Understanding Sandfly Sampling Methods: The Sticky Traps are attraction-based and not interceptive sampling tools of *Phlebotomus orientalis*

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Abstract

Background : It is generally assumed that sticky traps are interceptive sandfly sampling methods, although no previous experimental evidence has supported this assumption. In this study, we test this assumption experimentally for *Phlebotomus orientalis* , the principal vector of visceral leishmaniasis in East Africa, and provide an explanation for why their collection is highly male-biased.

Methods: Three field experiments were carried out in March 2016-June 2019, in Gedarif state, eastern Sudan. In a first experiment, we compared numbers of *P. orientalis* caught with sticky traps made of black, red, transparent, white, yellow, green and blue A4 size papers that were set simultaneously at different lunar light conditions. In a second and a third experiments, we compared numbers of *P. orientalis* captured on sticky traps placed side by side horizontally or vertically on the ground or horizontally on a stool. We also made observations on mating behaviour of sandflies following their landing on un-sticky papers placed on the ground.

Results: *Phlebotomus orientalis* showed significant attraction to white, yellow and transparent traps; with negligible numbers caught on the black and the red traps. Similarly, significantly higher numbers of *P. orientalis* were attracted to the horizontal traps, resulting in 8-fold increase in their yield as compared to the vertical traps. Placing the traps on the stools resulted in significant reduction of this attraction. In contrast to the sticky traps that captured only very few females, our observations indicated that when male sandflies land on un-sticky white paper they successfully lure females and copulate with them.

Conclusion: The study demonstrated that for *P. orientalis*, sticky traps are more attractant-based than interception-based sampling tools and supports the notion that males of this sandfly species probably utilize the bright surface of the papers of the traps to perform mating rituals to attract the females for copulation. However, their pre-mature death in the sticky oil hampers the completion of these rituals and thus the result in failure of the attraction of the females. The findings of the study have important implications for optimization of the ST design for vector surveillance purpose and understanding the behaviour of *P. orientalis* .

Background

Sandflies (Class: Insecta; Order: Diptera, Family: Psychodidae) are important haematophagus insects that transmit the pathogens causing leishmaniasis, bartonellosis, sandfly fever and vesicular stomatitis [1, 2]. The most important of these diseases is leishmaniasis, a multi-spectrum neglected tropical disease that can be manifested as long lasting cutaneous ulcers (cutaneous leishmaniasis, CL), mucocutaneous lesions (mucocutaneous leishmaniasis, MCL) or a visceral infection (visceral leishmaniasis, VL). Leishmaniasis has a wide global distribution with over one billion people at risk of infection in 98 countries. VL, caused by members of the L. donovani complex, is considered to be the most serious form of leishmaniasis. In absence of timely diagnosis and successful treatment, it has a fatal outcome. There are an estimated 50,000-200, 000 cases of VL annually, with 5–10% mortality rates each year. VL has a

wide distribution in Latin America, Africa, Europe and Asia, but 90% of cases occur in the seven high VL burden countries of Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan. Phlebotomus orientalis is the principal vector of VL in East Africa [5]. This sylvatic sand fly species is abundant in woodland habitat dominated by Balanites aegyptiaca and/or Acacia seyal trees that grow on black cotton soil. In village habitats, the vector rarely enters inside the huts/rooms but can be quite abundant in the courtyards of the houses (outdoor site) or in the peri-domestic habitats [6, 7]. In Sudan, Ethiopia and the Republic of South Sudan, P. orientalis shows marked seasonality, reaching peak abundance at late dry season (March-June) and disappearing during the months of heavy rains (August - September) [6–9].

Difficulties in finding the breeding sites of sandflies preclude studies on the immature stages under natural conditions. Thus, sampling adult populations and studying laboratory colonies provided most current information on the ecology and biology of sandflies [10]

Adult sandflies are small nocturnal insects with a weak jerky flight pattern, best described by short hops close to the ground level [11]. Whereas both sexes feed on sugar from plant origin, the females require a meal of vertebrate blood for egg nourishment and maturation. During the day the adults rest in humid places, which vary for different species, from inside human dwellings to crevices in trees and cracks in the soil [10]. Mating takes place near or on the host or in other lekking sites [12].

Efficacious, reliable and inexpensive traps are indispensable for monitoring sandfly populations, understanding their biology and ecology, and evaluation of control operations targeting the vectors of leishmaniasis. Such traps have been classified under two main categories; interceptive traps, which capture adults randomly by blocking their flight path; and attractant-based traps, which lures sandflies by light, animal baits or other attractants, such as CO_2 or kairomones [12]. While CDC light traps are the most commonly used attractant-based sandfly sampling devises, sticky paper traps have been considered as interceptive tools [12, 13].

Sandfly sticky traps are made of un-waxed card, or paper / metal sheets coated with castor oil or other viscous oil and placed vertically in the flight path of sandflies [14, 15]. In addition to the advantages of being inexpensive and easy to set up, no electrical power supply is needed to operate these traps and therefore, unlike the CDC traps, they hardly fail during operation. On the other hand, the sticky traps suffer the important disadvantage of sandfly male-biased collection, which has been observed with a number of species, including P. orientalis [6, 7, 9, 16, 17].

Although there is a near consensus that sandfly sticky traps are an interceptive sampling method, this classification is not based on experimental evidence and there are no literature records or data to support or refute this assumption. Nevertheless, the implications of this assumption are big; first and foremost, most field workers place these traps on vertical axes with the intention of intercepting the flies on their presumed flight path. As we will see from the results of the current manuscript, this method of trap placement must have led to very significant underestimation of P. orientalis vector abundance.

In this study we used a systematic experimental approach to test the notion that sticky-paper traps are interceptive sampling devices and address the intriguing question of their male-biased collection. First, we used coloured sticky paper traps to see whether sandflies are attracted to specific colour(s) or randomly trapped on any of the sticky papers. Secondly, we compared trap yields on sticky papers that were placed side by side horizontally or vertically on the ground. Here we tested the hypothesis that if sticky traps STs are interceptive, there will be higher numbers of sandflies on the vertical traps than on the horizontal traps. We then looked at effect of height from the ground on the yields of horizontally placed traps. Finally, by visual observation, we tested the hypothesis that male P. orientalis use the bright sticky traps as mating arena and that the reduction in the number of females in sticky trap collection is due to the premature death of males and their failure to complete the courtship behaviour that lures the females. The findings of this study have important implications, not only for optimization of the design of the sticky traps, but also for understanding the behaviour of P. orientalis and planning suitable control measures of this important vector of VL.

