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Vapor phase repellency and insecticidal activity of pyridinyl amides against anopheline mosquitoes

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A B S T R A C T

It is important to identify repellents that can provide reliable protection from arthropod biting and prevent arthropod-borne diseases, such as malaria. In the present study, the spatial repellent activity and toxicity of two novel pyridinyl amides (**1** and **2**) were evaluated against *Anopheles albimanus*, *Anopheles quadrimaculatus*, and *Anopheles gambiae*. In vapor repellency bioassays, compound **2** was generally more effective than DEET and 2-undecanone, while compound **1** was about as active as these standards. Overall, transfluthrin was the most active compound for inducing anopheline mosquito repellency, knockdown, and lethality. Although they were not the most active repellents, the two experimental amides produced the largest electroantennographic responses in female antennae. They also displayed modest toxicity to anopheline mosquitoes. Significant synergism of repellency was observed for the mixture of a pyrethroid-derived acid and the repellent 2-undecanone against anopheline mosquitoes, similar to that observed previously in *Aedes aegypti*. Overall, this study provides insight for further synthesis of alternative amide compounds for use as spatial treatments.

1. Introduction

Identifying new active compounds is essential to control vector populations and reduce the risk of acquiring resistance to commercially available active ingredients for mosquito control. DEET continues to be the gold standard repellent, and other skin-applied repellents include 2-undecanone, picaridin, and IR3535 (EPA, 2020). However, the effectiveness of DEET varies among mosquito species (Rutledge et al., 1978) and continuous usage of DEET can lead to resistance and reduced effectiveness, as observed in laboratory studies of *Anopheles albimanus* (Schreck, 1985) and *Aedes aegypti* (Stanczyk et al., 2010) mosquitoes. In addition, currently available spatial repellents that protect people against mosquitoes in a defined area predominately contain volatile pyrethroids (Norris & Coats, 2017), for which resistance is well known in mosquito species (Mutunga et al., 2015; Agramonte et al., 2017; Estep et al., 2017). For example, knockdown resistance (kdr) to pyrethroids has been documented in the Puerto Rico strain of *Ae. aegypti* (Agramonte et al., 2017; Estep et al., 2017) and the Akron strain of *Anopheles gambiae* (Mutunga et al., 2015). Moreover, resistance to pyrethroids may provide low levels of cross resistance to repellency provided by DEET and other contact and spatial repellents (Deletre et al., 2019; Yang et al., 2020a).

In order to better inform the search for new repellents useful for vector disease control, vapor phase repellency and toxicity was determined for two experimental pyridyl amides (Fig. 1), along with DEET and transfluthrin (TF) as positive controls on *An. albimanus*, *An. quadrimaculatus*, and *An. gambiae*. Behavior of mosquitoes was examined in the absence of human odor to eliminate confounding variables in the screen and to measure the innate potency of any repellent effects. Parallel assays determined the vapor phase knockdown and 24-h lethality of these compounds in the same apparatus to check for insecticidal action. Moreover, we observed previously that the pyrethroid-derived acid (1*R*-*trans*-permethrinic acid, TFA) was an effective synergist of established repellents, such as 2-undecanone in *Ae. aegypti* mosquitoes (Yang et al., 2020b). Thus, additional experiments were performed to see if any synergism occurred between 2-undecanone and TFA in anophelines. Finally, to gain insight into the physiological mechanisms of mosquito response to chemical repellents, the antennal olfactory system of *An. albimanus*, *An. quadrimaculatus*, and *An. gambiae* was screened against the compounds using EAG recordings.

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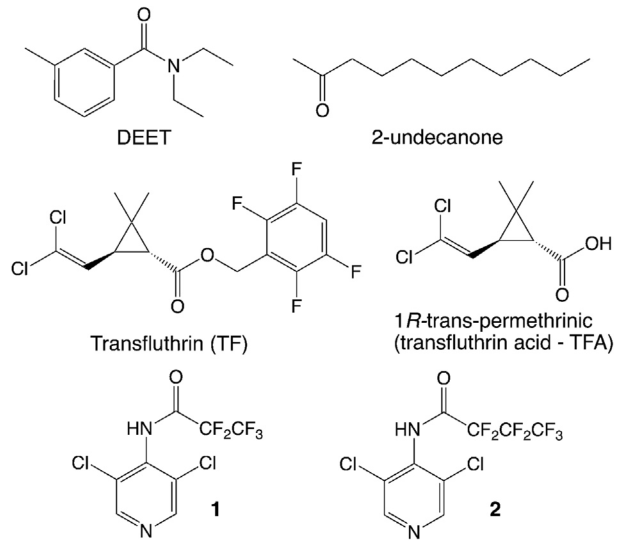

Fig. 1. Chemical structures of DEET, 2-undecanone, transfluthrin (TF), 1*R-trans*-permethrinic acid (TFA), and the experimental pyridinyl amides, *N*-(3,5-dichloropyridin-4-yl)-2,2,3,3,3-pentafluoropropanamide (**1**) and *N*-(3,5-dichloropyridin-4-yl)-2,2,3,3,4,4,4-heptafluorobutanamide (**2**).

2. Materials and methods

2.1. Chemicals

The DEET (97%), 2-undecanone (99%), transfluthrin (> 99%) (TF, Fig. 1), and acetone (> 99%) used were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). The 1*R-trans*-permethrinic acid, which we previously denoted (Yang et al., 2020b) as transfluthrin acid (TFA, Fig. 1) had > 95% purity and was purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). The two experimental pyridinyl amides, *N*-(3,5-dichloropyridin-4-yl)-2,2,3,3,3-pentafluoropropanamide (**1**, Fig. 1) and *N*-(3,5-dichloropyridin-4-yl)-2,2,3,3,4,4,4-heptafluorobutanamide (**2**, Fig. 1) were synthesized using methods similar to those described by Tsikolia et al. (2013) and Richoux et al. (2020).

