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Laboratory and semi-field evaluation of long-lasting insecticidal nets against leishmaniasis vector, *Phlebotomus* (*Phlebotomus*) *duboscqi* in Kenya

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Abstract

Background & objectives: Phlebotomine sandflies are vectors of leishmaniases and other diseases. Long-lasting insecticidal nets (LLINs) as possible tools for control have not been widely tested against them. The objective of this study was to determine the efficacy of Olyset® Net and PermaNet® LLINs alongside a local brand, K-O Tab® treated net (Supanet) against *Phlebotomus duboscqi* female sandflies.

Methods: Four replicates of unwashed and 20x washed Olyset Nets and PermaNets, K-O Tab-treated and untreated Supanet and 'no net' treatments were evaluated against sandflies within the laboratory by tunnel tests and in semi-field conditions in the greenhouse model for their efficacy.

Results: All bednets allowed entry of *P. duboscqi* sandflies and subsequent blood-feeding. Olyset net's blood feeding inhibition was significantly higher than that of Supanet in the laboratory but not in semi-field condition. Of the LLINs, only Olyset net had sandflies that could not feed significantly more than those of Supanet. Additionally, no significant efficacy difference was observed between LLINs washed 20x and unwashed ones. The only significant difference noted in number of sandflies that were found dead or paralyzed within bednets in the semi-field condition was between Olyset and K-O Tab treated Supanet. In the laboratory, unwashed Olyset had a significantly higher number of sandflies killed than all other bednet treatments.

Conclusion: Olyset net use in areas where sandflies are nuisance biters and/or disease vectors could be more beneficial in preventing sandfly bites than other tested bednets. It is recommended that mesh sizes of LLINs should be smaller for control of sandflies than those used for control of mosquitoes.

Key words Blood-feeding inhibition; Olyset® Net; PermaNet®; *Phlebotomus duboscqi*; Supanet®

Introduction

In Kenya, phlebotomine sandflies transmit visceral and cutaneous leishmaniases. Visceral leishmaniasis (VL), caused by *Leishmania donovani* is transmitted by *Phlebotomus martini* (Diptera: Psychodidae)[1]. *Phlebotomus duboscqi* sandflies transmit *L. major,* one of the causative agents of cutaneous leishmaniasis (CL)[2]. Many cases of leishmaniases still go unreported or undiagnosed, hence, the official statistics are currently not available for determining the actual number of cases[3].

The use of insecticide-treated nets (ITNs) may represent the most sustainable method of reducing intradomiciliary transmission of *Leishmania* in communities surrounded by forests, where diurnal resting sites of vectors are unknown or inaccessible[4]. Insecticide-treated nets act as 'baited traps' in which sandflies are attracted to exhaled carbon dioxide and host odour, and die after alighting on treated surfaces. They have been evaluated against phlebotomine sandflies in several countries including Italy[5], Burkina Faso[6], Sudan[7], Syria[8] and Kenya[9]. Insecticide-treated nets were shown to reduce cutaneous leishmaniasis transmitted by *P. sergenti* in Syria, Afghanistan and Turkey[10–12]. Moreover, VL cases in eastern Sudan reduced by ~27% after mass distribution programme of polyester ITNs of mesh size 25 holes/sq cm[13]. ITNs are occasionally provided by health ministries to protect populations at risk of CL in some Latin American localities where transmission occur in domestic environment, though the effectiveness of such strategies has rarely been evaluated[14].

Despite the fact that long-lasting insecticidal nets have been developed with treatments as well as insecticidal effects more evenly distributed than ITNs, they have not been widely tested against sandflies. However, they were found successful against malaria transmitting mosquitoes[15–19]. Though the degree of contact that sandflies may make with a treated surface is greater than that of mosquitoes[4], hence, increasing their susceptibility to contact insecticides, there is limited information on the efficacy of long-lasting nets in sandfly control[4,20].