Materials & Methods

Study Area

The study was conducted during three consecutive sandfly seasons, March-June 2016-2019, in the area of Belo Village (Nour-Elmadina) which is located on the south-western bank of River Rahad (12° 52’ 476” N 035° 09’ 039” E), ≈100 km south west of Gedarif town, Gedarif state, eastern Sudan (Fig. 1). The ecology of the area was described in previous publications [6, 18]. The soil is mainly black cotton soil (chromic vertizol), with mixed stretches of sandfly/clay soil (locally called “Azaza”) in some locations inside the village and near the river. *Balanites aegyptiaca* and *Acacia seyal* trees dominate the natural vegetation. Dense forests are found around the village at the east, south and western sides about 5 km from Dinder National Park. Citrus and neem (*Azadirachta indica*) are found near the river bank and inside the village, respectively. The climate is tropical continental with an annual rainfall of 800mm. The year is divided into three main seasons; a rainy relatively cool season (autumn: June – October), a dry relatively cool season (winter: November – February) and a dry hot season (summer: March- May). The annual mean maximum temperature varies from 28.3 to 38.1 °C, while the annual mean minimum temperature varies from 9.0 to 22.1 °C.

The inhabitants of the village belong to the Fulani tribe. Most villagers live in huts constructed of thatched grass or mud that are surrounded by a straw fence. The village population raises large number of goats, sheep, cattle and chicken. For religious reasons, the inhabitants don’t keep dogs anywhere in the village and cats are rarely seen.

Comparison of numbers of *P. orientalis* captured on different coloured sticky traps under different lunar light illumination

We conducted four field experiments in March-April 2016, March-June 2017 and April-June 2018 to compare numbers of *P. orientalis* captured on sticky paper traps that were prepared by coating differently

coloured A4 papers and A4 transparent plastic sheets with pure sesame oil. In the first experiment, a duplicate of 5 sets of sticky traps made of transparent plastic sheets and black, red, yellow, and white paper were placed vertically on wooden sticks at 3 cm above the ground in a 6m diameter circle on the ground between 18:00-06:00 hr during quarter lunar-half lunar nights (Fig.2). This experimental unit was replicated simultaneously in 4 different microhabitats; around an *Acacia seyal* tree, a *Balanites aegyptiaca* tree, a termite mound and an outdoor site at the periphery of Belo village. The experiment was repeated for 6 nights in April 2016 that spanned the period when the moon was quarter lunar to half lunar. Sandflies collected in each sticky trap were picked up by a fine brush and processed as described below. Samples from each circle were pooled according to the colour of the trap and the collection site.

The 4th experiment was conducted in full dark nights of April 2018 to compare numbers of *P. orientalis* captured on red, transparent, white, yellow, green and blue traps placed horizontally on the ground. The experimental design was similar to the second and third experiment described above; except for the spatial orientation of the traps, which were placed horizontally on the ground, supported in place by small stones. A total of 5 replicates were conducted in 3 nights. Sandflies were processed and recorded as described above.
In the second and the third experiments, the design was altered to include in each circle, blue and green A4 papers as additional coloured sticky traps. To keep the same circle diameter, the number of traps from each colour was reduced to one trap per circle. The two experiments were conducted under either full lunar (Experiment 3: 13-16th of the Lunar month in April 2017) or full dark nights (Experiment 4: 27-29th and the first night of two Lunar months in March and June 2017). Thus trapping was done for 4 dark nights and 8 lunar nights. Each night, a total of 10 circles, each containing one trap of each colour, were set between 18:00-06:00 hr in one site with mixed *A. seyal* and *B. aegyptiaca* trees at the periphery of Belo village. Sandflies were picked up and processed as described. Sandflies from all 10 sticky traps for each colour per night were pooled as a single sample.

Effects of vertical and horizontal orientations of sticky traps on their yields of *P. orientalis*:

We examined whether placing sticky traps in a vertical position to intercept a possible sandfly path will result in capturing higher numbers of *P. orientalis* than traps placed horizontally on the ground. In April 2017, ten sets of traps, each consisting of one horizontal and one vertical sticky traps, spaced at ½ m from each other, were placed in the study site described above, overnight from 18:00-0:06 hr. By next morning, sandflies on the traps were picked up and processed for subsequent identification as described. The sandfly yield of each 10 horizontal or 10 vertical traps were considered as trap night. The experiment was replicated for 6 nights and the numbers of *P. orientalis* per horizontal or vertical trap were compared using the statistical tests described below.

Effects of sticky traps height on their yield of *P. orientalis*:

An experiment was done during full dark nights in April 2018 and March 2019 to test the effect of the height from the ground on yields of horizontal traps as compared to vertical traps. Three sets of A4 size

sticky paper traps were placed horizontally on the ground, horizontally on 20 cm metal stools, or vertically on small sticks above the ground as described above. Each set consisted of 10 traps that were placed ½ m from each other in a straight line. To allow similar access to the surrounding area, the three lines of traps were set out in a triangle shape. Trapping was continued from 18:00 to 0600 hr. The experiment was replicated for 2 nights in March 2018 and 3 nights in March 2019. The positions of the three sets of traps were rotated with each new replicate, so that each trap received equal exposure to the surroundings. . Sandflies found in each set of traps were collected, sorted out and identified and then compared as described.

Observations on mating behaviour of sandflies as they land on non-sticky white papers placed horizontally on the ground.

A non-sticky clean white A4 paper were placed on the ground and used to observe whether both male and female *P. orientalis* and other sandflies land and copulate on its surface. No specific protocol was followed to document the behaviour of the sandflies. Attempts were made to capture the adult sandflies for further identification and counting, but these were unsuccessful. Nonetheless, a photograph was taken of unidentified male and female sandflies that were copulating on the paper.

Sandflies preservation and identification

Each morning, collected samples were removed from the sticky paper traps using small fine brushes; washed in a detergent and water, and finally preserved in 70% ethanol for subsequent processing. Preserved sandflies were sorted out by sex and genus under a binocular dissecting microscope. *Sergentomyia* sandflies were sorted and counted without further processing. *Phlebotomus* sandflies were mounted in PVA medium (BioQuip, CA, USA) and then identified to the species level, using standard morphological features described in relevant taxonomic keys [8, 13, 19, 20].

