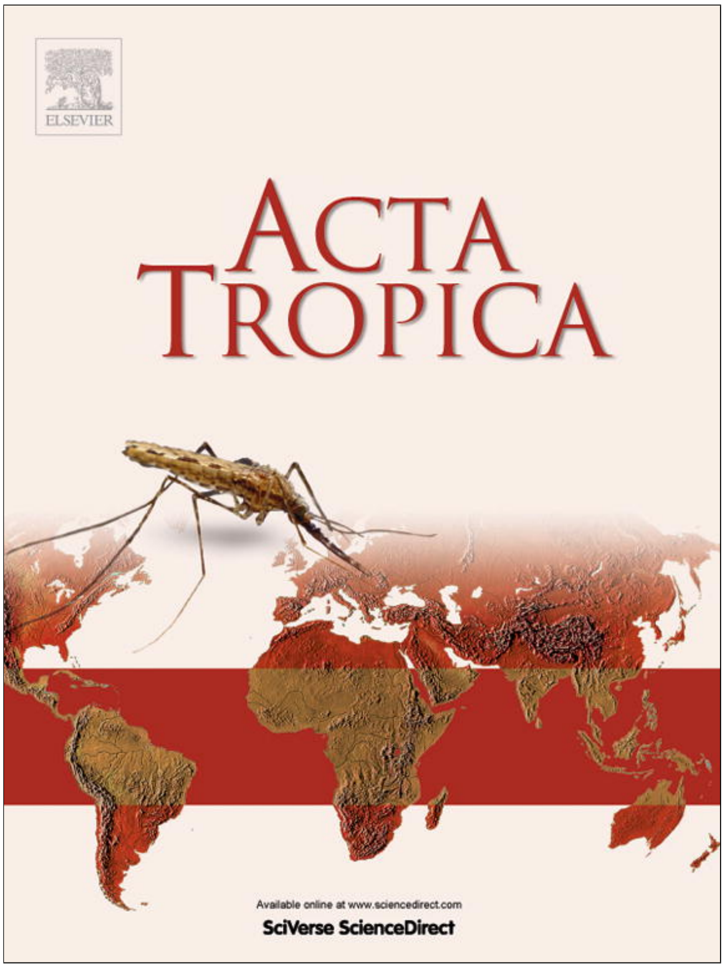
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Attraction of phlebotomine sand flies to baited and non-baited horizontal surfaces

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ABSTRACT

Female phlebotomine sand flies (Diptera: Psychodidae) transmit leishmaniasis as they engorge on vertebrate blood required for egg production. *Phlebotomus* (*Phlebotomus*) *papatasi* (*Scopoli, 1786*), the vector of *Leishmania major* (Yakimoff & Schokhor, 1914), the causative agent of cutaneous leishmaniasis (CL) were not attracted to large horizontal sticky traps (LHSTs) unless these were baited with CO_2 derived from dry ice or from fermenting sugar/yeast mixture (SYM). Attraction of *P. papatasi* males by CO_2 may indicate their tendency to mate on or near the blood-host. Male *P.* (*Larroussius*) *orientalis* (Parrot, 1936), the vector of visceral leishmaniasis (VL) in Ethiopia, were collected on LHSTs in large numbers. Although the number of females remained low, augmentation with SYM, increased the number of females by 800% while the number of males increased by only about 40%. Apparently, male *P. orientalis* utilize the horizontal surfaces for forming mating swarms. *P.* (*Paraphlebotomus*) *sergenti* (Parrot, 1917), is the vector of CL caused by *Leishmania tropica*. Although approximately twice as many *P. sergenti* males were caught on LHSTs as females, it appears that LHSTs were attractive to both sexes. Use of SYM baits is potentially useful for monitoring phlebotomine sand flies in places where dry ice is unobtainable or prohibitively expensive. LHSTs can provide an inexpensive alternative to CDC traps for monitoring some species of sand flies. Unfortunately, the numbers of female sand flies, crucial for estimating transmission of *Leishmania*, is usually low on LHSTs.

