Behavioural responses of *Phlebotomus duboscqi* to plant-derived volatile organic compounds

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Highlights

- Laboratory behavioural responses of *Phlebotomus duboscqi* evaluated to the volatile organic compounds linalool oxide, ocimene, *p*-cymene, *p*-cresol, and *m*-cresol, isolated from sand fly preferred host plants from the Fabaceae family
- In dose-response assays, the compounds singly and in blends increased the attractive responses of males and females over controls.
- Plant-derived attractants can potentially be exploited as lures to improve Afrotropical sand fly surveillance and control

Abstract

Phlebotomine sand flies are vectors of *Leishmania* parasites that cause leishmaniases. Both sexes of sand flies feed on plants primarily for sugars, although the chemical cues that mediate attraction to host plants remain largely unknown. Previously, using coupled gas chromatography/mass spectrometry (GC-MS), we identified several volatile organic compounds (VOCs) common to preferred host plants for selected Afrotropical sand flies from the Fabaceae family. Of the identified volatiles, the significance of the monoterpenes linalool oxide (LO), ocimene and *p*-cymene and the benzenoid *m*-cresol, *p*-cresol in sand fly behaviour is unknown. In olfactometer assays, we tested these compounds singly and in blends for their attractiveness to *Phlebotomus duboscqi,* cutaneous leishmaniasis vector in Kenya. In dose-response assays, single compounds increased the responses of males and females over controls, but their optimum attractive doses varied between the sexes. Two five-component blends, referred to as Blend-f and Blend-m for females and males respectively, were formulated and tested in dose-response assays against 1-octen-3-ol (positive control). Our results showed that males and females were significantly attracted to varying levels of the two blends. In pairwise assays, we evaluated the most attractive of these blends to each sex (i.e., Blend Am for male against Blend Bf for female), revealing that males were attracted to both blends at varying levels, while females were indifferent. Our results demonstrate that plant-derived VOCs can be exploited for sand fly management.

Keywords. Fabaceae, Kairomones, *Leishmania*, *Phlebotomus duboscqi,* Sand fly

Introduction

Phlebotomine sand flies are vectors of *Leishmania* parasites that cause leishmaniasis, a disease second only to malaria as a major vector-borne parasitic disease of global public health concern (WHO, 2017). Two forms of this neglected tropical disease occur in eastern Africa including Kenya –visceral (VL) and cutaneous (CL) leishmaniasis (WHO, 2020). Controlling the disease relies on prompt diagnosis and chemotherapy (Singh *et al*., 2016), although deeper understanding of sand fly behaviours can lead to new ways to limit parasite development and transmission in the vector.

Plant feeding is a common biological trait of both sexes of phlebotomine sand flies in. Plant meals primarily serve as sources of sugars for survival, and flight capacity to seek mates or hosts for a blood meal (Lima *et al*., 2016; Hassaballa *et al*., 2021a). In blood-feeding arthropods like mosquitoes, plant seeking is mediated by olfactory cues emitted by specific plant species (Nyasembe *et al*., 2012, 2018; Lahondère *et al*., 2020). Similar studies on chemical cues of plant origin that mediate sand fly attraction remain poorly elucidated although the attraction of *Phlebotomus papatasi* to different flowering plants has been demonstrated (Schlein & Yuval, 1987; Müller *et al*., 2011). In these studies, the volatile organic compounds (VOCs) eliciting attraction were not identified. However, other studies have shown that the plant volatiles 1-octen-3-ol, 1-nonanol and 1-heptanol are attractive to female of *Lutzomyia longipalpis* (Magalhães-Junior *et al*., 2014). Additionally, hexanol and octanol have been found to elicit attraction in *Nyssomyia neivai* (Machado *et al*., 2015). Notably, 1-octen-3-ol has been extensively studied in the field and demonstrated to show varied attraction for various sand fly species (Andrade *et al*., 2008; Pinto *et al*., 2011). These examples highlight the potential for attractants of plant origin for use in management and control of sand fly populations.

By examining plant feeding in wild-caught *Phlebotomus duboscqi* and employing molecular approaches, Hassaballa *et al.* (2021a) demonstrated selective feeding on several *Acacia* plant species (*Vachellia tortilis*, *Senegalia laeta* and *Vachellia nilotica*). Chemical analysis of the headspace volatile profiles of the plants identified the compounds (*Z*)-linalool oxide, (*E*)-β-ocimene, *p*-cymene, *p*-cresol, and *m*-cresol, as VOCs common to these plants. In this study, we tested the responses of *P. duboscqi* to these compounds in laboratory assays and identified candidate kairomones that could be used in the management of this CL vector.