2.2. Insects

Laboratory colonies of *An. albimanus* (from El Salvador, established in 1975) and *An. quadrimaculatus* (from Orlando, Florida, USA, established in 1952), maintained at the United States Department of Agriculture - Agriculture Research Service (USDA-ARS), Center for Medical, Agricultural & Veterinary Entomology (CMAVE), in Gainesville, Florida, USA were used. Laboratory rearing was accomplished for each mosquito species by placing about 1000 first-instar larvae into a plastic tray containing 3 l of well water. Each tray was placed inside an incubator at 25 ± 1 °C, 75 ± 5% relative humidity (RH), and a 14:10 h (light: dark, L:D) photoperiod. Larvae were fed daily with a mixture of 3:2 ground liver powder and brewer's yeast *ad libitum*. Pupae were collected from the rearing colony and emerging adults were maintained in an ambient temperature of 25–28 °C, relative humidity of 60–80%, and a 14:10 h (L:D) photoperiod. Adults were provided continuous access to 10% sucrose solution soaked in cotton balls. Non-blood-fed adult females 3–7 days post-emergence were used in bioassays.

Eggs of *Anopheles gambiae* wild-type (G3 insecticide-susceptible strain; MRA-112) were provided by BEI Resources under the CDC-MR4 programme. *Anopheles gambiae* were reared at the University of Florida, Emerging Pathogens Institute (Gainesville, FL, USA). The emerged larvae were fed daily with fish flakes (Tetra, Blacksburg, Virginia, USA) and maintained in incubators at 28 °C, 75% relative humidity, and 12:12 h (L:D) photoperiod. Pupae were collected and placed in paper containers containing water and secured with fine tulle for adult eclosion inside screened cages. Adults were provided continuous access to 10% sucrose solution soaked in cotton balls. Non-blood-fed adult females 3–7 days post-emergence were used in bioassays.

2.3. Vapor phase repellency bioassay

Spatial repellency was evaluated following the method of Jiang et al. (2019), as modified by Yang et al. (2020a). Since *Anopheles* mosquitoes prefer feeding in the evening and during the night (Sinka et al., 2010), testing was done at 17:00–22:00 h. The assay arena consisted of vertical glass tubes that were 12.5 cm long, with 2.5 cm diameter (TriKinetics Inc., Waltham, MA, USA) and contained a filter paper strip (12.5 × 3 cm) inside it for mosquitoes to rest upon. Adult mosquitoes were chilled on ice, 16 females were then transferred manually to the glass tube, and the ends were covered with a plastic mesh. Mosquitoes were allowed 15 min to acclimate before starting the bioassay. Each compound (30 mg) was dissolved in 1 ml of acetone, and circular filter papers were treated with 50 μl of the compound solution. Acetone alone (50 μl) was used as a negative control. After application, the filter papers were allowed 10 min for the acetone to evaporate and were then placed in clear conical polypropylene caps cut from 50 ml plastic centrifuge tubes. These were affixed to each end of the glass tubes, with the acetone control placed on the top end of the glass tube. The filter papers inside the caps were kept 0.5 cm away from the mesh of the glass tube to not allow mosquitoes to make direct contact with the compounds. Control experiments were set up with filter papers (treated with 50 μl acetone) on both sides on the glass tube.

Mosquitoes on the treated and control side were counted separately at 15, 30 and 60 min. A repellency ratio was calculated based on the number of mosquitoes present on the treated side of the midline divided by the total number of mosquitoes ($n = 16$) as described by Jiang et al. (2019). In addition, a post-assay behavioral test (PABT) was performed at the end of 1-h repellency bioassays to identify whether intoxication of mosquitoes prevented them from expressing a repellency response (Yang et al., 2020a). For the PABT, the cap of the control side was removed, and the glass tube was held vertically inside a mosquito cage while the bottom treated cap remained. Once the cap was removed, 30 min were allowed before counting the number of mosquitoes that remained in the tube. A control tube was run in parallel that was only treated with acetone. When the cap of the control tube was removed, all mosquitoes were observed to leave the tube in less than 10 min, showing no signs of intoxication. The repellency proportion was then calculated as: Corrected repellency proportion = (No. of mosquitoes on treated side–No. of mosquitoes failing the PABT)/(16 – No. of mosquitoes failing the PABT). Each assay tube comprised a replicate, and there were at least three and more typically four experimental replicates per treatment.

2.4. Repellency synergism assay

In previous work from our laboratory, TFA demonstrated synergistic repellency against *Ae. aegypti* mosquitoes when mixed with other contact and vapor phase repellents (Yang et al., 2020b). To determine whether synergism was also observed in *Anopheles* mosquitoes, TFA was mixed with the repellent 2-undecanone, because it gave one of the largest synergist ratios (11.6-fold) against *Ae. aegypti* (Yang et al., 2020b). Repellency bioassays were conducted as described above and evaluated the performance of 2-undecanone or TFA alone, and when a concentration range of 2-undecanone was mixed with an inactive amount of TFA (50 μg/cm^2).

2.5. Toxicity bioassay

Compounds were evaluated for their lethal effects on each anopheline species. The bioassays were performed with the same arenas, solvents, and treated filters as those described above, but in horizontal tubes as it gave more consistent mortality. Tubes were maintained at room temperature and knockdown activity at 1 h and 4 h was recorded, as well as mortality at 24 h. Knockdown was defined as an inability of mosquitoes to stand upright or be on their back or side flying along the bottom of glass tubes. Death was defined as the mosquitoes being immobile. In preliminary tests, it was observed that *An. albimanus* and *An. gambiae* had high control mortality after 24 h in control tubes (>20%). However, when a food source (cotton ball soaked in sugar water) was placed in the control tubes, there was no mortality observed after 24 h. Thus, all treated tubes for *An. albimanus* and *An. gambiae* had a sugar water cotton ball placed on the acetone end and the cap used to enclose the tube.