1. Introduction

Leishmaniasis endangers some 350 million people in 98 countries. Cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are the major clinical forms of the disease. CL manifests as a sore at the bite site of the infected sand fly and is usually self healing. VL, on the other hand, is a life-threatening systemic infection. An estimated 0.7–2.0 million cases of CL and 2–4 hundred thousand new cases of VL occur annually. Some 90% of global VL cases occur in the Indian sub-continent, East Africa and Brazil. CL is more widespread, with about one-third of cases occurring in the Mediterranean basin and the Middle East (Alvar et al., 2012; Desjeux, 2004). CL caused by *Leishmania tropica* and *Leishmania major* are emerging zoonotic diseases in Israel as well as other East Mediterranean countries (Jaffe et al., 2004; Postigo, 2010). VL, also known as Kala-Azar is considered an emerging disease in Ethiopia where it is frequently associated with HIV/AIDS, the leading cause of adult illness and death in that country (Anema and Ritmeijer, 2005; Hailu et al., 2007; Marlet et al., 2003). The causative agent of VL in the Indian subcontinent as well as East Africa is *Leishmania donovani* (Laveran & Mesnil, 1903) (Lukes et al., 2007).

The vectors of the leishmaniases are blood-sucking phlebotomine sand flies (Diptera: Psychodidae) belonging to two genera, *Phlebotomus* in the Old World, and *Lutzomyia* in the New World. There are some 700 known species of sand flies but only about 30 of those transmit leishmaniasis to humans. Like most hematophagous dipterans, female sand flies require vertebrate blood for egg production, and they transmit *Leishmania* during blood-feeding (Killick-Kendrick, 1999). The proven vector of *L. major*, a causative agent of CL in North Africa and the Middle East including Israel is *Phlebotomus papatasi* (Schlein et al., 1982b, 1984).

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L. tropica causes CL in urban anthroponotic foci as well as zoonotic foci in large parts of the Middle East (including Israel) and Asia. The most important vector of *L. tropica* throughout much of its distribution is *P. sergenti* (Alvar et al., 2012; Jacobson et al., 2003; Schnur et al., 2004). *Phlebotomus orientalis* is the proven vector of *L. donovani* in the extensive VL foci of Sudan and is most probably the vector in north Ethiopia as well (Elnaiem et al., 1998; Gebre-Michael et al., 2007; Hailu et al., 2007).

Sampling sand fly populations for assessing the risks of contracting leishmaniasis, is frequently accomplished using centers for disease control (CDC) miniature light traps with 6 V incandescent light bulbs. CDC traps are considered an especially effective trapping method when combined with a carbon dioxide (CO_2) source, which is particularly attractive to blood-questing females (Alexander, 2000).

Sticky traps are inexpensive and do not require electric power making them particularly valuable in remote regions of under-developed countries. They are thought to trap sand flies by passive interception rather than attraction and are, therefore, considered to yield unbiased samples (Alexander, 2000). Here we report on experiments using novel large horizontal sticky traps (LHSTs) with and without CO_2 or SYM, to study the bionomics of *P. papatasi* and *P. sergenti* in Israel and *P. orientalis* in Ethiopia.

2. Materials and methods

2.1. Study sites

Israel-Dead sea basin, Kibbutz Beit Ha'arava (31°59′ N, 31°46′ E), at 350 m below sea level. The Dead sea basin is an arid zone, with daily temperature that may reach 39 °C during the summer. The region belongs to the Saharo-Arabian phyto-geographical zone of desert vegetation infiltrated by plants of the Sudanese society (Zohary, 1973). The Dead sea basin is a well documented focus of CL caused by *L. major* and transmitted by *P. papatasi* (Jaffe et al., 2004; Schlein et al., 1984). Experiments were conducted on Marl soil, across a dry riverbed from date-palm plantation.

Kfar Adumim (31°49′ N, 35°20′ E) is a rural community located in the Judean Desert some 20 km east of Jerusalem (316 m above sea level). Climate is semiarid with 260 mm mean annual rainfall, and 20 °C mean annual temperature. Flora is predominated by perennial desert shrubs and annual grasses (Jaffe, 1988). The study area

