOR BLACK FLIES (DIPTERA: SIMULIIDAE)

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ABSTRACT. An in vitro repellent testing system for use with colony reared black flies, *Simulium vittatum*, is described. Postoviposition female *S. vittatum* were exposed to latex membranes treated with 15 µl of commercially available insect repellents every 2 h, up to 12 h. Repellents tested were the following: Repel® Plant-Based Lemon Eucalyptus Insect Repellent 2 (30% oil of lemon eucalyptus [OLE]); OFF!® Botanicals Insect Repellent IV (10% *p*-menthane-3,8-diol [PMD]); and Zevo™ On-Body (20% 3-[*N*-butyl-*N*-acetyl]-aminopropionic acid, ethyl ester, IR3535®). Untreated membranes served as control. The PMD and IR3535 had negative correlations between repellency rate and time (IR3535, m (slopes of mean repellencies over time) = −6.64; and PMD, m = −5.28), whereas OLE had none (m = 0). Statistical analysis demonstrated significance within all groups that included OLE or the control ($P < 0.00$), but none for groups consisting of PMD or IR3535 ($P = 0.31$).

KEY WORDS Insect repellent, IR3535, oil of lemon eucalyptus, *p*-menthane-3,8-diol, *Simulium vittatum*

INTRODUCTION

Black flies (Diptera: Simuliidae) are significant pests of man and animals. This pest status is further exacerbated due to the role in transmitting the filarial nematode that causes onchocerciasis (river blindness) in West Africa and in parts of Central and South America (Burnham 1998, Hoerauf et al. 2003, Enk 2006). In northeastern North America, large swarms of biting black flies can cause "black fly fever." This malady is caused by a negative reaction to black fly salivary compounds and may induce headache, fever, nausea, and swollen lymph nodes in the neck (Adler and McCreadie 2019). Commercial insect repellents have long been used to ward off swarming black flies; however, few techniques exist for testing the efficacy of these formulations against black flies in a laboratory setting. All previous related studies have been conducted in the field (Debboun et al. 2000, Tawatsin et al. 2006, Wilson et al. 2013) or with wild-caught black flies (Bernardo and Cupp 1986, Robert et al. 1992).

The University of Georgia Black Fly Research and Resource Center (Athens, GA) maintains the only known colony of black flies. The *Simulium vittatum* Zetterstedt colony provides a unique resource for experimentation by supplying a standardized test subject year-round. Colony protocol strives to support development through all life stages (egg, larvae, pupae, adult; Gray and Noblet 2014). Given sufficient larval nutrition, female *S. vittatum* are autogenous for the initial gonotrophic cycle (although will blood feed to produce subsequent egg batches; Adler et al. 2004). This biological trait is advantageous because postoviposition female *S. vittatum* are more likely to host seek and feed. To induce the highest biting rates, we try to use a high percentage of postovipositional females for repellent testing.