In this study, the efficacy of two brands of LLINs *vs* K-O Tab® treated and untreated bednets was tested against 2–3 day old female *P. duboscqi,* the vector for *L. major* in Kenya both in the laboratory and semi-field conditions. Differences in feeding success and survival of laboratory-reared sandflies that contacted treated bednets were determined.

Material & Methods

Laboratory studies: Experiments were conducted at the Centre for Biotechnology Research and Development (CBRD) laboratory of Kenya Medical Research Institute (KEMRI) from April to August 2007. *Phlebotomus duboscqi* sandflies were obtained from the sandfly colony maintained at the centre. The basic design of this experiment was based on that of World Health Organization Pesticide Evaluation Scheme with some modifications[21]. Experiments were carried out in the laboratory within tunnels constructed from glass cages with plaster of paris on their bases. Two such cages, one measuring 25 cm (width) x 25 cm (height) x 40 cm (length) and another 25 x 25 x 20 cm, were joined on their open ends with an adhesive tape to form a tunnel measuring 25 x 25 x 60 cm. Before tapping, a disposable cardboard frame with a bednet material was fitted in between the cages. The surface of the netting material 'available' to the sandflies was 400 cm^2.

The test bednet materials used were purchased from local stores and distributors. They included: Deltamethrin-treated PermaNet® (Vestergaard Frandsen, Denmark) of mesh size 25 holes/cm^2 and active ingredient of 55 mg/m^2, Permethrin-treated Olyset® Nets (Sumitomo Chemical Co. Ltd, Japan) of mesh size 9 holes/cm^2 and 1000 mg/m^2 of active ingredient and Supanet® (Deltamethrin K-O Tab® treated local bednet brand) of mesh size 25 holes/cm^2 and a target active ingredient concentration of 25 mg/m^2. Olyset and PermaNet bednets vigorously washed by hand 20 times and dried as recommended by manufacturers were also included[22]. Olyset nets were allowed over 15 days for insecticide regeneration[23] before use in the experiments. A non-treated Supanet® bednet and a 'no net' (without net fabric between the two sections of the tunnel) were used as controls.

In the shorter section of the tunnel, a restrained hamster acting as bait (host) was placed. Through the other end of the longer section of the tunnel, a total of one hundred 2–3 days old sugar and blood-starved *P. duboscqi* females were released at 0800 hrs[21]. They were free to fly in the tunnel but had to make contact with the piece of netting and locate the holes in it before passing through to reach the bait. Following their release in the tunnel, hungry sandfly probing ability was monitored during the first one hour using a video camera. After the second hour, sandflies were picked and counted separately from each section of the tunnel and immediate mortality was scored. Live females were placed in plastic cups supplied with sugar solution and delayed mortality was scored after 4, 6 and 24 h. During feeding success determination, only flies on the bait side of the tunnel were considered.

During tests, the cages were maintained at 27°C and 80–95% relative humidity. Blood feeding and inhibition was assessed by comparing the proportion of blood-fed females (both alive and dead) in treated and control tunnels. Overall mortality was calculated by pooling immediate and delayed mortalities of sandflies from the two sections of the tunnel. Each type of netting material was replicated four times using bednet pieces from four different bednets.

Semi-field studies: These studies were conducted in the greenhouse model Sapphire 960-4.25 m gutter height (Azrom Greenhouses, Israel) from November 2007 to June 2008. The structure together with its associated weather parameter measuring equipments have been described previously[24]. Before commencement of experiments, standard CDC light-traps (John W. Hock, TX, U.S.A.) were set up inside for two consecutive nights to ascertain sandfly absence.

Same bednet brands (total of six) that were used in the laboratory studies were tested in the greenhouse in this study. Untreated Supanet was used as a control. Labelled goats of about the same age and weight were caged inside each of the six bednets as they were most attractive to *P. duboscqi* sandflies in an animal bait comparative study[24]. Their use was approved by the Animal Care and Use Committee (ACUC) of Kenya Medical Research Institute.