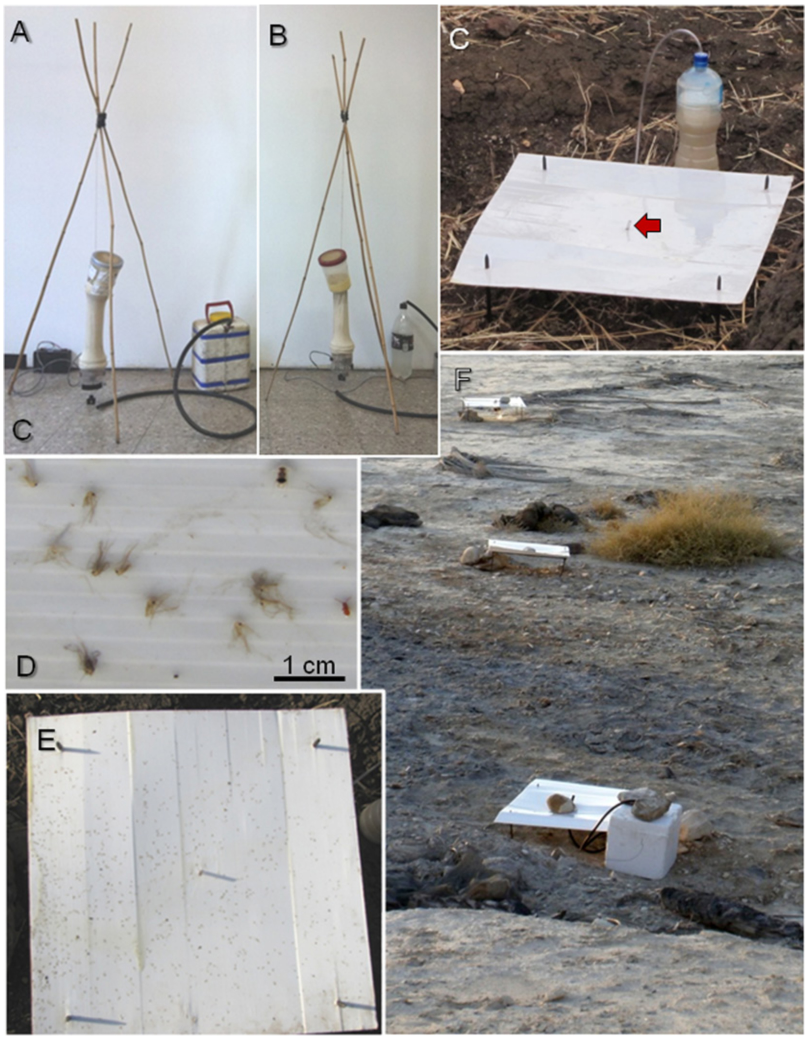

Fig. 1. (A, B) – CDC traps in the up-draft position with the opening some 10 cm above ground. (E) trap baited with dry ice inside poly styrofoam container. F – trap baited with SYM in a soft-drink bottle. (C) A large sticky trap (LHST) baited with SYM. Note the end of the emanation tube is protruding from the center of the white plastic board (arrow). The board is smeared with oil. (D) Phlebotomine sand flies (*Phlebotomus orientalis*) adhering to the oily surface of a LHST. Note the white background makes counting of sand flies relatively easy. (E) Hundreds of sand flies adhering to the oil on a LHST deployed over night in a fallow field in Ethiopia. (F) Experimental setup for testing LHSTs in the Jordan valley. The closest LHST is baited with dry ice (inside a poly Styrofoam box) as a source of CO_2.

was located on the rocky slopes to the south east of the village and in the gorge below. Caves and crevices serve as breeding sites for *P. sergenti*, the vector of *L. tropica* in Kfar Adumim (Moncaz et al., 2012; Schnur et al., 2004) and are inhabited by rock hyraxes (*Procavia capensis* [Pallas, 1766, Hyracoidea: Procaviidae]), the principal reservoir hosts of *L. tropica* in Israel (Svobodova et al., 2006; Talmi-Frank et al., 2010).