Statistical analysis:

Data were analyzed using SPSS statistical package (ver. 24.). Data were not normally distributed. Therefore, the nonparametric Kruskalis-Wallis Test, Wilcoxon signed- rank test and Man-Whitney test were used to compare the median number of sand fly specimens attracted to different colored sticky paper traps, at different experimental treatments.

Results

Species and sex composition of sandflies captured on different vertical colour sticky-paper traps.

A total of 10498 sandflies were captured on different colour sticky traps. The collection consisted of *P. orientalis* sandflies (16.42%), several species of *Sergentomyia* sandflies (83.1%) and negligible numbers of *P. papatasi* sandflies (0.5%). We did not conduct species identifications for the *Sergentomyia* sandflies, which we grouped together as total numbers of males and females.

For *P. orientalis*, almost all of the collection consisted of males (93.9 %), with a significantly smaller number of females (6.1%) (U = 8477.5, Z= -10.170, p< 0.000). In contrast, there was no significant difference between numbers of male and female *Sergentomyia* sandflies (males = 49.3%; females = 50.37%; (U = 1796.3, Z= -1.110, p= 0.267) (Fig.3). Similarly, for *P. papatasi* the total number of males and females were 26 and 27 respectively, with no significant difference between the two numbers (U = 18713.51, Z= -0.850, p= 0.396).

Comparison of the yield of coloured vertical sticky traps under different lunar light conditions:

Phlebotomus orientalis sandflies showed significant variations in numbers captured on different colour vertical sticky-paper traps. These numbers varied under different lunar conditions, but always, the black and red traps received the least number of sandflies.

The preliminary experiment, conducted under quarter to half lunar conditions in 2016, showed significant variation in numbers of *P. orientalis* captured on different coloured traps ($\chi2$ = 19.33, df=4, p< 0.001; Fig. 4a). The white, yellow and transparent sticky traps captured similar numbers of *P. orientalis*, which were significantly higher than the number of flies of the same species captured on the black traps (Z= -3.626, p = 0.000; Z= -3.341, p=0.001 and Z= -3.050, p=0.002, respectively). On the other hand, the numbers of *P. orientalis* captured on the red traps were similar to those captured on the black traps (Z= -1.228, p=0.219) and significantly less than the numbers captured on the transparent, white and yellow traps (Z= -2.425, p=0.015; Z= -3.683, p= 0.000 and Z= 3.226, p = 0.001, respectively).

In 2017, we repeated the experiment comparing the yields of different vertical coloured sticky traps under the contrasting light conditions of full lunar and full dark moon phases. In addition to the 5 sets of black, red, transparent, white and yellow traps, green and blue coloured sticky paper traps were also included in the comparison. Under both night light conditions, there were significant differences in numbers of *P. orientalis* captured on different coloured traps, albeit with higher statistical significance during the lunar nights than the dark nights ($\chi2$ = 30.098, df =6, p< 0.001) during lunar nights, Fig.4.b and $\chi2$ = 14.705, df=6, p= 0.023, Fig.4.c during dark nights).

In full lunar nights, the black and the red vertical sticky traps captured similarly low numbers of *P. orientalis*, which were significantly less than numbers captured on the white, the transparent and other coloured vertical traps (table 1). There were no significant differences between numbers of *P. orientalis* captured on the transparent, white, yellow, green and blue traps; although there was a clear tendency for higher yields of the vector on the white and the yellow traps ($\chi2$ = 5.773, df =4, p= 0.217) (Fig.4b).

Table 1

Summary of results of Wilcoxon's Ranked Signed paired Test analysis of numbers of P. orientalis captured on vertical coloured vertical sticky traps in both full lunar and full dark nights during 2017

	Black	**Red**	**Transparent**	**White**	**Yellow**	**Green**	**Blue**
Black		Z= -1.06 P = 0.29	Z= -1.83 P = 0.07	Z=-1.83 P = 0.07	Z=-1.84 P = 0.07	Z=-1.83 P = 0.07	Z= -1.60 P = 0.11
Red	Z = 0.65 P = 0.52		Z= -1.82 P = 0.07	Z= -1.46 P = 0.14	Z= -1.82 P = 0.07	Z= -1.82 P = 0.07	Z= -1.29 P = 0.20
Transparent	Z = 2.53 P = 0.01	Z = 2.38 P = 0.02		Z= -0.73 P = 0.47	Z= -1.83 P = 0.07	Z= -1.20 P = 0.27	Z= -1.83 P = 0.07
White	Z = 2.52 P = 0.01	Z = 2.38 P = 0.02	Z= -2.38 P = 0.02		Z=-1.46 P = 0.14	Z= -0.74 P = 0.46	Z= -1.46 P = 0.14
Yellow	Z = 2.52 P = 0.01	Z= -2.52 P = 0.01	Z= -0.07 P = 0.94	Z= -1.68 P = 0.09		Z=-1.60 P = 0.11	Z= -0.38 P = 0.71
Green	Z = 2.38 P = 0.012	Z= -2.21 P = 0.03	Z= -0.41 P = 0.69	Z= -1.35 P = 0.12	Z=-0.34 P = 0.74		Z= -1.46 P = 0.14
Blue	Z = 2.20 P = 0.03	Z=-2.04 P = 0.04	Z= -2.06 P = 0.04	Z=-2.52 P = 0.01	Z=-2.02 P = 0.04	Z= -1.36 P = 0.17	
Above diagonal = vertical sticky traps during full dark nights 2017							
Below diagonal = vertical sticky traps during full lunar nights 2017							

In full dark nights, only transparent, green and white traps captured significantly higher numbers than black traps (p = 0.003, 0.007 and 0.026, respectively). Although these coloured traps captured similar numbers of P. orientalis, only the transparent and the green traps yielded more P. orientalis than the red traps (Table 1).

In both dark and lunar nights, there were significantly higher numbers of males captured on different vertical coloured sticky traps than females. In lunar nights, these ratios appeared to be remarkably higher in case of white (49:1), yellow (12:1) and transparent traps (9:1) than for the same traps in dark nights and for all traps in both light conditions. Overall, the white traps seemed to have higher male biased sex ratios than other coloured traps (Table 2).