Materials and Methods

Sand fly colony

Both male and female *P. duboscqi* were obtained from a colony maintained in the laboratory at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus in Nairobi (1°13′12″S, 36°52′48″E; 1,600 m above sea level). The sand flies were maintained in cages covered with nets at a mean temperature of 25 ± 5 °C and 65-85% relative humidity, with a 12 h light: 12 h dark photocycle regime. Adult sand flies (2–3 day old) were used for behavioural assays and maintained on 5% glucose solution (*ad libitum*) (Sigma-Aldrich, St. Louis, Missouri, United States) and females were blood fed on anaesthetised mice for oviposition. They were kept separately in different cages in the laboratory 2-3 days after emergence.

Olfactometer assays

Choice assays were conducted to investigate the responses of male and female *P. duboscqi* to synthetic standards of VOCs identified as common to preferred host plants (Hassaballa *et al*., 2021a). These VOCs were linalool oxide (LO), ocimene, *m*-cresol, *p*-cresol and *p*-cymene. A Y-tube olfactometer (internal diameter 0.5 cm, stem length 6 cm, arm length 8.5 cm and a 60°

angle at the junction) was used and assays were conducted in a laboratory maintained at a temperature of 24 ± 1 °C; and 72% relative humidity. Charcoal-filtered clean air was passed through each arm of the olfactometer at a flow rate of 200 ml/min and pulled out of the stem of the Y- tube by a vacuum system at 300 ml/min using a battery-powered air entrainment kit (B. J. Pye, Kings Walden, UK). Lighting was provided in the observation Y-tube using a 220–240 V cool white fluorescent light. To allow the sand flies to adapt to the test environment, they were kept in the Y-tube room and starved for about 6 h before starting the experiment as described below.

Bioassay with chemicals

Evaluation of each of the compounds for attractiveness to sand flies was assessed in dose-response assays to establish the optimal attractive dose for each sex. A stock solution of each compound (1µg/ml) was prepared in dichloromethane (DCM, 99% Analytical grade: Sigma Aldrich, St. Louis, MO, USA). Based on the natural release rate (ng/plant/h; 0.8 – 1 ng/h) of the compounds to mimic odours released from the preferred host plant (*Vachellia tortilis*), five doses of each of these compounds were prepared at a concentration of 0.1, 0.2, 0.4, 0.8 and 1.6 ng/µl in DCM and each tested against a solvent (DCM) control consistent with those of a previous findings (Nyasembe et al., 2012).

Each treatment was dispensed by applying 20 µl of solution on a 1 × 1 cm strip of filter paper (Whatman No. 1). After evaporation of the solvent at room temperature (25 ^{O}C) for 30 s, the filter paper was placed inside one arm of the Y-tube. Into the other arm was an air-dried filter paper treated with 20 µl DCM only which served as control. For each test, a single sand fly was introduced into the Y-tube via the entrance stem and the choice made between the treatment and control (DCM), recorded within 5 min. When the insect had moved halfway through the Y-tube arm towards any of the odour sources within 5 min and spent at least 30 s in the chosen arm, it was recorded as a choice. The experiment using either sexes was replicated 20 times for each treatment dose and control. Before experiments with the compounds, sand fly response (both sexes) to the control solvent (DCM) vs control solvent (DCM) was tested to check if the olfactometer system was working without any bias using the same procedure as described above.

The filter papers (treatment and control) were replaced with fresh ones after five individual males and females had been tested. To avoid any bias, the position of the arms Y-tube containing the treatment and control were connected to the opposite arms after every five tested individuals. After every 10 replicates, a clean glass Y-tube and new test odour was used. Y-tubes were cleaned with Teepol odorless detergent and hot water, rinsed with distilled water and acetone to remove any contaminants and heated in an oven at 150 °C before use. All experiments were performed during the photophase 18:00 to 24:00 h of the night period when sand flies were active (Lane, 1993).

Based on the results obtained from the single compounds, a five-component blend (one for each sex) in the ratio (1:1:1:1:1) comprising optimal attractive dose of each compound was constituted and tested. Each blend (one for male, Blend-m and another for female, Blend-f) was evaluated against the known sand fly attractant 1-octen-3-ol (positive control) (Pinto *et al*., 2011; Tavares *et al*., 2018). The optimal attractive dose of 1-octen-3-ol for each sex was first determined and then used in subsequent assays against control (DCM). Additionally, to assess a dose-response effect, two blends prepared by doubling and halving the amounts of the individual compounds in BlendAm, the optimum dose for male and Blend-Af, the optimum dose for female, were tested (Table 1). Finally, the most attractive of these blends to each sex, were evaluated against each other in pairwise assays.