Ethiopia – Sheraro (14°24′09.69″ N–37°46′ 39.69″ E), a town in West-Northern Ethiopia, located in the Mi'irabawi Zone of the Tigray Region. The landform is composed of lowland plains with an elevation of ∼1028 m above sea level. The climate is generally sub-tropical-arid, with an extended dry period of nine to ten months and a maximum effective rainy season of 50–60 days. The rainfall pattern is predominantly uni-modal (June to early September) with a mean annual rainfall of ∼600 mm. The region experiences almost uniformly on average an annual temperature between 38 °C and 40 °C (http://www.ethiodemographyandhealth.org/Tigray.html). Experiments were conducted in fallow sorghum (*Sorghum bicolor* (L. Moench) fields characterized by deeply-cracked vertisols. Sparse remaining trees were mostly *Balanites aegiptica* (L. Delile, 1812), *Ziziphus spina christi* (L. Desf.), and *Acacia spp*. The entire district is recognized as an emerging focus of VL caused by *L. donovani* and transmitted by *P. orientalis* (Hailu et al., unpublished).

2.2. Traps

2.2.1. CDC traps

CDC Miniature light traps powered by 6 V batteries (John W Hock Co, Gainesville, FL), were positioned in an updraft orientation with the opening 10–15 cm above ground level to optimize their efficiency (Faiman et al., 2009). The traps were baited with either dry ice kept in an insulated container (Fig. 1E) or a mixture of bakers' yeast and sugar in a aqueous solution (Fig. 1A and B). Because of the nature of the terrain which is rocky with many vertical surfaces, CDC light traps in the Judean Desert were deployed down-draft with the opening about 40 cm above ground.

2.2.2. Large horizontal sticky traps (LHSTs)

Comprised white polypropylene boards (60 cm × 60 cm, Polygal Israel, http://www.polygal.co.il/) placed horizontally on square metal frames with short legs supporting the boards approximately 15 cm above ground. Preliminary studies showed that hardly any sand flies adhered to the bottom of sticky traps (Moncaz & Warburg, unpublished). Therefore, for the experiments described here, only the top sides of the boards were smeared with castor oil (Israel) or sesame oil (Ethiopia). The sand fly trapping efficiency of LHSTs with and without attractants was compared (Fig. 1C–F).

2.2.3. Baits

2.2.3.1. Dry ice. Stored in insulated containers. Sublimated CO_2 flowed through a 6 mm tube to the trap opening (CDC trap, Fig. 1A) or released above the surface of LHSTs (Fig. 1C). Dry ice was used only in experiments conducted in Israel since it was not available in the field site in Ethiopia.

2.2.4. Sugar/yeast mixture (SYM)

150 g food-grade sugar (sucrose) were dissolved in 1 L tap-water inside a 1.5 L plastic bottle. Fifteen grams (15 g) of dry bakers' yeast were added and mixed well shortly before deployment of the traps (Fig. 1B and C). CO_2 produced by fermentation, comprising mostly CO_2 mixed with putative additional attractants, flowed through a tube and was released close to the surface of the sticky trap.

2.3. Identification of sand flies

Sand flies captured using sticky traps were washed in 1% detergent solution and water to remove the oil and preserved in 70% ethanol. In the laboratory sand flies were mounted on microscope slides in Hoyer's mounting medium and identified based on the morphology of the pharynx and spermatheca of females and the external genitalia of males (Artemiev, 1980; Lewis, 1982; Perfil'ev, 1968).

2.4. Statistical methods

The numbers of sand flies trapped were tested for normality by 1-Sample Kolmogorov–Smirnov *Z* test (K–S). Thereafter, mean (±SE) trap yields were compared using 2- sample *t* test for data complying with normal distribution. Otherwise, Mann Whitney rank sum test was applied. One way ANOVA analysis was used to evaluate difference between lure supplemented LHSTs in the tests conducted at the Dead sea basin. All statistical analyses were carried out on GraphPad PRISM®, version 5 (San Diego, CA).

3. Results

3.1. Production of gasses by sugar/yeast mixture (SYM)

An experiment was conducted to determine the amount of gas (mostly CO_2) released by 1 L of SYM at 30 °C. The emitted CO_2 was allowed to flow into an inverted, graduated cylinder displacing a column of water. The volume was measured and the cylinder was refilled with water every hour. CO_2 production stabilized 2 h after preparation, and remained more or less constant mean = 1016 ± 96 ml/h for at least 11 h (Fig. 2). Derived from these results we estimate an a total of around 10 liters of CO_2 produced during the 10 h of darkness.