Table 2
Comparison of median numbers and sex ratios of Phlebotomus orientalis sandflies captured on different colour vertical sticky traps set out in full dark and full lunar nights in Belo village (Gedarif state, Sudan March – June 2017). ♂ =Males ♀ =Females

Trap colour	Full Dark Nights				Full Lunar Nights			
	Total	♂	♀	Male: female Sex Ratio	Total	♂	♀	Male: Female Sex ratio
Black	1.0	1.0	1.0	1.0:0.0	1.0	1.0	0.0	1.0:0.0
Red	3.0	2.5	0.5	5.0:1.0	0.5	0.5	0.0	1.0:0.0
Transparent	20	16.0	4.0	4.0:1.0	5.0	4.5	0.5	9.0:1.0
White	12.5	11.0	2.0	6.0:1.0	25.0	24.5	0.5	49.0:1.0
Yellow	4.5	4.5	0.0	4.5:1.0	6.5	6.0	0.5	12.0:1.0
Green	14.5	11.5	2.5	4.5:1.0	3.0	2.0	1.0	2.0:1.0
Blue	4.5	4.0	0.5	8:1	4.0	3.5	0.5	7:1.0

Comparison of numbers of P. orientalis sandflies captured on coloured sticky traps placed in horizontal position

In 2018, we conducted an experiment to compare collection of different color sticky paper traps when placed horizontally on the ground. The experiment utilized all coloured sticky traps described above except the black paper, which was not available at the time of the experiment. Interestingly, although the experiment was conducted under full dark conditions, the pattern of collection of P. orientalis on different coloured traps was more like the full lunar nights with significantly higher numbers of the vector captured on the white traps as compared to other colours (($\chi 2 = 35.017$, df = 5, $p < 0.000$). Z= -2.20, p = 0.03 for White – Transparent and Yellow – White; Z= -2.38, p = 0.02 for Green – White, Blue – White and White - Red. The red, green and blue sticky traps captured the lowest numbers of P. orientalis. (Fig. 5 and Table 3).

Table 3
Comparison of median numbers and sex ratios of P. orientalis sandflies captured on different colour horizontal sticky traps set out in full dark nights in Belo village (Gedarif state, Sudan, April 2018)

Trap colour	Horizontal trap on Full Dark Nights			
	Total	Males	Females	Male: female Sex Ratio
Red	0.5	0.5	0.0	0.5:0.0
Transparent	2.0	1.0	1.0	1.0:1.0
White	8.0	6.0	2.0	3.0:1.0
Yellow	3.5	2.5	1.0	2.5:1.0
Green	0.0	0.0	0.0	0.0:0.0
Blue	1.0	1.0	0.0	1.0:0.0

Effects of spatial orientations of white sticky paper traps on their yields of *P. orientalis* sandflies

The second set of experiments, testing the hypothesis that sticky paper traps are more attractant-based than interception-based sampling tools, compared yields of adjacently placed A4 white sticky paper traps placed in horizontal spatial orientation on the ground and traps placed vertically on the ground as described above. Remarkably, significantly higher numbers of *P. orientalis* were captured on the horizontal paper than on the vertical paper (Fig. 6; Z= - 2.201, p = 0.028), resulting in an 8-fold increase when the traps were placed horizontally on the ground (mean=79.14±24.80; median = 80.0) versus when the traps were placed vertically on the ground (mean=9.70 ±2.43, median=10).

The difference in spatial trap position did not affect the ratio of male and female *P. orientalis* captured on the sticky traps. However, this sex ratio appeared to be more biased for males in the horizontal than in the vertical traps (8.0:1.0 for horizontal and 2.3:1.0 in vertical traps) (Fig. 6).

Effects of sticky trap heights from the ground on their yields of *P. orientalis* sandflies

In a subsequent experiment, we examined whether differences seen in yields of horizontal and vertical traps were due to height from the ground level or actual trap spatial orientation. Therefore, three sets of traps were placed either horizontally on the ground, horizontally on 20 cm metal stools or vertically on the ground. A clear difference in the trap yield of the three positions was observed, with significantly highest numbers of *P. orientalis* captured on the horizontal traps placed on the ground, followed by the elevated horizontal traps and finally the vertical traps (Z= - 2.232, p= 0.026 for horizontal on the ground vs vertical;

Z= - 2.032, p= 0.042 for horizontal on the stool Vs vertical; and Z= - 0.552, p= 0.581 for horizontal on the ground vs horizontal on the stool). (Fig. 7).

Observations on courtship and mating behaviour of *P. orientalis* when landing on untreated white papers placed on the ground.

The results of the above sets of experiments led us to formulate a hypothesis to explain why most of the *P. orientalis* caught on the sticky traps are males. Here, we proposed that the bright surface of the sticky traps present an arena where the males can perform courtship rituals, including dance, love-songs and pheromone that attracts the females for copulation (Fig.8). However, due to their trapping in the sticky oil, the males fail to complete the courtship display and the attraction of the females. To test this hypothesis, we placed clear white papers on the ground at sunset and observed the behavior of sandflies as they land on this non-sticky paper. We saw large numbers of males landing on the paper and starting to perform an apparent courtship behavior; running on straight lines on the paper. Within a short time, female sandflies started to land on the paper and copulated with the males. Unfortunately, we did not have the tools to record the display and we did not manage to capture and identify male and female sandflies that landed on the paper.

Discussion

Properly designed traps are important tools for sampling disease vectors, studying their biology and ecology as well as evaluation of control operations. Understanding the mechanism by which these traps capture the target vector species is key to successful optimization of their performance and interpretation of their yields. Based on these mechanisms, sandfly traps have been classified under two broad categories, interceptive tools, e.g. sticky traps, and attractant-based tools, e.g. CDC light traps [12]. While the interception traps capture active sandflies in a given habitat without bias, the attractant-based tools lure sandflies by specific physical or chemical cues, such as light, CO_2, or host odors and kairomones [16, 21–25].

Sticky traps have been widely viewed as interceptive sampling tools and therefore in most studies they were intentionally placed in vertical positions to intercept a presumed sandfly flight path [12]. The results of our current study contradict this notion strongly and show that for P. orientalis the sticky traps are by far more attractant-based than interception-based sampling tools. Firstly, in the coloured sticky traps experiment, if the traps capture P. orientalis by interception, the collection on differently coloured papers would be distributed evenly. It must be emphasized that in this experiment we placed the coloured sticky papers on the vertical position to maximize any interception if existed. Clearly, interception was not the mechanism of trapping since the vector showed strong differential attraction to specific colours, with negligible numbers of flies captured on the black and red traps. This diminished collection of P. orientalis on the black and red papers provide a strong argument for active attraction since it is common knowledge that black does not reflect light and red light illicits minimum visual sensitivity by insects. Secondly, the interception mechanism implies that vertical placement of the sticky traps in the flight path

of P. orientalis should provide a higher yield than the horizontal orientation. The results of the experiment comparing yields of horizontal and vertical traps actually showed the complete opposite: an 8-fold increase in the collection of P. orientalis on horizontal compared to vertical traps.