2.6. Electroantennograms

Electroantennogram (EAG) measurements were determined by using methods as described in Yang et al. (2020a, 2020b). Female mosquitoes (5–7 days-old) were immobilized, the antennae were amputated at the base, and the distal tip was removed with a scalpel. The antenna was attached to two metal electrodes with a non-drying electrode gel (Spectra 360, Parker Laboratories, Fairfield, NJ, USA). The electrode pair was connected to a high-impedance DC amplifier, and signals were processed through a 4-channel serial-bus acquisition controller (IDAC4) and analyzed by a Dell microcomputer with EAG Pro software (Syntech, Hilversum, Netherlands).

Filter paper strips (0.8 × 5 cm^2) (Fisher Scientific, Pittsburgh, Pennsylvania, USA) were treated with 10 mg of the test compounds diluted in 10 μl of acetone. Each treated strip was allowed 1 min for the solvent to evaporate, and then placed into a Pasteur glass pipette (Fisher Scientific, Pittsburgh, Pennsylvania, USA). The tip of the pipette was inserted to one end of a “T” type glass connector, which was directed to the antennae. A continuous flow of charcoal filtered, and humidified air was provided at a rate of 0.6 l/min by a stimulus controller (CS-05, Syntech).

Stimuli were delivered in a 1 sec puff, and at least 120 sec intervals were given after each stimulation to allow for antennal recovery. Before recording any compound responses, a blank stimulus (air) response was assessed. A negative acetone control and a DEET positive control were then evaluated to identify that the antennae was responding properly. After an experimental compound was tested, a blank stimulus was tested again to confirm the antenna was still responding properly. The EAG response was measured as peak mV depolarization. Each response was replicated at least seven times for each compound on separate antennal preparations from each anopheline species. Bioassays were also performed later in the day (17:00–22:00 h). All items were handled using gloves to avoid exposure to human odors and to avoid contamination of the tubes or antennae throughout the experiments. The room conditions were 25 ± 1 °C and 75 ± 5% relative humidity.

2.7. Statistical analysis

Half maximal effective concentration (EC_{50}) values and repellency concentration-response curves for each compound were obtained by using a non-linear regression curve fit to a 4-parameter logistic equation with a variable slope, as provided by Prism 7 (GraphPad Prism7 Software, Inc. San Diego, California, USA). EC_{50} values were determined for each compound and compared within a species and each treatment across species, with non-overlap of 95% confidence limits considered to be statistically significant ($P < 0.05$).

EAG responses were calculated as corrected EAG values by taking the response value for a given chemical and subtracting the blank value (air puff without treatment) for each replicate. Comparisons were made between the tested compound and the matched acetone response for each replicate in a paired *t*-test. ANOVA with Tukey’s *post-hoc* test was used to evaluate significant differences among the compounds for each species.

Assessment of knockdown at 1 h and 4 h and mortality at 24 h had control mortality corrected by Abbott’s formula (Abbott, 1925). Concentration-response curves for each compound and the knockdown KC_{50} and lethality LC_{50} values were obtained by using non-linear regression to the 4-parameter logistic equation, as described above. KC_{50} values at 1 and 4 h, as well as the 24 h LC_{50} was determined for each compound in a species and each treatment across species, with non-overlap of 95% confidence limits considered to be statistically significant ($P < 0.05$).

3. Results

3.1. Vapor phase repellency

Vapor-phase repellency data was first analyzed across compounds within a species and then each compound was compared across the three anopheline species. In vapor-phase repellency assays on *An. albimanus*, all the tested compounds demonstrated concentration-dependent spatial repellency. However, complete repellency of *An. albimanus* was not observed for DEET, even at the highest concentration tested of 300 μg/cm^2 (Fig. 2A). The pyridinyl amides had EC_{50} values that were significantly different from DEET and were 3.3-fold (**1**) and 7.2-fold (**2**) more potent, but were not different from each other. Compounds **1** and **2** were most similar in activity to 2-undecanone, with repellency about 4-fold to 5-fold greater than DEET (Table 1). TF was by far the most active by at least 100-fold, which was observed for all the anopheline species (Table 1). For *An. quadrimaculatus*, DEET had a 1-h EC_{50} value of 101 μg/cm^2, while 2-undecanone and compound **2** were 2-fold and 2.7-fold more potent, respectively, whereas compound **1** showed the same repellent potency as DEET (Table 1). TF was again the most potent repellent, but concentrations above 1 μg/cm^2 were not tested as knockdown was observed (Fig. 2). For *An. gambiae*, DEET showed a 1-h EC_{50} value of 98 μg/cm^2, and for comparison, compound **1** and 2-undecanone were about twice as effective as DEET (Table 1). Compound **2** was significantly better at repelling *An. gambiae* mosquitoes, with roughly a 2-fold increase in potency compared to compound **1** and 2-undecanone, and a 4-fold increase in repellent potency compared to DEET (Table 1). In terms of overall compound performance within a species, the rank order of effectiveness was TF >>> Compound **2** ≅ 2-undecanone > Compound **1** > DEET.

When comparing the spatial repellency of each compound across the three *Anopheles* species, DEET was significantly less active (about 2.4-fold) at repelling *An. albimanus* in comparison to *An. quadrimaculatus* and *An. gambiae* (Table 1). Compounds **1** and **2** were significantly less effective at repelling *An. quadrimaculatus* mosquitoes than the other two species (Table 1), whereas 2-undecanone was fairly equipotent against all. TF repellency was significantly different among the three *Anopheles* mosquitoes, as well. The rank order of TF potency was *An. gambiae* > *An. albimanus* **>** *An. quadrimaculatus.* Overall, *An. quadrimaculatus* required 3-fold and 6-fold higher concentrations of transfluthrin than *An. albimanus* and *An. gambiae*, respectively (Table 1).