Table 1. Summary of blends used in Y-tube olfactometer assays

Sex	Blend type	Blend composition
Female	Blend-Af	LO (0.4) + ocimene (1.6) + *m*-cresol (0.8) + *p*-cresol (0.2) + *p*-cymene (0.8)
	Blend-Bf	LO (0.8) + ocimene (3.2) + *m*-cresol (1.6) + *p*-cresol (0.4) + *p*-cymene (1.6)
	Blend-Cf	LO (0.2) + ocimene (0.8) + *m*-cresol (0.4) + *p*-cresol (0.1) + *p*-cymene (0.4)
Male	Blend-Am	LO (0.4) + ocimene (0.2) + *m*-cresol (0.2) + *p*-cresol (0.8) + *p*-cymene (0.8)
	Blend-Bm	LO (0.8) + ocimene (0.4) + *m*-cresol (0.4) + *p*-cresol (1.6) + *p*-cymene (1.6)
	Blend-Cm	LO (0.2) + ocimene (0.1) + *m*-cresol (0.1) + *p*-cresol (0.4) + *p*-cymene (0.4)

Numbers in parenthesis indicate dose of each compound in ng/μl

Chemicals

The synthetic standards of linalool oxide (furanoid), ocimene mixture comprising (*E*)-β-ocimene, (*Z*)-β-ocimene, and allo-ocimene in a ratio of 22:2:1), *m*-cresol, *p*-cresol, *p*-cymene and 1-octen-3-ol used in the bioassays were all purchased from Sigma Aldrich. All the chemicals were at >97% purity.

Statistical analysis

Frequencies of count data from the Y-tube olfactometer assays were analysed using Chi squared ($\chi 2$) test to determine the significant difference of *P. duboscqi* female and male responses amongst different synthetic standards, singly and blends, against the controls. The statistical analyses were considered significant at $P < 0.05$ using R software v. 3.6.3 (R Core Team, 2020). There were no non-responders possibly attributed to the small size of the Y-tube used in this study.

Results

Both male and female *P. duboscqi* did not show a significant preference for the solvent controls used in pairwise comparisons ($\chi^2 = 0.2$, $df = 1$, $P = 0.65$) (Fig. 1). In dose-response assays, both sexes were attracted to the individual compounds at varying levels over controls comprising dichloromethane (DCM).

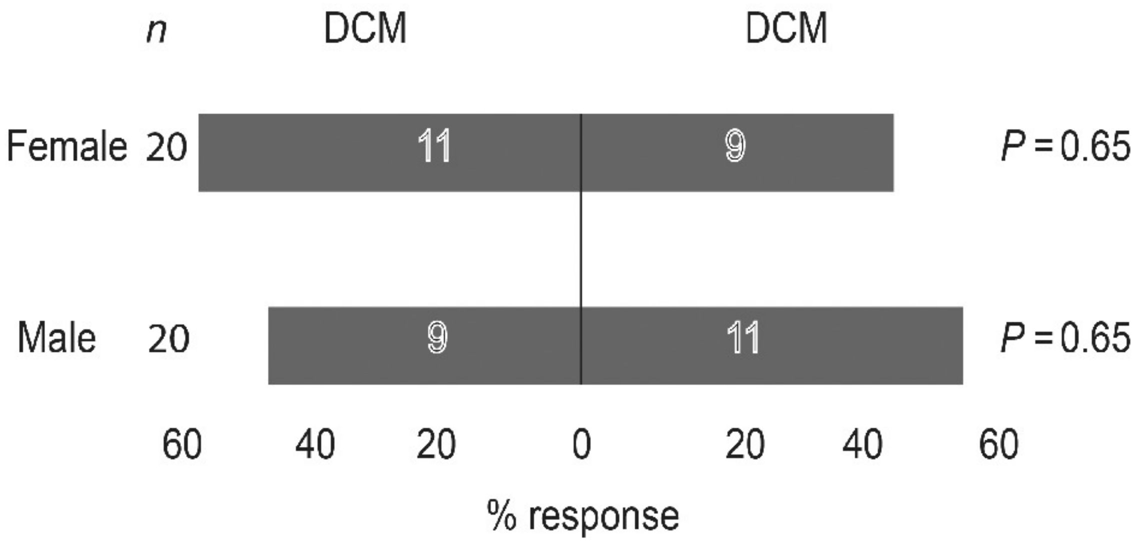

Fig. 1. Percentage (%) responses of *P. duboscqi* females and males to control solvent dichloromethane (DCM) vs. control solvent (DCM) in a Y-tube olfactometer. P-values indicate levels of significance between the treatments for each sex. Each pair of evaluation used a total of n=20 sand flies released singly per choice. Numbers in each bar represent those responding to either controls.