In the greenhouse, bednets were suspended from the inner frame of a portable sun shelter. Top of bednet was joined with a white cotton sheet forming the roof of the sun shelter. Caged goats in bednets were placed on a white sheet of cloth on the floor on top of plastic sheets. The six bednets were placed 3.4 m from each other in Latin square design and rotated on each experimental day in clockwise direction to avoid bias due to position. The goats were rotated in anticlockwise direction to ensure each goat slept under all the bednets at the end of the rotation. After a full rotation of bednets through the six positions in six days, a different set of same nets was introduced for another rotation until four sets of each bednet type had been tested.

On every experimental day, 500 starved insectary-bred female *P. duboscqi* sandflies raised in a nearby insectary were released at the centre of the design 3.2 m from each bednet at 1800 hrs. To differentiate sandflies used on various days, they were dusted with fluorescent dyes (Day-glo colour Corp. Cleveland, Ohio, U.S.A.) of different colours. At 0600 hrs the following day, sandflies captured within and without the bednets were scored as follows; dead outside the bednet, alive outside the bednet, dead inside the bednet and alive inside the bednet. Only sandflies marked with dye colour used the previous day were scored.

To determine whether the sandflies were dead or not, they were observed for any movements under a dissection microscope. They were visually distinguished as to whether they had blood-fed or not. Blood feeding rate was used to determine the feeding success. All sandflies collected alive inside the bednets were supplied with sugar solution soaked in cotton in a moist environment. These were initially monitored for 1 h then over another period of 11 h for determination of mortality rates.

Experiments were conducted under the following environmental conditions: temperature and relative humidity conditions inside and outside the greenhouse during the experimental nights were as follows: temperature outside—range 18.71–32.34°C, mean 24.01°C; temperature inside—range 15.62– 31.52°C, mean 21.97°C; relative humidity outside—range 20.5–90%, mean 61.41%, relative humidity inside—range 20.9–98%, mean 70.84%.

Data management: Data were entered in MS Excel and thereafter imported into STATA 9.2, (STATACORP, TX, U.S.A.) for analysis. Results of feeding success through the various bednets were compared using Kruskal-Wallis to assess the sandfly exclusion efficacy/blood feeding inhibition of a given type of bednet. Numbers of *P. duboscqi* sandflies that fed through pairs of individual bednets were compared by Wilcoxon rank-sum test.

Results

Laboratory studies

Blood-feeding rate of P. duboscqi sandflies in different bednets: Only sandflies found on the host side of the tunnel were considered. The numbers that fed under bednet brands differed significantly (χ^2 = 18.732, p <0.05). The 'no net' tunnels had the highest number of sandflies that were blood-fed and this was significant when compared with each of the rest of the treatments (p <0.05) (Fig. 1). The number of sandflies that were blood-fed in PermaNet and Olyset net types did not differ significantly (z = –0.155, p >0.05) and 20x washed LLINs did not lead to significant increase in blood feeding by sandflies (PermaNet: z = –1.191, p >0.05; Olyset net: z = –0.588, p >0.05). The Olyset net allowed significantly lower rate of blood feeding than conventionally K-O Tab-treated Supanet, (z = 2.071, p <0.05) but not PermaNet (z = 1.183; p >0.05). The difference between treated and untreated Supanet was satistically significant (z = –2.021, p <0.05).