3.2. Repellency synergism of 2-undecanone with TFA

For 1-h synergism assays, TFA was initially tested alone at 50 and 100 μg/cm^2 on filter papers, and there was a clear trend of concentration-dependent repellency in all three species (Fig. 3A). However, there was not a statistically significant repellent effect of 50 μg/cm^2 TFA among the three species. A small, but statistically significant repellency by TFA exposure was observed at 100 μg/cm^2 compared to the acetone control for *An. gambiae* (ANOVA, $F_{(2, 18)} = 12.53$, $P = 0.0053$), but not for *An. albimanus* or *An. quadrimaculatus*. When an inactive amount of TFA (50 μg/cm^2) was mixed with 2-undecanone, the concentration-response curves for 2-undecanone were shifted to lower concentrations, and statistically significant synergistic effects were observed (Fig. 3B–D). The 1-h EC_{50} values of 2-undecanone on *An. albimanus* and *An. gambiae* were reduced about 6-fold by TFA (Table 1). In *An. quadrimaculatus*, there was a large change in response slope and synergism was also observed, with the 1-h EC_{50} value of 2-undecanone decreased by a factor of four (Table 1).

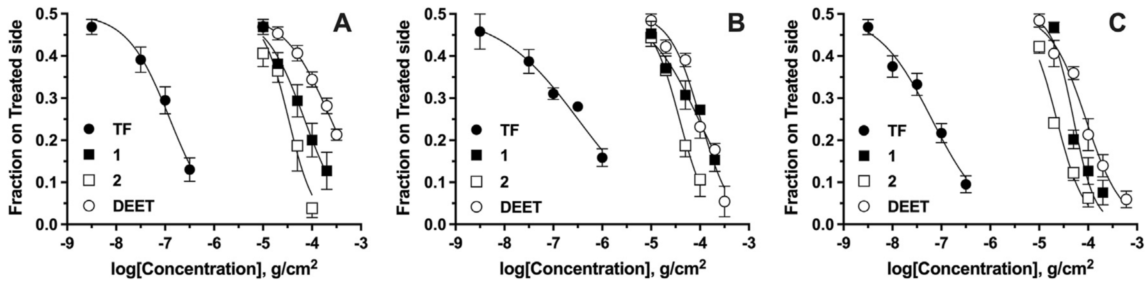

Fig. 2. Concentration-response curves for 1 h vapor repellency of transfluthrin (TF), experimental compounds **1** and **2**, and DEET against *An. albimanus* (**A**), *An. quadrimaculatus* (**B**) and *An. gambiae* (**C**) in vertical tubes. Symbols are means ± SEM. 2-Undecanone was omitted from the plots for clarity and its repellency alone is plotted in Fig. 3.

Table 1
Repellency of compounds against adult anopheline females

Compound	1-h EC_{50} repellency values, μg/cm^2 (95% CI)		
	An. albimanus	*An. quadrimaculatus*	*An. gambiae*
DEET	237 (200–293)^{aA} Slope = −0.9; R^2 = 0.90	101 (84–121)^{aB} Slope = −1.4; R^2 = 0.91	98 (77–125)^{aB} Slope = −1.1; R^2 = 0.90
2-UND	50 (38–67)^{bcA} Slope = −0.9; R^2 = 0.83	50 (45–55)^{bA} Slope = −2.4; R^2 = 0.97	43 (36–51)^{bA} Slope = −1.9; R^2 = 0.87
2-UND + TFA	*7.6 (5.7–10.5)^{dA}; SR = 6.6 Slope = −0.9; R^2 = 0.76	*13 (9–31)^{cB}; SR = 3.8 Slope = −0.8; R^2 = 0.80	*6.6 (4.6–9.0)^{cC}; SR = 6.5 Slope = −0.9; R^2 = 0.75
1	70 (52–95)^{bAB} Slope = −1.1; R^2 = 0.80	93 (69–132)^{aA} Slope = −0.9; R^2 = 0.78	50 (41–62)^{bB} Slope = −2.0; R^2 = 0.91
2	33 (25–44)^{cAB} Slope = −1.7; R^2 = 0.81	38 (31–46)^{bA} Slope = −1.5; R^2 = 0.90	23 (19–27)^{dB} Slope = −1.6; R^2 = 0.93
TF	0.12 (0.09–0.17)^{dA} Slope = −1.0; R^2 = 0.86	0.38 (0.22–0.74)^{dB} Slope = −0.5; R^2 = 0.83	0.06 (0.05–0.08)^{eC} Slope = −0.8; R^2 = 0.90

Notes: Lower case and capitalized superscript letters indicate significant difference ($P < 0.05$) among compounds for each species (column) and across the different mosquito species for each compound (row), respectively (ANOVA with Tukey's mean separation test, $P < 0.05$). EC_{50} values for the different compounds not labeled by the same lowercase superscript letter (within a column, across compounds within a species) or uppercase superscript letter (within a row, across the three species for each compound) are significantly different ($P < 0.05$). For 2-undecanone (2-UND) and 2-UD + TFA, asterisks indicate a significant difference in the EC_{50} values (two-tailed *t*-test, $P < 0.05$). Synergist ratio (SR) = EC_{50} 2-UD/(EC_{50} 2-UD + TFA).