Overall, the comparison of numbers of P. orientalis captured on differently coloured sticky traps showed that the vector was more attracted to the white, yellow, transparent and green traps than to the blue and red traps. The attraction was clearly influenced by the lunar cycle. More numbers of P. orientalis were attracted to the white and yellow papers during quarter lunar, half lunar and full lunar nights while in full dark nights the transparent and green papers appeared to result in higher yields of the vector. Nonetheless, it must be stated that there was a lack of statistical significance in the results of the dark nights experiment on vertical traps, and this deserves revisiting by more replicates than the 4 nights used for the experiment. Interestingly, the spatial orientation of the traps seemed to alter the differential attraction of P. orientalis, since in dark nights white and yellow horizontal traps attracted significantly more numbers of the vector than all other coloured traps. Thus, it seems that variations of the intensity of reflected light that results from the lunar cycle and the trap position can influence the colour preference of P. orientalis.

Apart from attempts to bait sticky traps with coloured chemical light sticks [21], there are no previous published reports on attraction of sandflies to differently coloured sticky traps that can be used for comparison with current results. Burkett et al (2007) found that although all sticky traps baited with colours of chemical light sticks in the visible spectrum captured significantly more sandflies than did the infrared baited sticky traps and un-baited sticky traps, there was no significant difference in the yield of traps baited with differently coloured light sticks. It must be stressed that no species identification was done on the sandflies caught by these authors; a problem that seriously compromises the results and the conclusion of this study. On the other hand, a number of field and laboratory studies demonstrated that sandflies have true colour vision and could be differentially attracted to specific wavelengths in the UV and blue-green-yellow region [26, 27]. For example, studies showed that sandflies are more attracted to CDC traps baited with UV light than standard CDC traps that are baited with incandescent light [21]. The attraction may also be influenced by the intensity of the light. In contrast to wavelength sensitivity ranges reported by Mellor & Hamilton (2003), recent work indicates that for high-intensity light emitted from diodes, red light is more attractive to sandflies than white light, blue light or green light [28]. It must be stressed that in our study we had no means to control for light intensity other than conducting the experiments under low intensity (dark lunar nights) and higher intensities (full lunar nights).

Despite the variations of the collection at different lunar phases, there was an overall higher attraction of P. orientalis to white papers than other coloured traps. This preference was probably due to the fact that the light reflected by white colour is brighter and contains all ranges of the spectrum. It is also probable that the white Xerox paper used in the study may reflect more UV light which is known to attract sandflies, other Dipterans and most other insects [29]. Cronin & Bok (2016) suggested that most insect species include UV wavelength in their range of spectral sensitivity because of its adaptive ecological value as signal for detecting sources of sugar, interspecific communication and orientation during flight [30].

Therefore, it is recommended that for routine surveillance of P. orientalis white sticky traps are used to optimize sandfly collection.

The results of the experiment comparing yields of the vertical and horizontal traps correspond closely to the findings of Gebresillassie et al. (2015) who reported that, in agricultural fields of NW Ethiopia, horizontally placed A4 size sticky paper traps captured significantly higher numbers of P. orientalis than similar traps that were hung vertically at 30 cm above the ground [7]. However, the differences between the horizontal and the vertical traps detected in this previous study could also be due to elevation from the ground where more sandfly flight activity takes place [11]. The results of current experiments comparing yields of vertical and horizontal traps also support the findings of Moncaz et al. (2013) who showed that un-baited Large Horizontal Traps (LHT) with sticky surfaces made of 60 × 60 cm white polypropylene boards coated with sesame oil and placed on a metal frame 15 cm above the ground captured large numbers of male P. orientalis [16]. Although the experiment conducted by these authors did not include direct comparison with vertical traps, large numbers of P. orientalis captured on these LHT stand out as evidence of attraction, especially when compared to literature reports of traps laid on the vertical axis.

To achieve further progress towards optimization of the sticky traps for sampling P. orientalis, we compared numbers of P. orientalis captured on traps set vertically on the ground, or horizontally on the ground or horizontally on stools set at 15 cm above the ground. The data confirmed that the horizontal sticky traps attract higher numbers of P. orientalis than the vertical traps and showed that higher numbers of the vector are captured on horizontal traps set at the ground level than horizontal traps set on stools, at 15 cm above the ground level. Based on these results, it is recommended that for routine surveillance of P. orientalis the standard sticky traps should be laid on the ground instead of being placed on stools or laid vertically.

Remarkably, most P. orientalis individuals captured on the sticky traps during the current study were males. This is a common observation, which has been reported in most previous studies on this vector [6, 7, 9, 16, 17, 31]. Moncaz et al., 2013 addressed this phenomenon in more detail and reported that although baiting LHT's with CO_2, emanating from Yeast Sugar extract, increased the number of females 8-fold and the number of males by 0.4-fold, the number of females captured on LHT's remained extremely low as compared to number of males [16]. The authors related this observation to the previous report of Ashford (1974) who suggested that P. orientalis males utilize horizontal surfaces to form mating swarms [32]. We support the view that the high attraction of males to the sticky traps is due to mating behaviour. However, instead of the swarming hypothesis, we suggest that the males land individually on the surface of the bright horizontal surfaces to be visible to the females and perform courtship behaviour. To avoid disturbing the sandflies during our observations, we had to stay about one metre away from the paper and therefore we were not able to see detailed courtship behaviour. However, the landing on the surface of the paper and the straight-line running of the males appears to display visual signals for the females, which landed and mated with them within a short time. It is suggested that future field and laboratory studies should utilize infra-red video and sound recordings and chemical analysis that can allow further

observations on the mating behaviour of P. orientalis. Similar studies on other species of sandflies revealed a number of courtship rituals, including dancing, love songs, body touch, wing flapping and release of sex pheromones that attract and/or stimulate the females to copulate (23, 25, 33, 34, 35).