3.2. Production of CO_2 from dry ice kept in insulated poly-styrofoam containers (used for LHSTs)

To estimate the amount of CO_2 produced, the container with dry ice was weighed in the evening just before the traps were set and once again during collection some 10 h later. On average, approximately 30 g/h CO_2 were discharged (n = 12). Assuming ideal gas molar volume of 22.45 L and that the emissions comprised almost entirely CO_2 (MW 44 g/mol), this volume represents 15.3 L/h or 153 during the night.

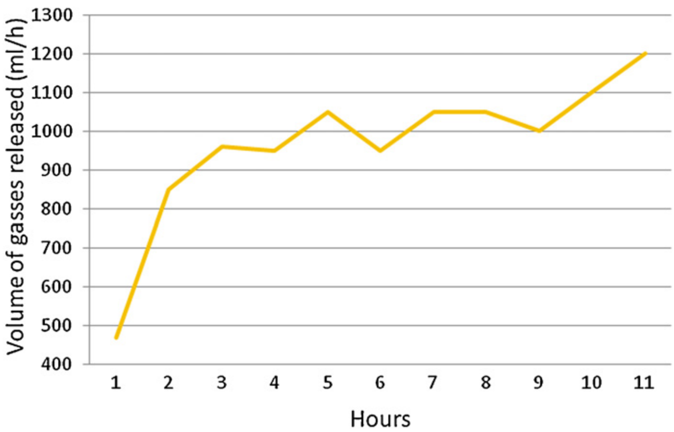

Fig. 2. The volume of gas emitted by a mixture of 150 g sugar and 15 g dry bakers' yeast mixture in 1 L of water at 30 °C. The rates of emissions stabilized after approximately 2–3 h producing 800–1200 ml of gas per hour.

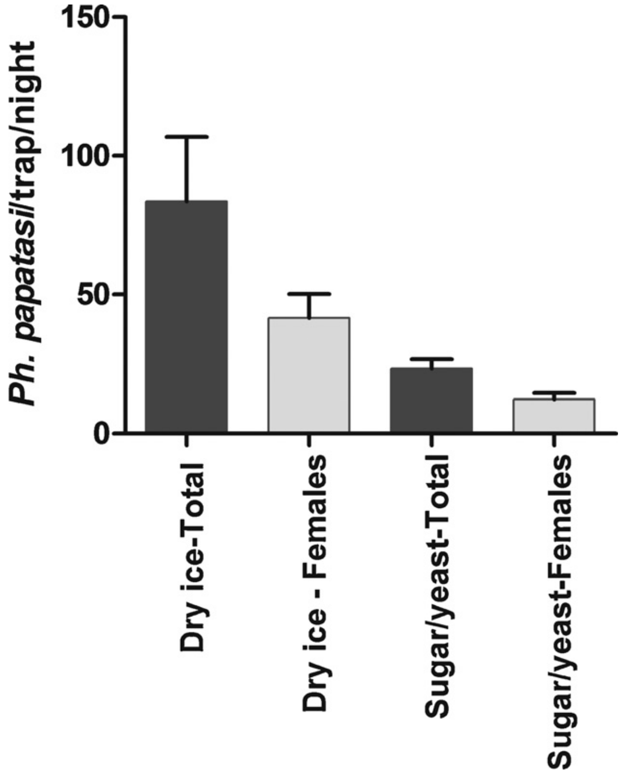

Fig. 3. *Phlebotomus papatasi* sand flies captured by baited CDC traps. The 2 columns on the left represent the catch of CO_2-baited traps while the 2 columns on the right represent catches of traps baited with emanations from SYM (16 trap nights each). The proportions of males and females captured was approximately equal in both types of attractants. The dry ice produced approximately 66 L CO_2/h while the SYM produced approximately 1 L CO_2/h.

3.3. CO_2-baited traps, Dead sea

To obtain a baseline comparison between dry-ice and SYM, we evaluated the efficacy of CDC traps with these baits for trapping sand flies. Four CDC traps were supplemented with dry ice and four with SYM. Traps were positioned 10 m apart, left overnight (approximately 10 h) and sand flies were collected the subsequent morning. In these experiments the dry ice containers used were different from the ones described above. Approximately 130 g CO_2/h (=66.3 L/h) were released near dry-ice baited traps.