In this protocol, 3 nondeet-based commercially available insect repellents were assessed. The products were the following: Repel® Plant-Based Lemon Eucalyptus Insect Repellent 2 (30% oil of lemon eucalyptus [OLE]); OFF!® Botanicals Insect Repellent IV (10% *p*-menthane-3,8-diol [PMD]); and Zevo™ On-Body (20% 3-[*N*-butyl-*N*-acetyl]-aminopropionic acid, ethyl ester, IR3535®). The OLE, previously known as Quwenling, is a waste product produced through hydrodistillation of essential oils from the leaves and twigs of *Corymbia citriodora* Hooker (Collins et al. 1993, Carroll and Loye 2006b, Moore 2015). The spent product contains PMD. The OLE naturally contains PMD; however, PMD can also be chemically synthesized from citronella for use in insect repellent products, seen in OFF! Botanicals Insect Repellent IV (Kurnia et al. 2020, Xuan-Tien et al. 2024). As an active ingredient, PMD has been shown to repel an array of hematophagous invertebrates, including mosquitoes, midges, black flies, stable flies, ticks, and land leeches (Trigg and Hill 1996, Carroll and Loye 2006b, Jaenson et al. 2006, Wilson et al. 2013, Kirton 2013, Frances et al. 2014). The active ingredient (3-[*N*-butyl-*N*-acetyl]-amino-propionic acid, ethyl ester, IR3535) is a synthetic molecule derived from the nonessential amino acid β-alanine (Puccetti 2007) and has been shown to repel mosquitoes, sand flies, fleas, lice, and ticks (Cilek et al. 2004, Naucke et al. 2006, Bohlmann 2008, Carroll 2008, Carroll et al. 2010, Weeks et al. 2019). Both PMD and IR3535 primarily achieve repellency via inhibition of odorant receptors. In *Aedes aegypti* (L.), PMD is antagonistic on olfactory proteins AaOR2-Orco and AaOR8-Orco, whereas IR3535 is antagonistic on olfactory proteins AaOR2-Orco, AaOR8-Orco, and AaOR10-Orco (Dickens and Bohbot 2015).

To evaluate these repellents, our repellent testing protocol uses on a Rutledge-style water-jacketed glass membrane feeder system. These systems are

widely used in vector biology research to induce feeding in a variety of insects (Owens 1981, Durvasula et al. 2014, Feldlaufer et al. 2014, Mahmood and Colacicco-Mayhugh 2014, Dias et al. 2021) and to assess repellent efficacies against mosquitoes (Rutledge et al. 1976, Cockcroft et al. 1998, Rutledge and Gupta 2004, Waka et al. 2004, Dube et al. 2011, Rutledge et al. 2015). One previous study assessed deet-based repellents against black flies using this system (Bernardo and Cupp 1986). Our protocol builds upon this existing work to establish an effective, in vitro methodology for evaluation of many commercially available insect repellents against black flies. We hypothesize that this novel protocol will be a useful tool for future research in the prevention of onchocerciasis.

MATERIALS AND METHODS

Our testing protocol used 4 water-jacketed membrane feeders connected in succession to a recirculating water bath (ISO Temp 1016S, Fisher Scientific, Atlanta, GA). The recirculating water bath is operated at 37°C to simulate human body temperature. The feeders are connected to each other and the water bath with Tygon tubing (1.5 cm in diameter). Each water-jacketed membrane feeder (made in the University of Georgia glass-blowing shop) is a 5 cm in diameter (19.6 cm^2 surface area), hollow-walled glass bell with 2 fittings, 1 on each side, and a top port (Fig. 1). The 2 fittings allow warm water, provided by the recirculating water bath, to be pumped through the walls of the bell, thereby warming the environment within the bell. The top port allows for addition of liquid. When the bell opening is covered with a membrane, any liquid added through the port is contained behind the membrane and is warmed by the recirculating water bath.

The membranes used in these experiments were latex, cut from powder-free examination gloves (SKINTX®; GD Care Inc., Azusa, CA). The cut pieces of membrane were washed manually with a 10% solution of laboratory detergent (Sparkleen™ 1; Fisher Scientific Co., Pittsburgh, PA), rinsed with hot water, and blotted dry with paper towels. The latex membranes were securely attached to the membrane feeders with #32 rubber bands. All treatments, including an untreated control, were assigned randomly to 1 of 4 membrane feeders. In a fume hood, 15 µl of each repellent was pipetted onto the surface (19.2 cm^2) of a corresponding membrane and spread uniformly across with a gloved finger.