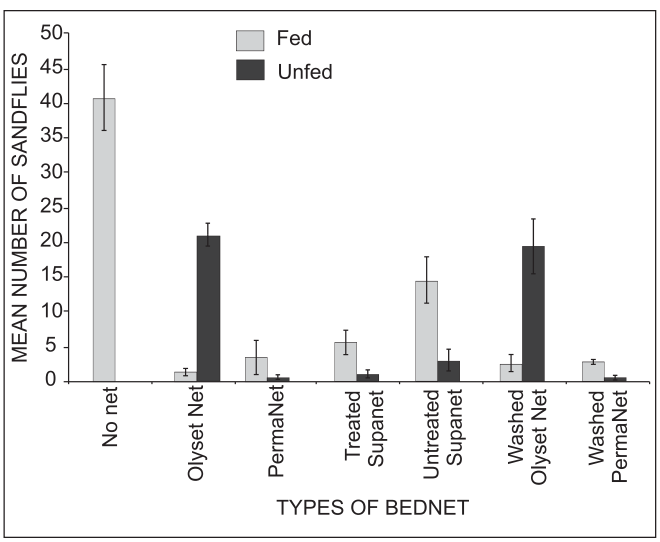

Fig. 1: The number (mean ± SE) of fed and unfed sandflies found within the bednets and 'no net' control in the laboratory

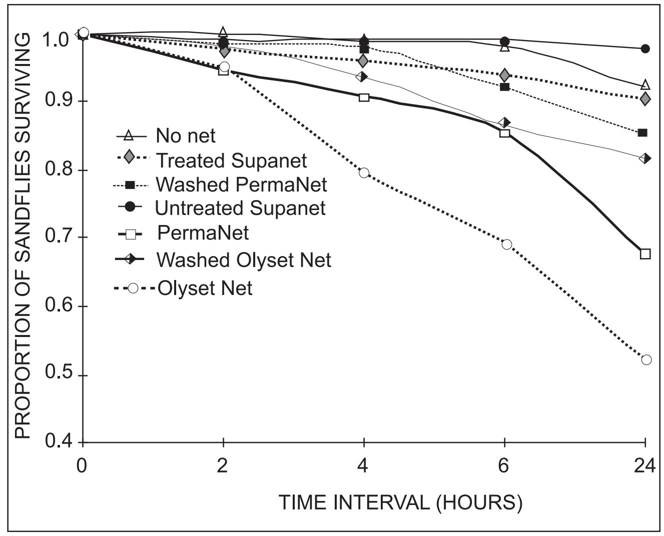

Fig. 2: Survival curves of total number of sandflies found inside and outside bednets in laboratory experiments

The numbers of unfed sandflies were significantly different in all bednet treatments (χ^2 = 20.252, p <0.05) (Fig. 1). The number of unfed sandflies was significantly higher in Olyset nets than that of all others (p <0.05) except washed Olyset net. There were no differences between the rest of the other bednet combinations. PermaNet had the lowest number of unfed sandflies.

Survival analysis, mortality rates and paralysis: A log rank test of homogeneity by bednet type revealed significant differences in survival rates of all sandflies (those that crossed the net barrier plus those that did not) over 24 h in tested bednets (p <0.05). When only Olyset and PermaNet bednets were compared, the difference in the rates was still significant (p <0.001). The lowest proportion of sandflies survived was in Olyset net (Fig. 2). The same case was observed when only flies that crossed the net barrier were compared except the differences between unwashed and washed PermaNet which were insignificant (p >0.05).

Table 1. Mortality rates, mean ± SE of dead and paralyzed sandflies found inside and outside of different bednets after 24 h in the laboratory

Type of bednet	Mortality rate (%)*		No. of sandflies within			No. of sandflies without		
	Sandflies within	Sandflies within + Sandflies without	Dead	Paralyzed	Total	Dead	Paralyzed	Total
Olyset net	53.4	47.75	11.7± 2.9	13.25 ±5.2	25± 6.4	36±10.8	33±10.6	69±10.1
Washed Olyset net	17.52	18.25	4.25±3.1	15.5±3.4	19.7±5.4	14±4.8	32.25±5.5	46.25±2.3
PermaNet	6.25	32.25	0.25±0.2	0	0.25±0.2	32±9.2	22.25±2.9	54.25±11.3
Washed PermaNet	7.69	14.5	0.25±0.2	0	0.25±0.2	14.5±6.2	6±4.7	20.5±10.3
Treated Supanet	7.69	9.75	0.5±0.5	0	0.5±0.5	9.25±3.6	4±2.1	13.25±3.7
Untreated Supanet	6.66	2.25	0.5±0.5	0	0.5±0.5	1.75±0.8	0	1.75±0.8

*Mortality rates for sandflies within and without bednets were not calculated for ‘no net’ owing to lack of net barrier to distinguish the two sides.