3.3. Electroantennograms

Assuming that repellency occurred *via* antennal perception, electroantennograms (EAG) were recorded for five compounds in an applied odorant format and compared within each species (Fig. 4). For all the anophelines, every compound gave a statistically significant EAG response compared to matched acetone controls. Further, the EAG responses to DEET, TFA, and TF were not different from each other and experimental compound **2** was always the most active. For *An. albimanus*, the experimental compounds **1** and **2** had significantly greater EAG responses than DEET and the other compounds, all of which did not differ from each other (Fig. 4A). For *An. quadrimaculatus*, the rank order of effectiveness of the three most active compounds was **2** > **1** = 2-undecanone, which were all more active than DEET, TFA and TF. These three compounds responded equivalently, but TF was less active than compound **1** but equal in activity to 2-undecanone (Fig. 4B). For *An. gambiae*, the experimental compound **2** had the largest EAG responses, and it was significantly greater than all the other treatments (Fig. 4C). The rest of the compounds showed no statistically significant differences among them.

3.4. Knockdown effect at 1 and 4 hours

Although the pyridinyl amides were envisioned as spatial repellents, it was appropriate to also screen them for knockdown and lethal effects. The experimental amides and DEET showed little knockdown (< 50% at 100 μg/cm^2) in this exposure paradigm, with one exception. Compound **2** had a 4-h KC_{50} of 48 (39–59) μg/cm^2 (slope = 1.8, R^2 = 0.89) on *An. albimanus*. For 2-undecanone, concentration-response curves for knockdown at 1 h and 4 h, and mortality at 24 h showed nearly identical curves at all time-points and species (Supplementary Fig. S1). Overall, the 1-h and 4-h KC_{50} values ranged from 67 μg/cm^2 to 93 μg/cm^2 across the anophelines, displaying a difference of only about 39%, although the values for *An. quadrimaculatus* were significantly higher than the other two species at both time-points (Table 2). In contrast, concentration-response curves for TF knockdown at 1 h and 4 h with transfluthrin differed significantly and are shown in Fig. 5 and Table 2. KC_{50} values after 4 h of TF exposure were significantly less than at 1 h across all species, typically by about 2-fold to 3.5-fold. Moreover, the KC_{50} values for the anophelines were significantly different from each other at both time-points, except for the 4-h KC_{50} of *An. quadrimaculatus* and *An. gambiae* (Table 2). Finally, as expected, TF was a much more active knockdown agent than 2-undecanone.

3.5. Lethality at 24 hours

Vapor-phase mortality in 24 h exposures was also assessed for these repellents. Inspection of Fig. 6 shows differing response potencies and slopes for compounds **1**, **2**, DEET and 2-undecanone, with **2** generally the most potent and **1** having the lowest slope value (Table 3). For *An. albimanus*, both experimental compounds were not significantly different from DEET, but were more active than 2-undecanone (Table 3). For *An. quadrimaculatus*, the toxicity of experimental compound **1** and 2-undecanone were comparable to DEET, whereas the experimental compound **2** (LC_{50} = 13 μg/cm^2) had about a 6-fold increase in efficacy (Table 3). For *An. gambiae*, both experimental compounds and 2-undecanone were significantly different from DEET (1.5-fold to 3.4-fold). In all cases, TF was by far the most toxic compound, and its potency varied significantly across the three species, from 2-fold to 4-fold (Table 3).

4. Discussion

4.1. Vapor repellency

The novel pyridinyl amide **1** was about as active as 2-undecanone and demonstrated significantly higher spatial repellency against *An. albimanus* and *An. gambiae* when compared to DEET. Compound **2** exhibited significantly greater efficacy as a spatial repellent in all *Anopheles* mosquitoes, showing a 3-fold to 7-fold increase in repellency compared to the DEET standard. Perhaps compound **2** has higher volatility or more

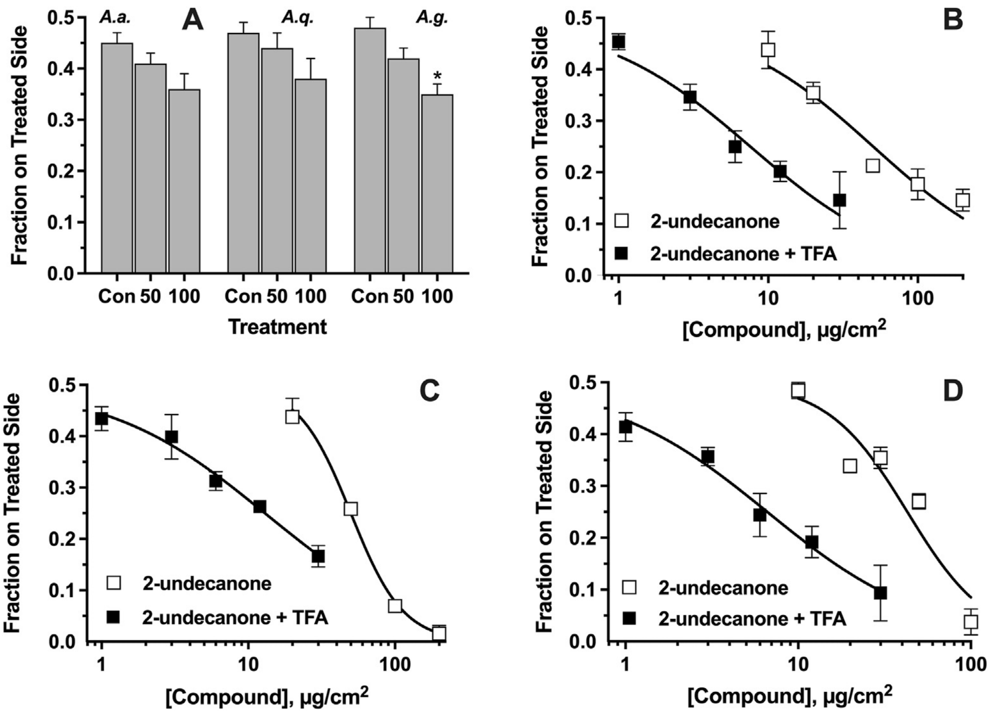

Fig. 3. Vapor phase repellency (1 h) of TFA alone (**A**) and with increasing concentrations of 2-undecanone against *An. albimanus* (**B**), *An. quadrimaculatus* (**C**) and *An. gambiae* (**D**) in vertical tubes. Bars (**A**) or symbols (**B**–**D**) are means ± SEM. In (**A**) Con = acetone controls and 50 and 100 are concentrations of 1*R*-*trans*-permethrinic acid (TFA) in μg/cm^2; asterisk indicates repellency significantly different from control. Concentrations of 2-undecanone > 30 μg/cm^2 caused knockdown. *Abbreviations*: A.a., *Anopheles albimanus*; A.g., *Anopheles gambiae*; A.q., *Anopheles quadrimaculatus*.