Between repellency evaluations, the water-jacketed membrane feeders were suspended on a resting board (4 × 14 × 65 cm) board with 4 (9-cm-diameter) holes cut into it. After treatment application, the feeders were suspended on the resting board for 2 h preexperimental start. During this time, 10 ml of a 10% sucrose solution, created in-lab using standard table sugar and distilled water, was pipetted through the top port into the membrane feeder. No additional chemical attractants were added. Repellency evaluations were begun after 2 h due to formulations of IR3535, PMD, and OLE maintaining high levels of repellency against hematophagous flies through this time frame (Carroll and Loye 2006a,b, Naucke et al. 2007). Also, the 2-h incubation period allowed sufficient warming of the sucrose solution. A humidifier (AirCare® MA0800; Essick Air Products Inc., Tacoma, WA) was operated in the laboratory to increase relative humidity. Relative humidities ranged from 23.5% to 26.2%. Temperatures in the laboratory were relatively warm, ranging from 21°C to 25°C.

Fig. 1. One of 4 water-jacketed membrane feeders with fittings on either side and a top port for addition of liquid. The feeder is affixed with a latex membrane and set atop an adult feeding container. The nylon mesh top is pulled tightly to support the weight of the feeder, while enabling flies to feed through it.

The adult feeding containers, where the flies were confined during repellency evaluations, were modified, pint-sized, paper food containers (Neptune Containers, Newark, NJ). The center of the lid was removed, and a thin, 100% nylon mesh (Casa Solid Tulle Bright White; Joann Fabrics and Crafts, Hudson, OH) was secured to the lid rim with a strip of duct tape. The mesh was attached tightly, so the membrane feeder sat flatly on the screen and in complete contact with the membrane surface (Fig. 2). The bottom of the containers was also removed, and the surfaces were covered with a transparent vinyl sheet (Frost King, Mahwah, NJ) that was attached with hot glue. The transparent vinyl sheet allows for direct observation of the flies and the treated membrane surface. A hole (2 × 2 cm) was cut in the side of the container and covered with overlapping panels of latex dental dam (Coltene Elasti-Dam, Cuyahoga Falls, OH) to allow loading of flies via aspiration.

The center of the water-jacketed membrane feeder repellent testing system was a viewing frame. The viewing frame (61 cm high, 69.5 cm wide, and 38 cm deep) was constructed of 1.5-cm thick plywood, coated with polyurethane, and screwed together with 3-cm wood screws. The frame had 4 (9-cm-diameter) holes cut out to hold the adult feeding containers. The frame was situated on a standard desk, and the 61-cm height allowed the person evaluating biting rates to view the membranes

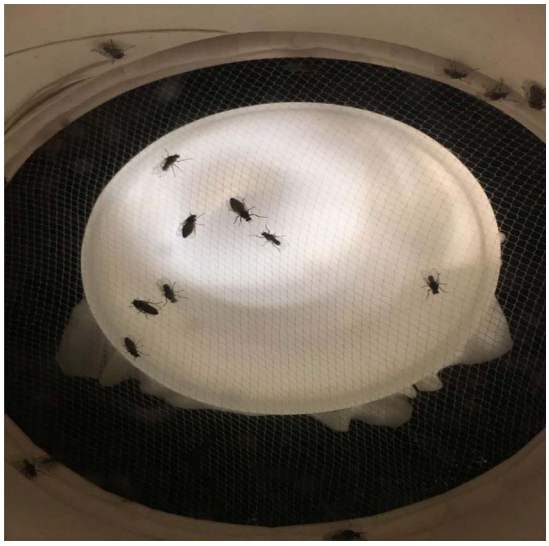

Fig. 2. Flies observed feeding on a treated membrane through the transparent vinyl bottom of an adult feeding container.

through the clear vinyl bottom of the adult feeding containers. Due to the *S. vittatum* colony's strong phototactic nature, light was limited during repellency evaluations to only enter through the feeder or membrane (Figs. 2 and 3). The viewing frame was shrouded with black fabric on all exposed sides, and a disc of black fabric was placed around the membrane feeder.