We propose that the failure of these traps to capture the females is mainly due to the premature death of the males on the oil and the consequent failure to perform the courtship behaviour and and/or love songs and release attractive pheromones. Our observations supported this hypothesis as we routinely saw many male sandflies (most probably P. orientalis) landing on the clean un-sticky white papers that we placed horizontally on the ground and successfully completing the dance and copulating with the females.

It is recommended that future studies should use the experimental approach described in the current study to determine how sticky traps capture other important sandfly species and whether the observed behaviour of P. orientalis is common within the genus or the subgenus levels. In a previous report, Moncaz et al., (2013) showed that the behaviour of P. orientalis contrasted sharply with behaviour of P. papatasi, which was not attracted to large horizontal surfaces (LHT's) unless baited with CO_2 derived from dry ice or fermenting yeast/sugar mixtures. On the other hand, the authors reported that the large horizontal surfaces seemed to attract both sexes of P. sergenti, although the number of males captured on the traps was twice the number of females [16].

In conclusion, the findings of this study should provide an important insight into the mechanism by which sticky traps capture P. orientalis and help in optimizing their design for routine surveillance of the vector and studies on ecology and control of leishmaniasis. The re-classification of the sticky traps as an attractant-based rather than interceptive sampling method of P. orientalis should help guide the research on further improvements of this important sandfly sampling method. Future studies should also be done to find solutions that can help overcome the disadvantage of lack of females in the collection, by utilization of CO_2 and other host odors and kairomones [16, 36, 37]. Studies are also urgently needed to investigate the release of sex pheromones in P. orientalis. As have been done for Lu. longipalpis [23, 25, 38], these pheromones can be characterized, synthesized and successfully applied to lure both males and females of the vector to the traps. More recently, a study showed that when co-located with insecticides, synthetic sex pheromones significantly reduced the incidence of canine visceral leishmaniasis and the abundance of Lu. Longipalpis [39].

Abbreviations

VL
Visceral leishmaniasis

Declarations

Availability of data and material:

All data used to generate the conclusions of this article are included within the paper. Raw data can be shared with other researchers upon specific request.

Ethics approval and consent to participate

The study described in this manuscript did not involve vertebrate animals or human subject.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests related to the study.

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Authors' contributions:

DAE conceived the study, participated in the experimental design, data collection and analysis and drafted the manuscript. AKA participated in the experimental design data collection and analysis and manuscript drafting. OD, BMB, TGMG and MAH participated in the experimental design and data collection. HE, BE and OFO participated in data interpretation and manuscript review. MDB and JA contributed to data interpretation and manuscript review. NK participated in conceiving the study, data analysis and interpretation and manuscript drafting. All authors reviewed and approved the final version of the manuscript.

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Figures

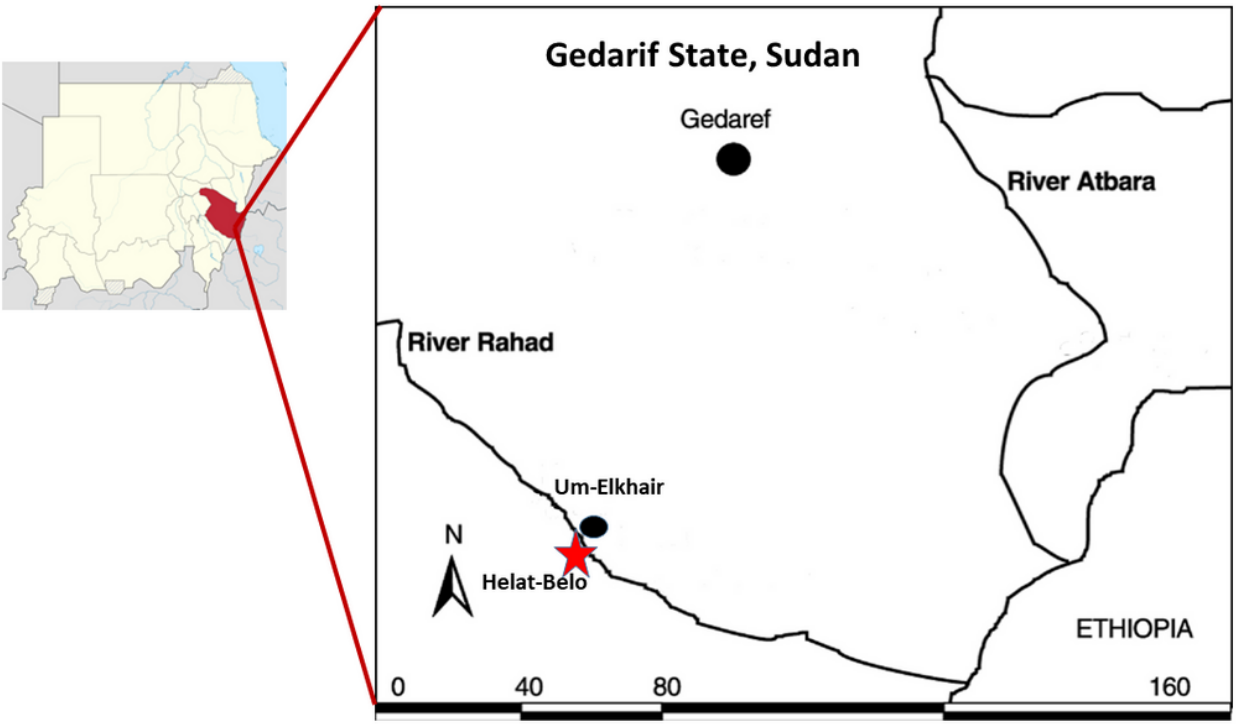

Fig.1:

Figure 1

Map showing location of study area around Belo village, Gedarif state, Sudan.

Fig.2:

Figure 2

Set-up of an experiment comparing number of sandflies captured on different coloured sticky traps. Traps were set vertically around an Acacia seyal tree on a black cotton soil site at the periphery of Belo village, Gedarif state, eastern Sudan; March/ 2016.