A total of 521 sand flies were trapped by eight CDC traps. Of these, 389 (75%) were trapped in dry ice-baited traps (4trap/nights, mean = 97 sand flies/trap), while 132 sand flies (25%) were trapped in SYM-baited traps (4 trap nights, mean = 33 sand flies/trap). The predominant species in all catches was *P. papatasi*; 86% of the sand flies trapped in dry ice-baited traps, and 70% of sand flies trapped in SYM-baited traps. Male/female ratio among the trapped *P. papatasi* was approximately 1:1 (166/168 in dry ice and 49/44 in SYM-baited traps) (Fig. 3).

3.4. Large horizontal sticky traps (LHSTs), Dead sea

A total of 2387 sand flies was trapped during six nights. Of these, 2232 (94%) were *P. papatasi* while the rest comprised *Sergentomyia* spp. Un-baited LHSTs trapped very few sand flies (2.4 ± 3.1 *P. papatasi*/trap/night). LHSTs baited with SYM trapped significantly more (108 ± 28 *P. papatasi*/trap/night, $P < 0.05$). Dry ice-baited LHSTs were more efficient, trapping an average of 250 ± 47 *P. papatasi*/trap/night. The differences in trapping efficiency between the three trap-types were significant (Fig. 4, $P < 0.05$).

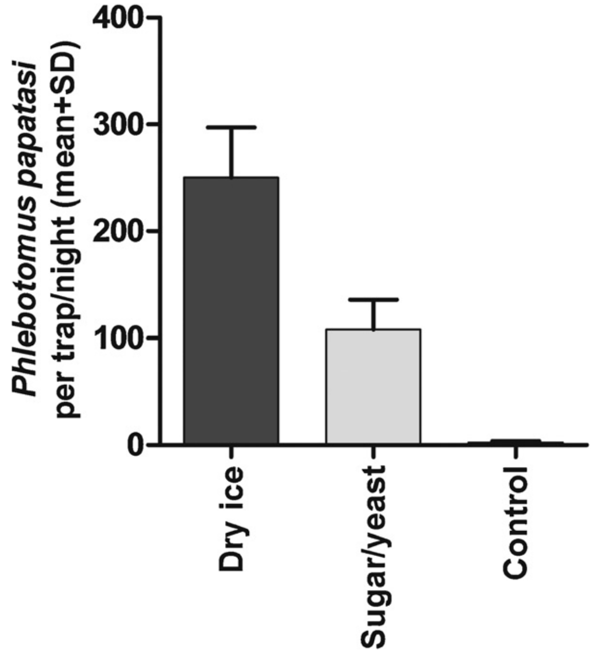

Fig. 4. *Phlebotomus papatasi* sand flies captured by LHSTs near the Dead sea. CO_2 (dry ice)-baited LHSTs (left bar) trapped significantly more sand flies than SYM-baited LHSTs (central bar). Both of the baited LHSTs trapped more sand flies than control non-baited LHSTs (left bar).

As demonstrated using baited-CDC traps, both CO_2 and SYM were attractive to male and female *P. papatasi*. Dry ice-baited LHSTs captured 64% females while SYM-baited LHSTs trapped 67% females. The deviation from 1:1 ratios were not statistically significant ($P = 0.12$ for SYM baited LHSTs, and $P = 0.08$ for dry ice-baited LHSTs). A total of eight male and 7 female *P. papatasi* were trapped during 6 trap/nights in non-baited LHSTs.

3.5. LHSTs, Judean Desert

Non-baited LHSTs were deployed on a rocky slope close to cave openings near Kfar Adumim. A total of 1400 *P. sergenti* were trapped during 32 trap/nights (mean = 127.3 trap/night). Of these, 920 (66%) were male and 480 (34%) were female. The proportions of male to female sand flies was significantly different from 1:1 (*t* test, $P = 0.0012$) (Fig. 5).