Of the five compounds, LO was optimally attractive to females and males at 0.4 ng/µl ($\chi^2 = 7.2$, $df = 1$, $P < 0.007$) and 0.4 ng/µl ($\chi^2 = 12.8$, $df = 1$, $P < 0.0003$) respectively (Fig. 2; Fig. 3) (Table 2). Ocimene was optimally attractive to females at 1.6 ng/µl ($\chi^2 = 12.8$, $df = 1$, $P < 0.0003$), and males at 0.2 ng/µl ($\chi^2 = 7.2$, $df = 1$, $P < 0.007$) (Fig. 2; Fig. 3) (Table 2). *m*-Cresol was optimally attractive to females at 0.8 ng/µl ($\chi^2 = 5$, $df = 1$, $P < 0.025$), and to males at 0.2 ng/µl ($\chi^2 = 9.8$, $df = 1$, $P < 0.002$) (Fig. 2; Fig. 3) (Table 2). *p*-Cresol was optimally attractive to females at 0.2 ng/µl ($\chi^2 = 5$, $df = 1$, $P < 0.025$), and to males at 0.8 ng/µl ($\chi^2 = 7.2$, $df = 1$, P

< 0.007) (Fig. 2; Fig. 3) (Table 2). *p*-Cymene was optimally attractive to females at 0.8 ng/µl (χ^2= 7.2, *df* = 1, $P < 0.007$), and to males at 0.8 ng/µl ($\chi^2 = 5$, *df* = 1, $P < 0.025$) (Fig. 2; Fig. 3) (Table 2). Likewise, 1-octen-3-ol was optimally attractive to females at 0.4 ng/µl (χ^2= 7.2, *df* = 1, $P < 0.007$), and to males 0.4 ng/µl ($\chi^2 = 5$, *df* = 1, $P < 0.025$) (Fig. 2; Fig. 3) (Table 2).