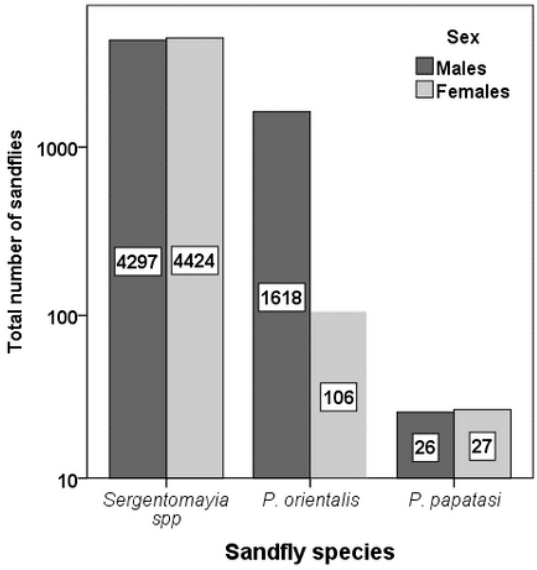

Fig. 3

Figure 3

Comparison of numbers of male and female sandflies captured on vertical sticky-paper traps. Data provides summary of different coloured traps set in Belo village, Rahad region, eastern Sudan in March – June (2016-2017).

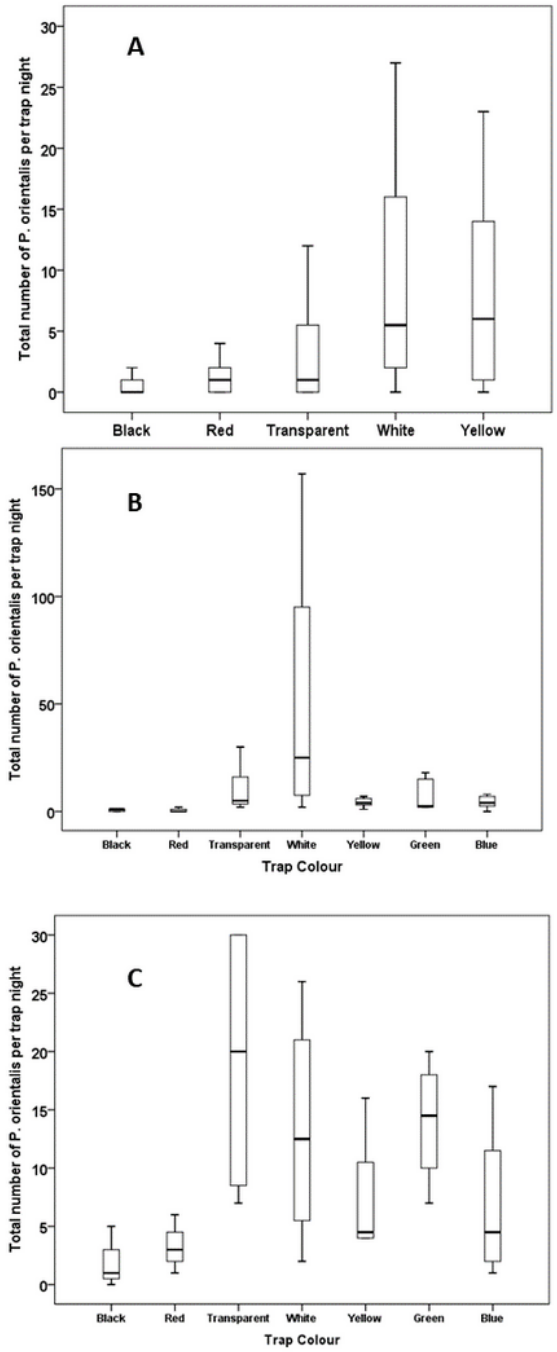

Fig:4:

Figure 4

Numbers of Phlebotomus orientalis sandflies captured on coloured sticky traps, during different lunar phases. Each trap night is the total number of male and female sandflies captured on 10 A4 size paper or transparent plastic sheet placed vertically on the ground, between 18:00 -06:00Hr at different microhabitats in Belo village, Rahad region, eastern Sudan. The figure is a boxplot with horizontal lines

representing the median. A: quarter lunar – half lunar nights (April 2016); B: Full-lunar nights (March-June 2017); C: Full dark Nights (April 2017).

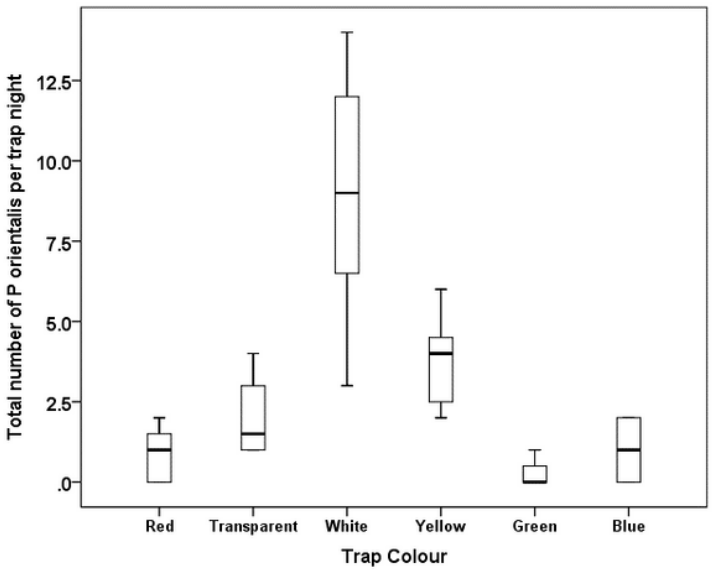

Fig.5

Figure 5

Numbers of Phlebotomus orientalis sandflies captured on horizontal coloured sticky traps, during full dark nights. Each trap night is the sandfly yield of 10 A4 size paper or transparent plastic sheet placed horizontally on the ground, between 18:00 -06:00Hr at different microhabitats in Belo village, Rahad region, eastern Sudan (March-June 2018). The figure is a boxplot with horizontal lines representing the median.