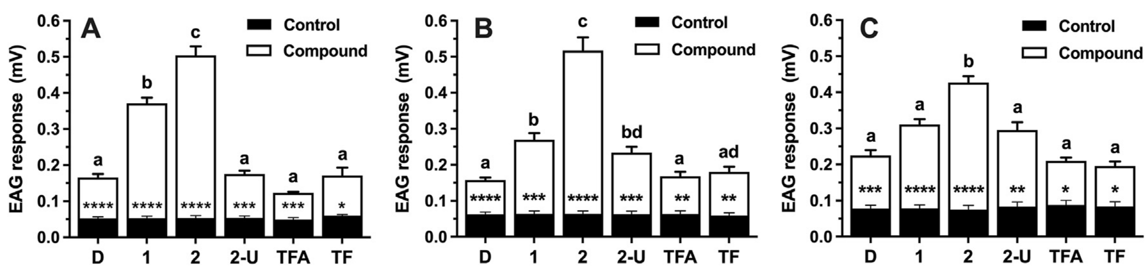

Fig. 4. Electroantennogram responses to 10 μl of acetone controls (black bars), DEET (D), experimental compounds **1** and **2**, 2-undecanone (2-U), 1*R*-*trans*-permethrinic acid (TFA), and transfluthrin (TF) against *An. albimanus* (**A**), *An. quadrimaculatus* (**B**) and *An. gambiae* (**C**). Stacked bars are means ± SEM. Asterisks indicate statistical significance of a compound's EAG response at 10 mg in comparison to matched acetone controls in a paired *t*-test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001). Letters indicate statistical comparisons across compounds by ANOVA with Tukey's *post-hoc* test, where bars not labeled by the same letter are significantly different (*P* < 0.05).

Table 2
Knockdown of anopheline adult females, with KC_{50} values (μg/cm^2) calculated after 1 and 4 h of exposure. The 95% confidence interval (95% CI) along with response slope and the coefficient of determination (R^2) are given

	An. albimanus		*An. quadrimaculatus*		*An. gambiae*	
	1-h KC_{50}	4-h KC_{50}	1-h KC_{50}	4-h KC_{50}	1-h KC_{50}	4-h KC_{50}
2-UND	69^{a}	67^{A}	93^{b}	91^{B}	76^{a}	75^{A}
95% CI	65–73	62–73	83–105	85–96	71–81	70–80
Slope	6.7	6.1	3.0	4.0	5.2	5.6
R^2	0.96	0.94	0.96	0.99	0.96	0.96
TF	0.13^{a}	0.06^{A*}	1.30^{b}	0.50^{B*}	2.00^{c}	0.57^{B*}
95% CI	0.1–0.2	0.05–0.07	1.2–1.5	0.4–0.6	1.7–2.4	0.4–0.7
Slope	1.4	1.2	3.4	1.6	1.3	2.2
R^2	0.96	0.96	0.93	0.96	0.95	0.94

Notes: EC_{50} values at 1 h for the different species not labeled by the same lowercase superscript letter (within a row) or at 4 h by uppercase superscript letter (also within a row) are significantly different. For TF, 4-h KC_{50} values marked with an asterisk were different from 1-h values, as judged by non-overlap of the 95% confidence intervals.

potent or efficacious activity on the odorant receptor complex due to its additional difluoro-methylene group. Interestingly, there was variability in response to each compound tested between the mosquito species, with a significant 2-fold decrease in spatial repellency of DEET against *An. albimanus* in comparison to *An. gambiae* and *An. quadrimaculatus*. Similarly, Robert et al. (1991) also reported a 2-fold decrease in repellency of DEET observed in *An. albimanus*, when compared to *An. gambiae* in contact repellency bioassays using rabbits. In addition, the three species of *Anopheles* had significantly different spatial repellency response to transfluthrin. *Anopheles quadrimaculatus* showed the lowest sensitivity to TF when compared to the other species. Thus, different mosquito species respond differently to repellents, as other studies have also reported (Rutledge et al., 1978; Robert et al., 1991; Coleman et al., 1993). These results demonstrate the importance of testing a wide range of mosquito species to observe the full range of a repellent's activity.

Although new chemistry is the preferred approach for developing new repellents, an alternative is to seek possible synergists for currently available compounds. In our laboratory, synergistic effects were observed in *Ae. aegypti* when the pyrethroid derived acid, TFA, was coapplied with a variety of other repellents (Yang et al., 2020b). Further,

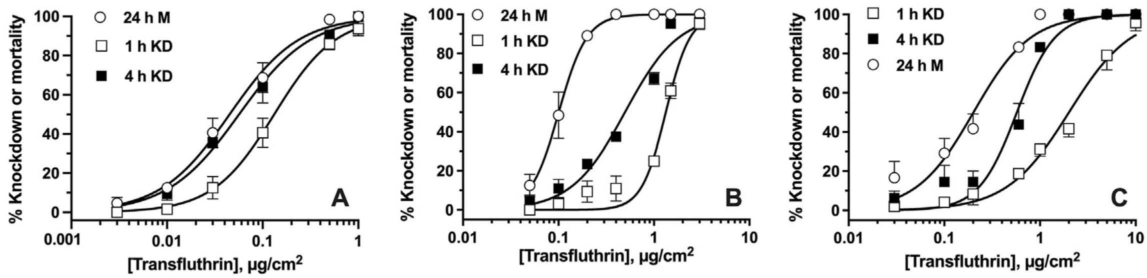

Fig. 5. Vapor phase toxicity concentration-response curves for transfluthrin against *An. albimanus* (**A**), *An. quadrimaculatus* (**B**) and *An. gambiae* (**C**) in horizontal tube. Symbols are means ± SEM. *Abbreviations*: M, mortality; KD, knockdown.