Table 1 shows mortality rates and number of dead and paralyzed sandflies found outside and inside the different bednets after 24 h in the laboratory. The lowest rates were observed in untreated Supanet whereas the highest were in Olyset nets. Numbers of dead and paralyzed sandflies were significantly higher in unwashed Olyset net than in unwashed PermaNet (p <0.05). Similarly these number of sandflies in washed Olyset net was significantly higher than in unwashed PermaNet (z = –2.366, p <0.05) and treated Supanet (z = –2.366, p <0.05). There was no difference in number of dead and paralyzed sandflies between the washed and unwashed LLINs (PermaNet: p >0.05; Olyset net: p >0.05). The same result was observed between treated and untreated Supanet (p >0.05).

Prevention of the flies entering through the net was estimated by the mortality and paralysis in the chamber outside the net. Both numbers of dead and sum of dead and paralyzed sandflies differed significantly among the bednets (Dead: χ^2 = 2.205, p <0.05, Sum: χ^2 = 18.510, p <0.05). Unwashed Olyset and unwashed PermaNet equally prevented sandflies from entering the bednets (z = 0.866, p >0.05). Unwashed PermaNet significantly prevented entry of sandflies more than the washed one (z = 2.021, p <0.05). This was not the case when the unwashed and washed Olyset nets were compared (z = 1.888, p >0.05). Both unwashed LLINs significantly prevented more sandfly entry into bednets than the treated Supanet (PermaNet: z = 2.309, p <0.05, Olyset: z = 2.309, p <0.05). Treated Supanet prevented entry of more sandflies than untreated one (z = 2.309, p <0.05).

Effect of bednet treatments on sandfly probing: Probing activity captured by a video camera showed that the number of times sandflies probed before feeding was 4–9 under Olyset net and if feeding ensued sandflies only fed partially without full engorgement. One to two probing attempts preceded feeding to engorgement in ‘no net’, treated and untreated Supanets. The number of probing patterns before feeding in PermaNet was haphazard ranging from 1 to 11. Most sandflies after passing through the Olyset net were unable to recognize the host (hamster).

Semi-field studies

Blood-feeding rate of P. duboscqi sandflies in different bednets: The number of sandflies that fed did not differ significantly among the bednets (χ^2 = 1.652, p >0.05). However, unwashed Olyset nets had the lowest number of sandflies that were blood-fed whereas untreated Supanet had the highest numbers (Fig. 3).

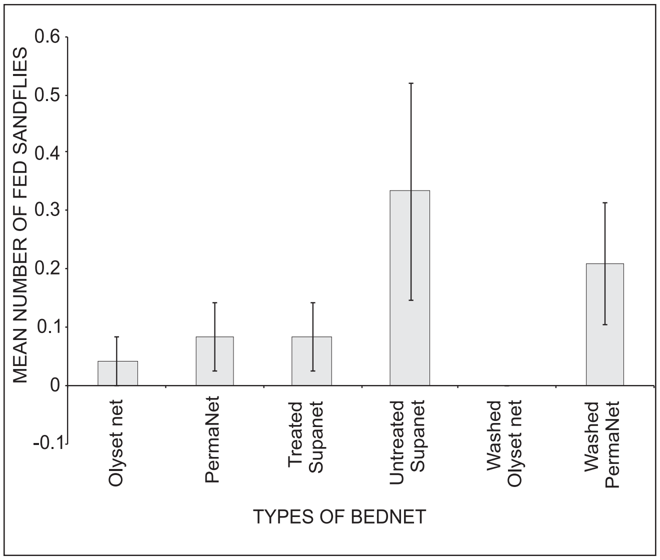

Fig. 3: Number (mean ± SE) of fed sandflies found within the bednets in greenhouse

On the other hand, the number sandflies that did not feed differed significantly (χ^2 = 23.580, p <0.05). Olyset nets had the highest number of unfed sandflies followed by PermaNets. There were no differences among other bednets except between unwashed Olyset and treated Supanet (z = –2.231, p <0.05).