Prior to the first observation, 40 postovipositional female flies were aspirated into each of the adult feeding containers. The flies were collected from the colony oviposition chambers after approximately 26 h. Determination of sex and gravidity was conducted visually while aspirating. Females with narrow or deflated abdomens were selected because they were more likely to be postovipositional.

Adult feeding containers with 40 flies each were placed in the 9-cm holes in the top of the viewing frame. Each membrane feeder was placed on the mesh surface of its corresponding adult feeding container. After a 5-min feeding window, biting rates were assessed. Biting was determined by viewing a stationary fly on the membrane with its mouthparts actively engaged against the mesh and membrane (Fig. 2). Flies that were moving were not scored as biting. After repellency evaluations were completed, the membrane feeders were moved to the resting board, and the containers of flies were removed. Every 2 h, up to 12 h, fresh adult feeding containers were loaded with 40 new flies, and biting rates were evaluated. The use of fresh containers per repellency evaluation ensured residual repellent volatiles did not interfere with biting rates. The use of new flies per repellency evaluation enabled the most accurate biting rates as time increased. Three replicates of this protocol were completed for data analysis.

Fig. 3. The water-jacketed membrane feeder apparatus and viewing frame. The connected water-jacketed membrane feeders sit atop 4 adult feeding containers loaded with 40 female flies each and connected to the water bath on the right of the image. The viewing frame supports the apparatus and is shrouded below and on top.

Statistical analysis

Data analysis was conducted through analysis of covariance (ANCOVA; repellent treatment as a categoric variable and time as a continuous variable), followed by post hoc Tukey's honest significant difference (HSD) tests to isolate specific differences among treatments. Statistical significance was based on a 95% confidence interval ($\alpha = 0.05$). Goodness of fit was determined by R^2. Poisson family general linear models (GLMs) were generated to predict values for hour 12 of replicate 1 because that test was ended at 10 h because membranes became compromised. Data were also assessed using mixed effects and repeated measures analysis of variance, but we did not use those analyses because a significant interaction existed between time and treatment. The ANCOVA incorporated the progressive and linear impact of time into treatment contrasts. The statistical software RStudio (Versions 2024.12 and 2025.05, Posit Team, Boston, MA) was used to generate all analyses. Installed packages implemented to organize and format data were broom, emmeans, ez, dplyr, knitr, and tidyr. Integrated RStudio functions used were avo, glm, and TukeyHSD.

RESULTS

The ANCOVA testing indicated a significant difference in repellency rates among treatments (Fig. 4 and Table 1). Tukey tests indicated that the 30% OLE had greater repellency than any of the other treatments (Table 1). The 10% PMD and 20% IR3535 treatments differed from the untreated control but not from each other (Table 1). The magnitude of the differences among treatments increased over 12 h (being the least at hour 2 and the most at hour 12). Percentage of variation explained by the model was very high ($R^2 = 0.82$). Because biting rates in the untreated control increased over the 12 h of the experiment (Fig. 4), we adjusted repellency rates of