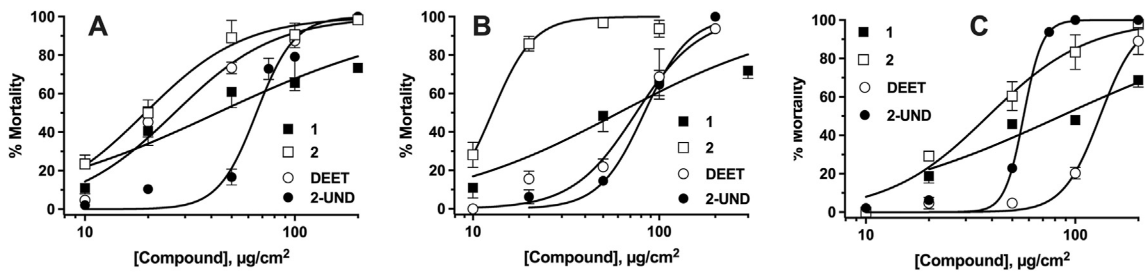

Fig. 6. Vapor phase toxicity concentration-response curves for experimental compounds **1** and **2**, DEET, and 2-undecanone (2-UND) against *An. albimanus* (**A**), *An. quadrimaculatus* (**B**) and *An. gambiae* (**C**) in horizontal tubes. Symbols are means of ± SEM.

Table 3
Lethality of 2-undecanone (2-UND), experimental compounds **1** and **2**, transfluthrin (TF), and DEET against *An. albimanus*, *An. quadrimaculatus* and *An. gambiae*. LC_{50} values (μg/cm^2) after 24 h exposure are given, with 95% confidence interval (CI) in parentheses

Compound	24-h LC_{50} values, μg/cm^2 (95% CI)		
	An. albimanus	*An. quadrimaculatus*	*An. gambiae*
DEET	27 (22–32)^{aA} Slope = 1.8; R^2 = 0.92	75 (61–91)^{aB} Slope = 2.5; R^2 = 0.87	131 (119–145)^{aC} Slope = 4.9; R^2 = 0.95
2-UND	66 (60–72)^{bA} Slope = 4.8; R^2 = 0.93	83 (74–93)^{aA} Slope = 3.6; R^2 = 0.97	57 (54–59)^{bB} Slope = 9.7; R^2 = 0.99
1	43 (31–60)^{aA} Slope = 0.87; R^2 = 0.76	61 (39–94)^{aB} Slope = 0.9; R^2 = 0.76	85 (67–111)^{cB} Slope = 0.86; R^2 = 0.88
2	19 (16–24)^{aA} Slope = 1.9; R^2 = 0.87	13 (11–14)^{bB} Slope = 3.9; R^2 = 0.92	38 (31–47)^{dC} Slope = 1.8; R^2 = 0.94
TF	0.05 (0.04–0.06)^{cA} Slope = 1.2; R^2 = 0.95	0.1 (0.09–0.11)^{cB} Slope = 3.0; R^2 = 0.93	0.2 (0.16–.25)^{eC} Slope = 1.5; R^2 = 0.93

Notes: EC_{50} values within a column not labeled by the same lowercase superscript letter or uppercase superscript letter within a row are significantly different ($P < 0.05$). The slope of the line and the coefficient of determination (R^2) for the curve fit are also shown.

it was demonstrated that TFA has two modes of action: it assists the volatilization of compounds and also enhances responses on the antennae. In the present study, we find that TFA can also synergize the action of 2-undecanone on anophelines, and likely other repellents, as well. Because pyrethroid acids have no ability to increase or block nerve firing when applied to the *Drosophila melanogaster* larval central nervous system (Yang et al., 2020b), its synergistic effects on 2-undecanone at the antennal level is unlikely to result from an agonist action on the voltage-sensitive sodium channel. The identification of the site(s) and mode(s) of action of pyrethroid acids on the insect olfactory system awaits further investigation.

4.2. Electroantennograms

In the present study, the antennal olfactory response of three anophelines to the experimental compounds and standard repellents were assessed. The results showed that all the compounds tested exhibited a significantly greater response than the acetone control in the three species of *Anopheles*. Although EAG responses do not indicate whether a compound will be an effective repellent, some studies have observed a correlation between electrophysiological and behavioral response of repellents (Stanczyk et al., 2010; Deletre et al., 2015; Yang et al., 2020a). Deletre et al. (2015) observed a correlation for essential oils against *An. gambiae*, where compounds such as cinnamaldehyde and citronellal both exhibited strong EAG response and were active repellents and irritants. In our study, experimental compound **2** induced the largest EAG response of all the compounds in the three *Anopheles* spp. and in behavioral bioassays also demonstrated promising repellent activity. In contrast, only a small EAG response was observed for TF, and it was by far the most active repellent and conversely, TFA gave an EAG response in *Ae. aegypti* antennae, but did not show statistically significant repellency (Yang et al., 2020b). Similarly, strong repellency and irritancy were also observed in *An. gambiae* for carvacrol, yet there was little EAG response to this compound (Deletre et al., 2015). The weak olfactory response of *Anopheles* species to TF, especially in *An. gambiae*, is similar to results of other studies. Liu et al. (2013) have reported weak olfactory responses of *Culex quinquefasciatus* to permethrin and allethrin in single sensillum recordings, and a recent study with transfluthrin found no significant EAG response to this compound, with previous positive results possibility related to impurities (Andreazza et al., 2021). Yang et al. (2021) screened antennae, mouthparts, and wings, as well as tarsi of the pro-, meso-, and metathoracic legs of *Ae. aegypti* mosquitoes and found that all but the hindleg tarsi were sensitive to TF, but not exceptionally so. A small number of, but behaviorally important receptors, may contribute to pyrethroid-induced spatial repellency, in addition to their action on sodium channels.