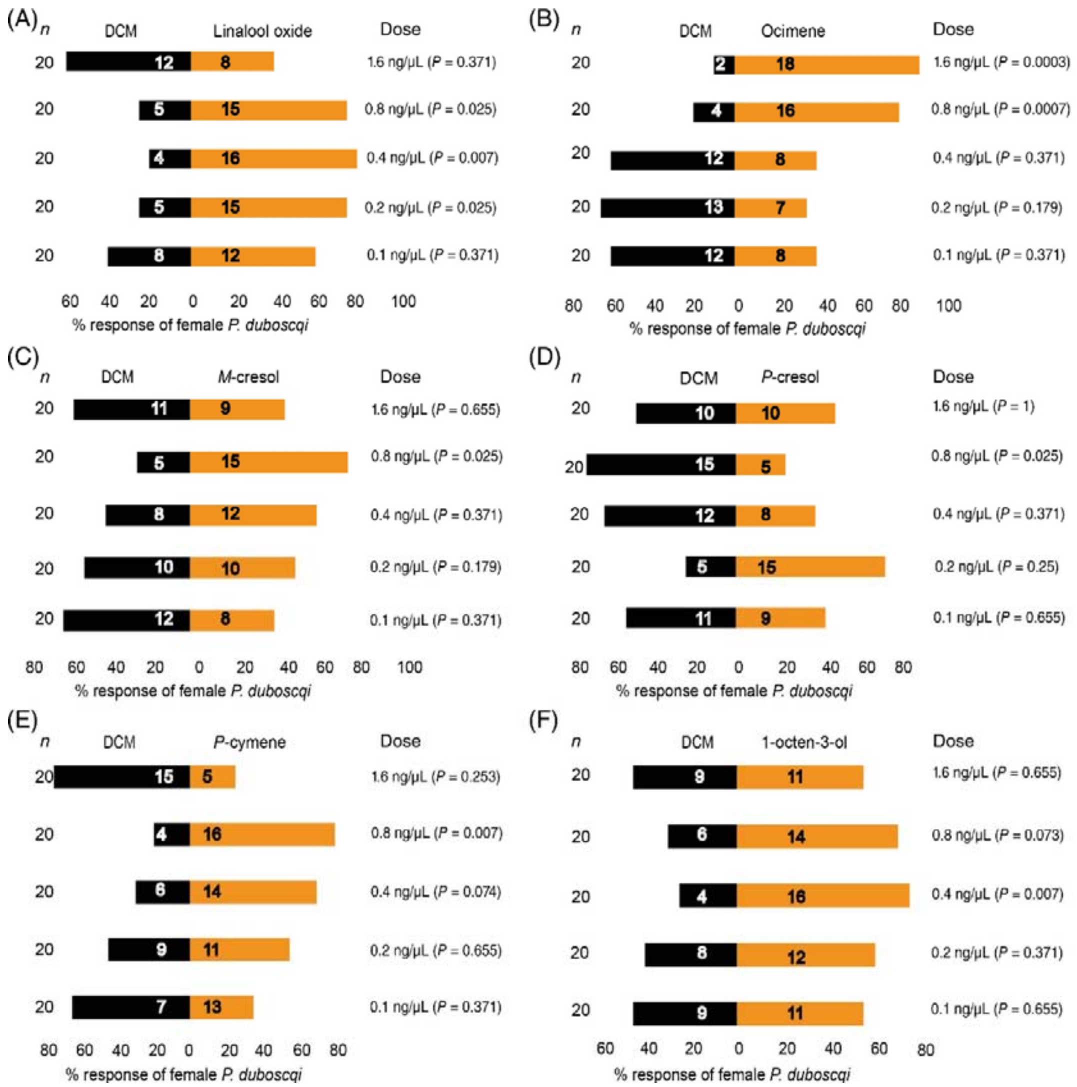

Fig. 2. Percentage (%) responses of females of *P. duboscqi* to odour components of sand fly host plants (A) linalool oxide, (B) ocimene, (C) *m*-cresol, (D) *p*-cresol, (E) *p*-cymene, and (F) 1-octen-3-ol, all against DCM = dichloromethane (control solvent) in a Y-tube olfactometer. P-values indicate levels of significance between each treatment and control at α=0.05. Each pair of evaluation used a total of n=20 sand flies for each compound dose released singly per choice. Numbers in each bar represent those responding to the treatment or control.