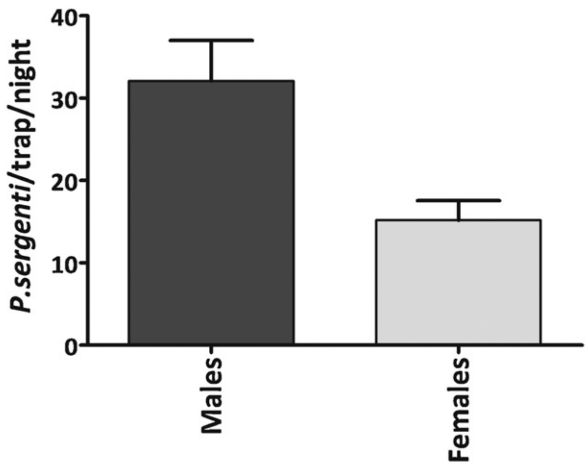

Fig. 5. *Phlebotomus sergenti* sand flies captured by non-baited LHSTs in the Judean desert. LHSTs trapped significantly more males (left bar) than females (right bar).

Table 1
Comparison between non-baited (unshaded cells) and sugar/yeast mixture (SYM)-baited (shaded cells) LHSTs. Experiments conducted in open fallow fields in the Sheraro district of Northern Ethiopia.

	Total phlebotominae		Phlebotomus orientalis	
	Mean ± SD non-baited LHSTs	Mean (%increase) SYM baited LHSTs	Mean ± SD non-baited LHSTs	Mean (%increase) SYM baited LHSTs
Male	65.0	65.0(0)[a]	41.9	55.9(33.4)
Female	8.1	15.0(85.2)[b]	1.0	9.0(800)
Total	73.1	80.0 (9.4)	42.9	63.9(48.9)

[a] The numbers of *Sergentomyia* males decreased.
[b] Increase attributable to Sergentomyia clydei and Sergentomyia schwetzi females.

3.6. CDC light traps, Judean Desert

Six CDC light traps with incandescent bulbs were deployed near cave capturing a total of 1372 sand flies (mean of 229/trap/night [175 males and 54 females]). The proportions of male to female sand flies were significantly different from 1:1 (paired *t* test, $P = 0.001$) (data not shown).

3.7. LHSTs, Ethiopia (Sheraro)

A total of 2064 sand flies were trapped on LHSTs. Of these, 1452 (70.3%) were *P. orientalis*. The balance was made up of *Sergentomyia spp.* and rare specimens of *P. rodhaini Parrot, 1930. P. heischi* (Kirk and Lewis,1950) and a single specimen of *P. elgonensis* (Ngoka, Madel & Mutinga, 1975). Non-baited LHSTs captured an average of 43 *P. orientalis* per trap per night (13 trap/nights). LHSTs baited with SYM captured an average of 64 *P. orientalis* per trap per night (14 trap/nights). Female *P. orientalis* were captured only in small numbers. However, significantly more females were trapped on SYM-baited LHSTs (mean of 9 *P. orientalis* females per trap per night) than on non-baited LHSTs (mean of 1 *P. orientalis* female per trap per night) (Table 1).

4. Discussion

CO_2 baited CDC traps are the gold standard for trapping mosquitoes and phlebotomine sand flies (Alexander, 2000; Hoel et al., 2010; Pinto et al., 2001; Silver, 2007). Since emission of CO_2 imitates the emanations from potential hosts, mostly the blood-feeding females are attracted to CO_2-baited traps (Guerenstein and Hildebrand, 2008; Orshan et al., 2010; Silver, 2007). However, the males of certain mosquito, as well as sand fly species, are attracted to CO_2 as well, presumably because mating occurs on or close to the host (Alexander, 2000; Garcia et al., 1989).

Males of *P. argentipes* (Annandale & Brunetti, 1908), the vector of VL in the Indian sub-continent and *Lutzomyia (Lutzomyia) longipalpis* (Lutz & Neiva, 1912), the vector of VL in Latin America, were shown to actively protect an area of skin of the blood-host and mate with females that come to feed. This behavioral ritual is termed lekking (Jarvis and Rutledge, 1992; Lane et al., 1990). In addition, it is not uncommon for male sand flies to approach blood-hosts remaining on plants or rocks in their vicinity, mating with females that approach the host or leave it having engorged on blood (Ashford, 1974; Miles and Foster, 1976).

In our experiments, CO_2 and was equally attractive to male and female *P. papatasi* using either CDC traps or LHSTs. Although absolute numbers were smaller, the same was true of SYM baited traps as well (Figs. 3 and 4). In all likelihood, the superiority of the dry ice over SYM stems from the fact that dry ice containers released 66.2 L/h CO_2, which is almost two orders of magnitude more than the 1.02 L/h CO_2 released from SYM bottles.