4.3. Knockdown and toxicity

The experimental compounds showed little knockdown activity, similar to DEET, with the exception of experimental compound **2** against

An. albimanus in 4-h exposures. In 24-h toxicity bioassays, compound **2** demonstrated insecticidal activity greater than DEET, and against *An. quadrimaculatus* had 6-fold greater efficacy. Interestingly, compound **1** was not as effective as a toxicant when compared to compound **2**, similar to its reduced effectiveness in the repellency and EAG bioassays. This broad correlation suggests that antennal effects, repellency, and lethality are mediated by the same, or closely related target site(s) for the amides.

Consistent with our findings of low DEET knockdown and toxicity, Deletre et al. (2013) used a high-throughput screening system (HITSS) and reported that DEET (55 nmol/cm^2) did not cause a knockdown response in *An. gambiae* and did not show a toxic effect. In contrast, Grieco et al. (2005) used the HITSS and showed that 25 nmol/cm^2 exposures to *Ae. aegypti* had 52% knockdown in 1 h and 48% mortality at 24 h. This difference may reflect a true species-dependent sensitivity of the mosquitoes being tested (*Aedes vs Anopheles*). However, Pridgeon et al. (2009) showed that in topical bioassays, DEET was toxic and had higher insecticidal activities than other repellents such as IR3535 and picaridin against *An. albimanus*, *An. quadrimaculatus*, *Ae. aegypti*, and *C. quinquefasciatus*.

Little is known about the toxic effects of 2-undecanone on mosquitoes, whereas mortality of 2-undecanone is commonly reported in herbivorous insect species (Farrar & Kennedy, 1987; Lin et al., 1987). It was previously shown that 2-undecanone had larvicidal activity against fourth-instar larvae of *Aedes albopictus* with a 24-h $LC_{50} = 10$ μg/ml (Liu et al., 2015), and to first-instar larvae of *Ae. aegypti* and *An. quadrimaculatus*, with a 24-h LC_{50} value of 14 μg/ml in both species (Ali et al., 2013). In the present study 2-undecanone was about as toxic as DEET against adult *Anopheles* mosquitoes.

TF showed active knockdown and toxicity, and there was some variability in response to transfluthrin between species, with *An. albimanus* exhibiting the highest sensitivity. Potent knockdown and mortality have been reported previously for TF in mosquitoes, and in *An. quadrimaculatus*, TF was one of the most active pyrethroids, exhibiting high vapor toxicity (Bibbs et al., 2018). However, resistance to TF has also been reported by Wagman et al. (2015) in vapor exposure repellency assays of wild caught *Ae. aegypti* from Belize. Agramonte et al. (2017) documented a 29-fold resistance ratio to TF in a permethrin-resistant Puerto Rico (PR) strain of *Ae. aegypti*, based on topical bioassays. In addition, Yang et al. (2020a) reported 48-fold resistance to transfluthrin using vapor phase toxicity bioassays in PR mosquitoes, which are known to carry both kdr and metabolic mechanisms (Estep et al., 2017). Other studies have shown that TF showed little cross resistance in a metabolically resistant (FUMOZ-R) strain of *An. funestus*, and the P450 inhibiting synergist piperonyl butoxide had little toxicity enhancing effect (Horstmann & Sonneck, 2016). Thus, although transfluthrin may be an effective toxicant in laboratory-reared mosquitoes, its effectiveness is probably reduced in at least some wild mosquito populations. Further investigation is warranted to compare the performance of these repellents against *Anopheles* mosquitoes found in the field.

5. Conclusions

The spatial repellent activity and toxicity of two novel pyridinyl amides (**1** and **2**) were evaluated against *Anopheles albimanus*, *Anopheles quadrimaculatus*, and *Anopheles gambiae* and compared to the activity of three commercial standards. As expected, transfluthrin was overall the most active compound for inducing repellency, knockdown, and lethality. Compound **2** was generally a more effective repellent than DEET and 2-undecanone, while compound **1** was about as active as these compounds. The two experimental amides produced the largest electroantennographic responses in female antennae, despite the fact that they were not the most active repellents. The amides displayed modest toxicity to anopheline mosquitoes. Significant synergism of repellency was observed for the mixture of a pyrethroid-derived acid and the repellent 2-undecanone against anopheline mosquitoes, similar to the synergism observed in *Aedes aegypti* (Yang et al., 2020b). The findings of this study suggest that amide compounds are useful lead molecules for the continued development of spatial treatments.

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Ethical approval

Not applicable.

CRediT author statement

Ingeborg Cuba: conceptualization, investigation, formal analysis, writing - original draft, editing. Gary Richoux: investigation, writing - review and editing. Edmund Norris: investigation, formal analysis, writing - review and editing. Ulrich Bernier: funding acquisition, writing - review and editing. Kenneth Linthicum: resources, writing - review and editing. Jeffrey Bloomquist: conceptualization, funding acquisition, project administration, supervision, writing - review and editing. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://doi.org/10.1016/j.crpvbd.2021.100062.

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