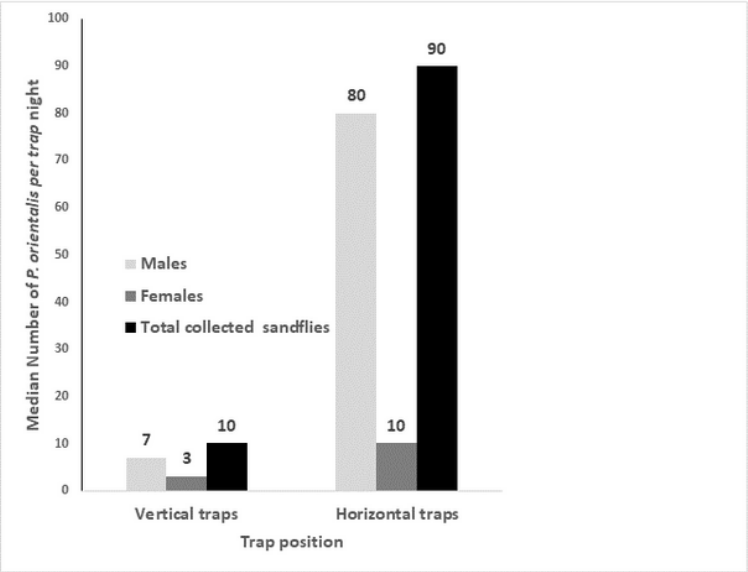

Fig.6

Figure 6

Comparison of sex ratios of Phlebotomus orientalis sandflies captured on vertical and horizontal sticky traps. Each trap night is the sandfly yield of 10 A4 size paper placed vertically or horizontally on the ground, between 18:00 -06:00Hr at the periphery of Belo village, Gedarif state, eastern Sudan (April 2017).

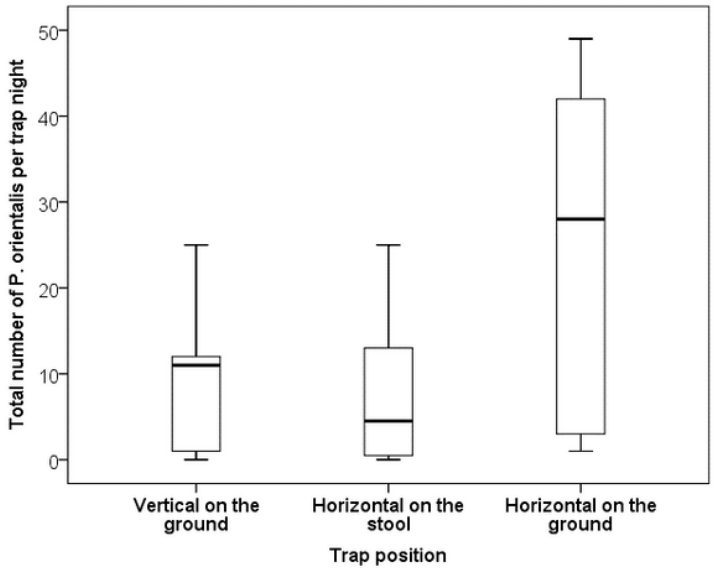

Fig. 7

Figure 7

Effects of spatial orientation and height on collections of P. orientalis sandflies by sticky traps. Experiment conducted in April 2018 in Belo village, eastern Sudan.

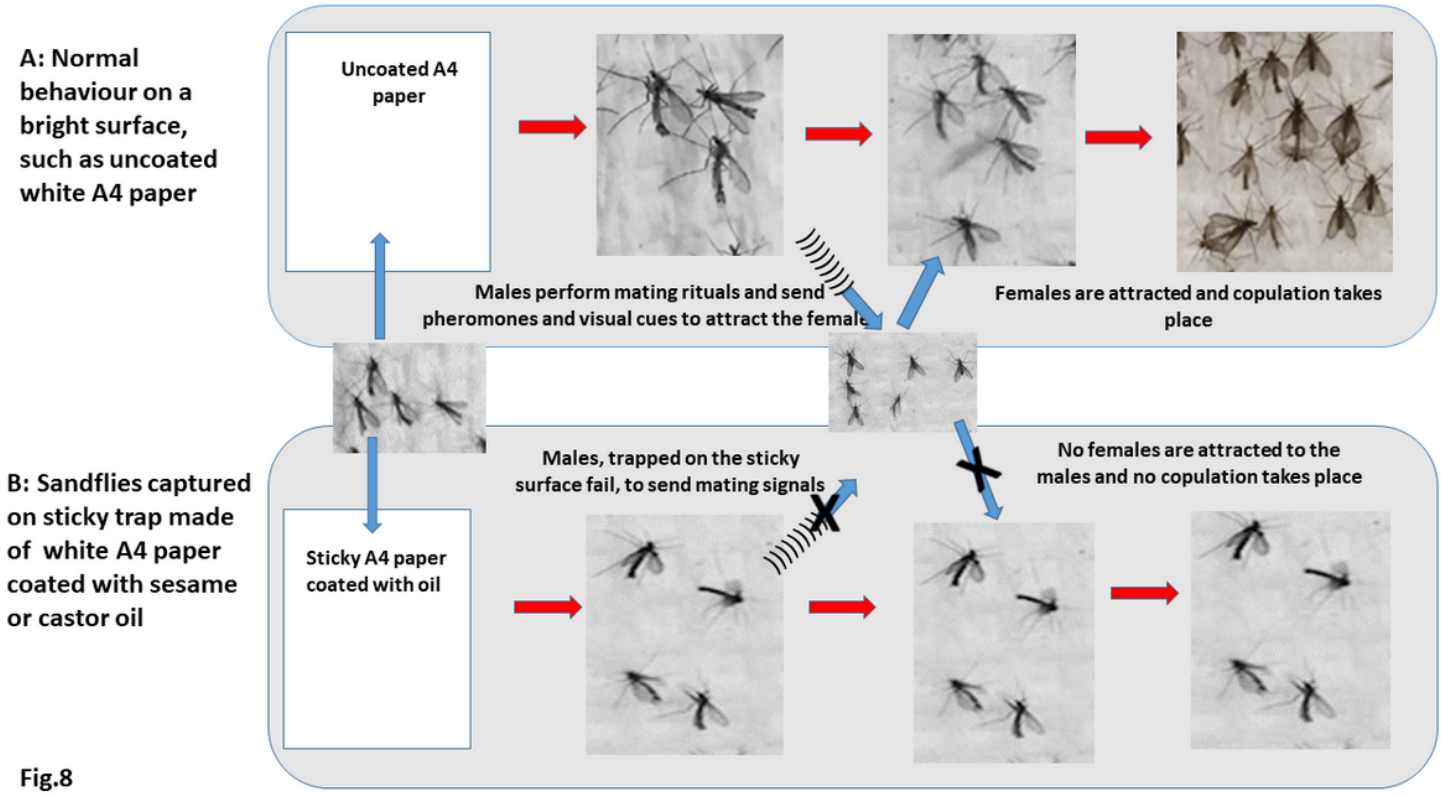

Fig.8

Figure 8

Hypothetical illustration of how white sticky traps capture Phlebotomus orientalis and why the sandfly collection by these traps consists of males mainly.