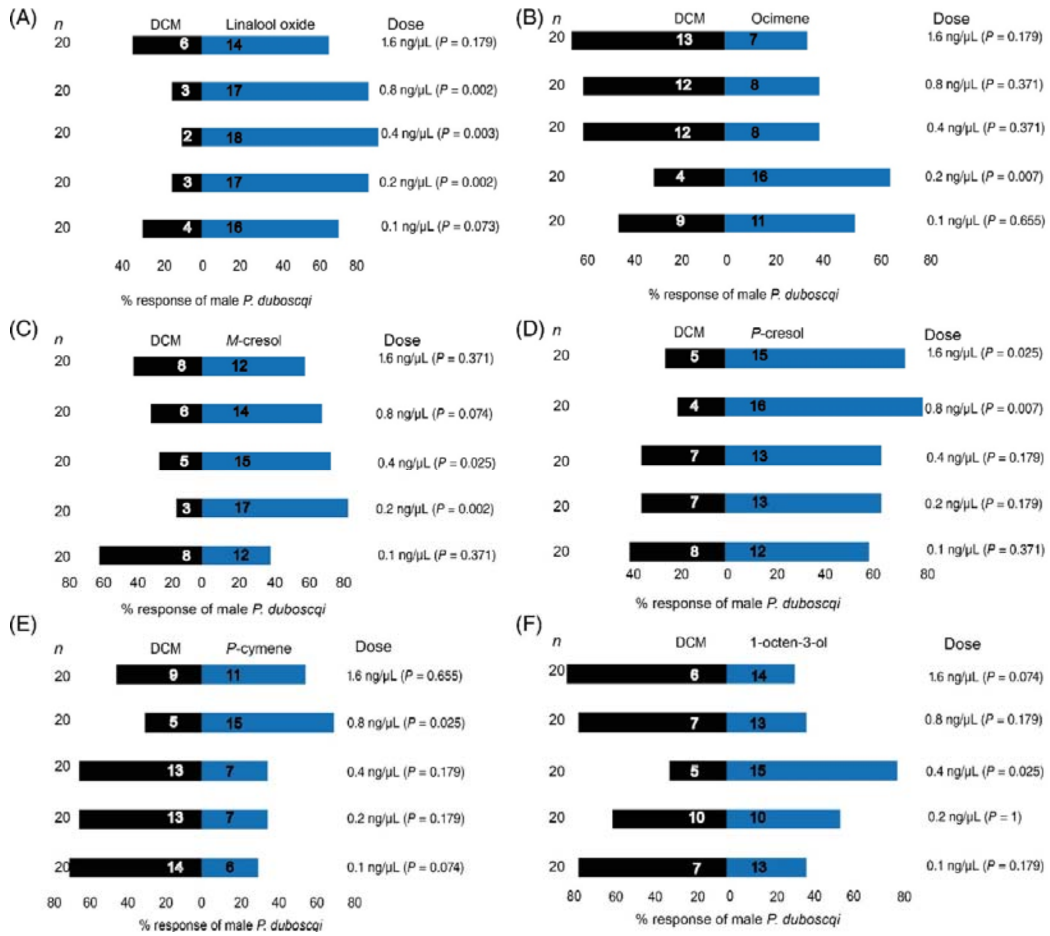

Fig. 3. Percentage (%) responses of male *P. duboscqi* to odour components of sand fly host plants (A) linalool oxide, (B) ocimene, (C) *m*-cresol, (D) *p*-cresol, (E) *p*-cymene, and (F) 1-octen-3-ol, all against DCM = dichloromethane (control solvent) in a Y-tube olfactometer. P-values indicate levels of significance between each treatment and control at α=0.05. Each pair of evaluation used a total of n=20 sand flies for each compound dose released singly per choice. Numbers in each bar represent those responding to the treatment or control.

Table 2. Summary of behavioural responses of *P. duboscqi* females and males to plant volatile organic compounds.

	Female choice				Male choice			
Compound name	optimal attractive dose	χ^2	df	*P. value*	optimal attractive dose	χ^2	df	*P. value*
Linalool oxide (LO)	0.4 ng/ml	7.2	1	0.007	0.4 ng/ml	12.8	1	0.0003
Ocimene	1.6 ng/ml	12.8	1	0.0003	0.2 ng/ml	7.2	1	0.007
m-Cresol	0.8 ng/ml	5	1	0.025	0.2 ng/ml	9.8	1	0.002
p-Cresol	0.2 ng/ml	5	1	0.025	0.8 ng/ml	7.2	1	0.007
p-Cymene	0.8 ng/ml	7.2	1	0.007	0.8 ng/ml	5	1	0.025
1-octen-3-ol	0.4 ng/ml	7.2	1	0.007	0.4 ng/ml	5	1	0.025