Non-baited LHSTs were not attractive to either female or male *P. papatasi*. The mating behavior of this species has not been studied directly. However, courtship rituals were studied showing that unlike *Lu. longipalpis, P. papatasi* males do not demarcate nor patrol lekking patches (Chelbi et al., 2012). On the other hand, it has frequently been noted that males are attracted to CO_2 (Beavers et al., 2004) or potential hosts (Schlein et al., 1982a). Moreover, male *P. papatasi* are frequently found resting in-doors in close proximity to blood-fed females (Sirak-Wizeman et al., 2008; Srinivasan et al., 1993). In addition, large numbers of *P. papatasi* including many males were attracted to turkey sheds in the Jordan Valley. Based on these observations, one may assume that mating occurs on or in close proximity to the host

Richard Ashford, an entomologist working in Ethiopia, observed that rather large numbers of male *P. orientalis* routinely landed upon his open notebook, and that occasional females that landed amidst the many males would rapidly be engaged in copulation. He deduced that *P. orientalis* males were using the horizontal surface of his open note-book to establish mating swarms (Ashford, 1974). Our results from Ethiopia show that *P. orientalis* males are attracted to [horizontal] LHSTs even when those are not baited (Table 1). The locations where these experiments were conducted were in open fallow fields of black-cotton soil (vertisol), with no rocks and very few trees. There were no objects that resemble LHSTs to explain where the *P. orientalis* males congregate on those nights when our traps are not there. Nevertheless, we presume that the LHSTs represent suitable venues for mating swarms of *P. orientalis* males but that females arrive there only when attracted by SYM and even so, only in small numbers (Table 1). Thus, horizontal LHSTs can be very useful for monitoring *P. orientalis* populations but not for trapping large number of females' even when baited with SYM. Interestingly, a similar male congregation was observed in Israel when a single LHST deployed in a fallow field in central Israel, trapped 50 male *P. (Larroussius) tobbi* (Adler and Theodor, 1930) and no females (Moncaz and Warburg–unpublished). Both *P. orientalis* and *P. tobbi* belong to the subgenus *P. Larroussius* perhaps explaining their similar behavior.

Non-baited LHSTs were used to trap *P. sergenti*, the vector of *L. tropica*. Results indicate a third behavioral mode of this species with regards to LHSTs. Both sexes of *P. sergenti* were attracted to LHSTs albeit twice as many males than females were trapped (Fig. 5). Unlike the field site in Ethiopia, The one near Kfar Adumim is a rocky slope with many light-colored rocks and stones. Therefore, one may hypothesize that males utilize such surfaces for swarms and or a different behavior associated with courting and/or mating. In fact, one doesn't need to assume that the LHSTs are very attractive because these slopes contain breeding habitats of *P. sergenti* and the density of sand flies in these habitats is exceptionally high (Moncaz et al., 2012; Orshan, 2011). This hypothesis is backed by the finding that large numbers of *P. sergenti* and similar male/female ratios were also obtained using CDC light traps. Thus use of LHSTs can replace light traps in situations where electricity or availability of expensive traps is a constraint (Reza and Mansour, 2006).

Carbon dioxide (CO_2) produced by yeast fermentation, has been studied under controlled conditions in the laboratory and utilized successfully for monitoring mosquitoes in the field (Saitoh et al., 2004; Smallegange et al., 2010). CO_2, is very attractive to female sand flies (Alexander, 2000; Beavers et al., 2004; Orshan, 2011). Experiments reported here document a significant increase in the numbers of *P. orientalis* (both males and females) captured in SYM-baited LHSTs as compared with un-baited control LHSTs (Table 1). Moreover, preliminary data indicates that SYM-baited CDC traps capture significantly more *P. orientalis* females than light traps (Kirstein, un-published). Both sugar and bakers' yeast are available almost anywhere in the world where people bake bread. The cost of these ingredients is negligible in the context of scientific research and their deployment as an attractive mix requires no more than empty plastic bottles, flexible tubes and some water. Therefore, SYM baited CDC traps or LHSTs, can be a practical solution for monitoring sand flies in areas where dry ice is not available.

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