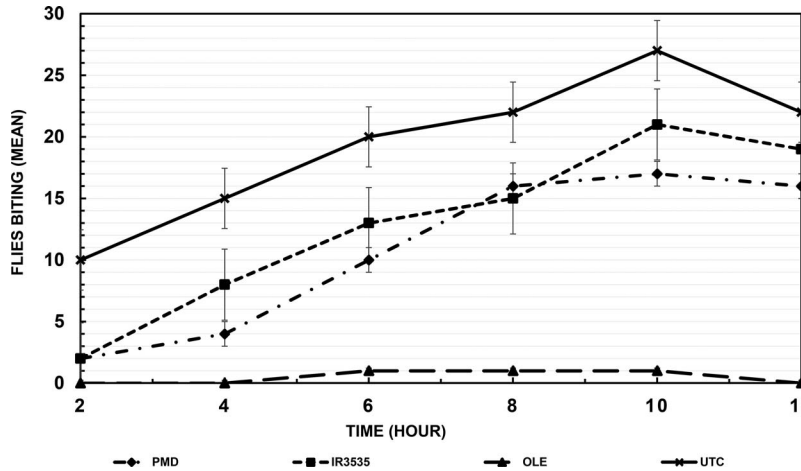

Fig. 4. Mean biting rates ($N = 3$) for 20% 3-[*N*-butyl-*N*-acetyl]-aminopropionic acid, ethyl ester, IR3535® (IR3535), 30% oil of lemon eucalyptus (OLE), 10% *p*-menthane-3,8-diol (PMD), and the untreated control against postoviposition female black flies over 12 h (UTC). Slopes: IR3535, m = 1.7; OLE, m = 0; PMD, m = 1.4; and UTC, m = 1.2.

each commercial product as a percentage of the control in Fig. 5. Positive slopes in biting rate are observed for all non-OLE treatments, including the control (IR3535, m = 1.7; PMD, m = 1.4; UTC, m = 1.2; Fig. 4). Conversely, negative slopes in repellency rate are observed for all non-OLE products (IR3535, m = −6.64; PMD, m = −5.28; Fig. 5), demonstrating negative correlations between repellency and time. The OLE produced a flat slope (m = 0) for biting and repellency rate, with slight variation in the median portion of the data set (Figs. 4 and 5). No degradation in repellency was observed over the time allowed in these experiments.

DISCUSSION

Our protocol development demonstrated that 15 µl is adequate for uniform membrane coverage, while still allowing failure within 12 h (15 µl per 19.6 cm^2) in most cases. This is higher than the optimal dose per unit area in contemporary studies on artificial membranes against mosquitoes (10 µl per 19.625 cm^2; Waka et al. 2004, Dube et al. 2011). We used 15 µl due to observed feeding on the farthest edges of the membranes at lower volumes. The assumption is that below 15 µl, repellents did not spread evenly across the membrane surface. In contrast, the volume used in this protocol was lower than studies on human skin against mosquitoes (1–1.5 ml per 600 cm^2; Carroll and Loyle 2006a,b; and 50 µl per 16 cm^2; Peng et al. 2022), an d black flies (25 µl per 6.6 cm^2; Robert et al. 1992; and 2 ml per 710–780 cm^2; Tawatsin et al. 2006). This, however, is expected because human skin reacts differently to repellent products than latex through texture, evaporation, and absorption (Barradas et al. 2013, Tavares et al. 2018).

Table 1. Analysis of covariance and Tukey honest significant difference among treatment groups.

Treatment[1]	Difference	Lower confidence interval	Upper confidence interval	*P* adjusted
UTC–IR3535	6.33	2.95	9.71	0.00***
UTC–OLE	18.94	15.56	22.32	0.00***
UTC–PMD	8.55	5.17	11.93	0.00***
PMD–IR3535	−2.22	−5.59	1.15	0.31
PMD–OLE	10.38	7.01	13.76	0.00***
OLE–IR3535	−12.61	−15.98	−9.23	0.00***

[1] UTC, untreated control; IR3535, 20% 3-[*N*-butyl-*N*-acetyl]-aminopropionic acid, ethyl ester, IR3535; OLE, 30% oil of lemon eucalyptus; PMD, 10% *p*-menthane-3,8-diol.

*** High statistical significance ($P < 0.00$).

In our testing system, the 30% OLE treatment clearly exhibited the greatest repellency, and its effectiveness did not wane over the 12-h testing period. Carroll and Loye (2006b) showed 20% OLE to be equally effective as 30% deet against mosquitoes in both laboratory and field tests and provided decent to good repellency against biting Leptoconops carteri Hoffman (Carroll and Loye 2006a). Consequently, it is not surprising that a 30% concentration of OLE was also effective against black flies. The other materials exhibited some repellence, as compared with the untreated control, but the effectiveness declined significantly over the 12-h testing period. Differences in treatments are supported by laboratory testing against mosquitoes in Carroll and Loye (2006b), when complete protection times (CPT) between 10% PMD and 20% OLE were substantially significant (PMD, 124 ± 108 min and OLE, 307 ± 144 min), and Cilek et al. (2004) when CPT for 20% IR3535 was 167.3 ± 12.3 min.