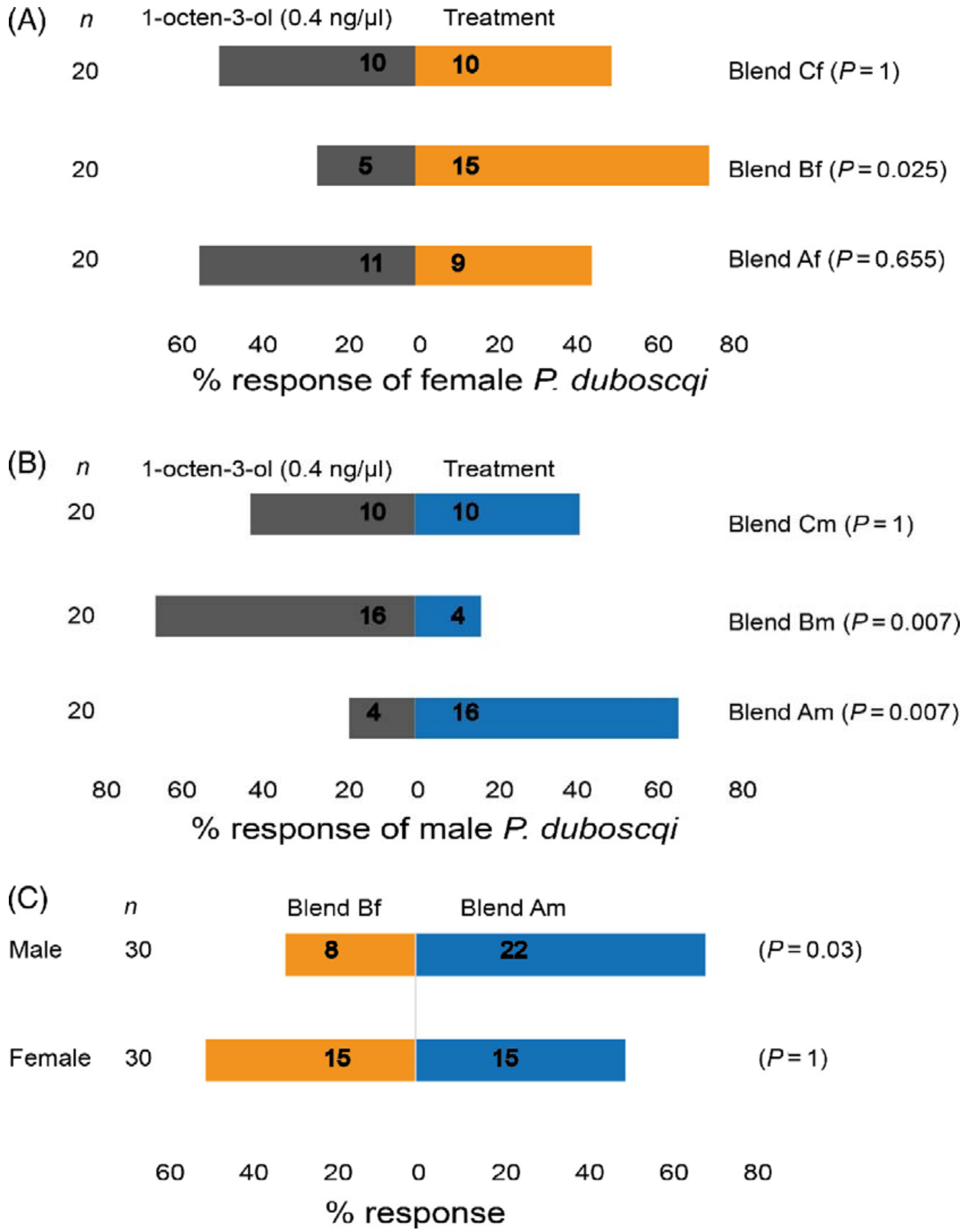

Fig. 4. Percentage (%) responses of *P. duboscqi* A) females and B) males, to different blends each tested against the positive control (1-octen-3-ol). C) % response to the most attractive blend for female (double dose) relative to Blend Am (the most attractive blend for male, optimal dose). P-values indicate levels of significance between each treatment and control at α=0.05. Each pair of evaluation used a total of N=20 (Fig. 4A, B) or N=30 (Fig. 4C) sand flies released singly per choice. Details of blend composition are provided in Table 1. Numbers in each bar represent those responding to the treatment or control.

Tests with the optimal attractive doses of each compound formulated in a blend recorded the following results: females were attracted to two-fold the optimal attractant doses (Blend Bf) compared to the positive control (1-octen-3-ol) (Blend Bf; χ^2 =5, *df*=1, $P < 0.025$) (Fig. 4A). In contrast, males were highly attracted to the blend at the optimal attractant dose (Blend Am) tested against 1-octen-3-ol (Blend Am; χ^2 = 7.2, *df*= 1, $P < 0.007$) (Fig. 4B).

On the other hand, Blend Am and Blend Bf elicited significant attractive responses in males and females, respectively (Fig. 4A and B). On the other hand, Blend Am and Blend Bf elicited no significant attractive responses in females (χ^2= 0, *df* = 1, P = 1), but males were highly attracted to Blend Am (χ^2= 4.5, *df* = 1, $P < 0.034$) (Fig. 4C).

Discussion

We evaluated the behavioural responses of male and female *P. duboscqi* to the plant-derived VOCs linalool oxide (LO), ocimene, *p*-cymene, *p*-cresol, and *m*-cresol in laboratory olfactometer assays. When tested individually against a negative control, each compound elicited a positive response although in a dose-dependent manner. These findings are in agreement with previous studies with VOCs, as demonstrated for sand flies (Machado *et al*., 2015) and mosquitoes (Nyasembe *et al*., 2012; Tchouassi *et al*., 2013; Jacob *et al*., 2018; Tchouassi *et al*., 2019).

Linalool oxide has been posited as a generalist plant-based lure, having been found to increase trap catches of the malaria vectors *Anopheles gambiae* sl (Nyasembe *et al*., 2012, 2015; Jacob *et al*., 2018), the dengue vector *Aedes aegypti* (Nyasembe *et al*., 2015; Omondi *et al*., 2019) and the flood water *Aedes* mosquito primary vectors of Rift Valley fever (Nyasembe *et al*., 2015). In the current study, the monoterpene LO was the most attractive volatile

>80%for both sexes of *P. duboscqi,* confirming LO as an attractant for a wide range of blood-feeding insects including wild sand flies. Andersson and Dobson (2003) noted that the release of LO from plants may reflect adaptations to attract lepidopteran pollinators. As for disease vectors such as sand flies, detection of LO in their crop is indicative of sugar meal source containing this compound.