Biting rate on the untreated control increased over time, an observation that has been made regularly throughout our testing. We suggest two explanations for this trend: physiologic or ovipositional status and residual saliva stimulating feeding via the invitation

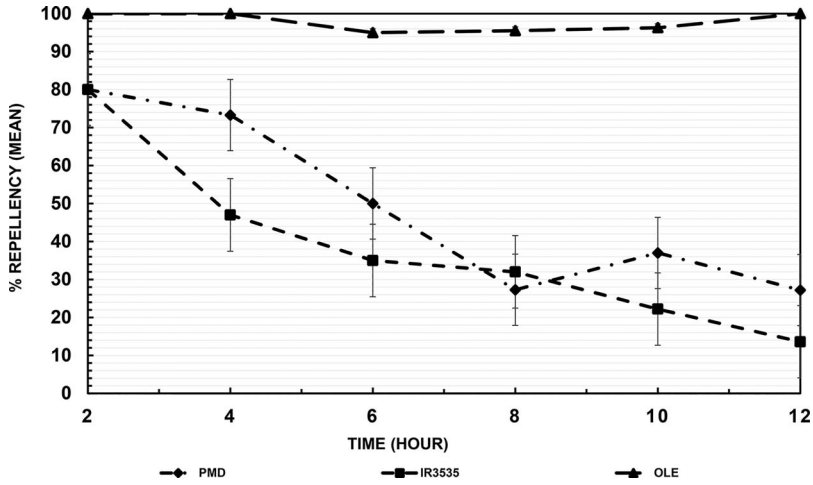

Fig. 5. Mean percentage of repellency ($N = 3$) of 20% 3-[*N*-butyl-*N*-acetyl]-aminopropionic acid, ethyl ester, IR3535® (IR3535), 30% oil of lemon eucalyptus (OLE), and 10% *p*-menthane-3,8-diol (PMD), and the untreated control against post-oviposition female black flies over 12 h (UTC). Slopes: IR3535, m = −6.64; PMD, m = −5.28; and OLE, m = 0.

effect (McCall and Lemoh, 1996). The further into the 12-h testing period that flies are collected, the more time they have to oviposit, yielding higher rates of postovipositional flies. Also, older flies have higher rates of dehydration, which may make them more inclined to feed. No matter the cause, this phenomenon is likely observed, to some extent, in all treatments.

This study had a few limitations, which may have affected results. The low number of replicates ($N = 3$) could have limited accuracy of the formulations' repellency trends over time, and, in turn, the statistical relationships to one another. To solve this limitation, future experiments will use 5 replicates. Formulations of deet performed inconsistently in this system at higher concentrations (>7%) and above 6 (Kerr, unpublished data). These inconsistencies were not observed until testing higher concentrations and may be related to the plasticizing nature of deet negatively affecting latex membrane integrity (AFPMB [Armed Forces Pest Management Board] 2015). Latex membranes were initially chosen due to the materials' rigor and consistent use in maintenance of triatome colonies (Durvasula et al. 2014). The durability of the membrane is critical as membrane failures typically terminate testing for the day and are highly counterproductive. Future experiments will evaluate other membrane options. Despite these limitations, we believe the described protocol remains a valuable, novel tool.

Repellent testing systems exist for use against colony-reared mosquitoes (Klun et al. 2005, Deng et al. 2014, Ali et al. 2017); however, no such system exists for black flies. The objective of this study was to establish an effective, in vitro repellent testing system for use against colony-reared black flies. The preliminary data shown here demonstrate the effectiveness of our protocol and prove its potential for future use with other insect repellents.

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