Our results also show differential attraction of both sexes of *P. duboscqi* to the monoterpenes ocimene and *p*-cymene and the benzenoids *p*-cresol, and *m*-cresol. Whereas the optimal attractive dose for ocimene was 0.2 ng/µl for males and 1.6 ng/µl for females, it was two-four-fold higher/lower for *p*-cymene for both sexes. Likewise, whereas *p*-cresol was optimally attractive at 0.2 ng/µl for females and 0.8 ng/µl for males, it was four-fold higher or lower for *m*-cresol for both sexes. These findings are consistent with previous results found for *Stomoxys calcitrans* L. reported by Tangtrakulwanich *et al*. (2015). The results also emphasize that differences in odour quality and quantity may determine sand fly responses to plant volatiles (Nyasembe *et al*., 2012). Interestingly, *p*-cymene, *p*-cresol and *m*-cresol have been reported as components of the volatiles emitted by fresh livestock dung and termites mounds (Hassaballa *et al*., 2021b). They have also been reported to attract gravid *Stomoxys calcitrans* L. in the field (Tangtrakulwanich *et al*., 2015; Baleba *et al*., 2019).

Several studies have shown that most insects are more attracted to blends of compounds than to the individual components in the blend (Nyasembe *et al*., 2012; Jacob *et al*., 2018; Baleba *et al*., 2019). Additionally, modelling studies have confirmed that insects sensory systems encode odourant mixtures more efficiently than single odourants (Chan *et al*., 2018). In the present study, blends of the compounds at the optimal attractive doses elicited significantly higher responses in both sexes than those found for 1-octen-3-ol, which agree with these previous studies. Interestingly, males discriminated more strongly a male specific blend than the one optimally attractive to females (Fig. 4C). In contrast, females did not exhibit any preference between such blends (Fig. 4C). Whether the males are more sensitive to odour quality and quantity than their female counterparts are unclear. However, these results suggest that either sex may require different blend concentrations to attract them in the field, which would require field evaluation.

Differences in behavioural responses to VOCs between sexes have been observed in other blood-feeding insects such as tsetse flies (Olaide *et al*., 2019) and mosquito disease vectors (Tchouassi *et al*., 2019). Electrophysiological and field studies on the compounds tested in the present study are needed to shed light on this. Inherent biological and life cycle differences could also account for the sex-specific variation in response to the compounds since it is known that females generally engage in more diverse olfactory mediated activities, e.g., host feeding, oviposition, plant feeding, mating than males (Nyasembe *et al*. 2012, Tchouassi *et al*. 2013; 2019).

A few shortcomings of the present study are worth highlighting. The blends tested represented only one host plant. As such male and female responses may vary for representative blends for ratios in the headspace of the different host plant species. Hence, additional experiments are needed to test the representative blends for these different host plants. The approach adapted was guided by the interest to develop potent blends by combining optimally attractive compounds at different doses as done previously (Tchouassi *et al*., 2013). Further field evaluation of how the blends perform against the individual compounds and not only 1-octen-3-ol are needed. More so, investigation is needed on the effect of the blends on other sand fly species.

Conclusion

We conclude that the plant-derived VOCs tested in this study act as kairomones and can potentially be developed into attractant lures to improve outdoor trapping and management of sand fly vector of leishmaniasis populations.

Acknowledgements

We acknowledge Steve Kiplagat for his valuable contributions rearing and supply of sand flies used in the experiments. Special thanks to Dr Merid Getahun for behavioural assay for providing some chemical standards used in this study. We wish to thank also Mr. Basilio Njiru for technical support. I.B.H was supported by a German Academic Exchange Service (DAAD) In-Region Postgraduate Scholarship (Grant number 91672086).

This study was partly supported by the project, Combatting Arthropod Pests for better Health, Food and Climate Resilience (Project number: RAF-3058 KEN-18/0005) funded by Norwegian Agency for Development Cooperation (Norad). We gratefully acknowledge the financial support for this research by the following organizations and agencies: UK's Foreign, Commonwealth & Development Office (FCDO); the Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); the Federal Democratic Republic of Ethiopia; and the Government of the Republic of Kenya. The views expressed herein do not necessarily reflect the official opinion of the donors.

Author contributions

Conception and design of the study, IBH and DPT; methodology, IBH and DPT; performed behavioural experiments and statistical analysis, IBH; interpretation of data and drafting the manuscript IBH, BT and DPT, reviewing and editing IBH, DM, DKM, CLS, BT and DPT;

supervision, CLS, BT and DPT. All authors reviewed and final approval the final version to be submitted.

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