Sandfly mortality and paralysis: There were significant differences among bednets with regard to the number of dead sandflies found within the bednet (χ^2 = 31.247, p <0.0001). Table 2 shows mortality rates and numbers of sandflies found inside and outside of different bednets after 24 h under semi-field conditions. Olyset nets had the highest number of dead sandflies within followed by washed PermaNet. The only significant difference among the bednets was in the numbers of dead sandflies in treated Supanet and unwashed Olyset (z = –2.274, p <0.05). The outcome was the same when the numbers of the sum of dead and paralyzed sandflies were compared.

Mortality rates of sandflies caught in the bednet after 24 h were 99% in Olyset nets and 41% in untreated Supanet. There were only paralyzed sandflies inside the washed PermaNet and unwashed Olyset nets and no live sand flies were observed. With regard to the sandflies found on the white flooring sheet outside bednets, unwashed Olyset net and treated Supanet had the highest number of dead sandflies. Untreated Supanet had the lowest number and this differed significantly when compared with other bednet treatments (p < 0.05) except PermaNet (p > 0.05). Similarly, there was no difference among the dead flies outside other bednets (χ^2 = 2.526, p >0.05).

Bednet entry rate by sandflies: Number of sandflies that entered different bednet treatments differed sig-

Table 2. Mortality rates and numbers of sandflies found inside and outside of different bednets after 24 h under semi-field conditions

Type of bednet	% Mortality of sandflies inside	Mean ± SE of sandflies inside			Mean ± SE of dead sandflies outside[b]
		Dead	Paralyzed	Total	
Treated Supanet	93	3.5±1.15	0.16±0.07	3.6±1.2	1.3±0.86
Untreated Supanet	41[a]	0.58±0.14	0	0.58±0.14	0.3±0.17
Olyset net	99	7.42±1.54	0.20±0.10	7.45±1.58	1.37±0.53
PermaNet	92	3.96±0.88	0.29±0.09	4.25±0.88	0.58±0.18
Washed Olyset net	99	7.42±2.02	0	7.42±2.02	0.92±0.04
Washed PermaNet	93	4.46±0.78	0.33±0.14	4.79±0.87	0.75±0.25

[a]The number of sandflies found alive in untreated Supanet bednets was likely to have been higher than what was recorded since sandflies in these bednets were observed to easily fly out thus pushing up the mortality rate. [b]Only dead sandflies are indicated because there were no paralyzed sandflies.

nificantly (χ^2 = 20.721, p <0.001). However, without considering the number of sandflies in Supanet bednets, there were no differences among the remaining bednets (χ^2 = 2.308, p >0.05). Attraction into the bednets by sandflies (total numbers) by the six goats used in the experiment was not different (χ^2 = 2.238, p >0.05). Fluorescent dyes used to mark sandflies had no effect on the total number of flies that entered the bednets (χ^2 = 0.471, p >0.05).

Discussion

Results from this study showed that both LLINs tested did not completely inhibit *P. duboscqi* female sandflies from taking blood-meals. This could be because of the mesh sizes which are large enough to allow sandfly entry and that the presence of the insecticide alone might not have been enough to prevent sandfly host-seeking. In the laboratory, Olyset net proved to be better than PermaNet in preventing blood feeding. The number of sandflies that blood-fed under this bednet was significantly less than those under K-O Tab treated Supanet. The difference in these numbers was not significant in PermaNet *vs* Supanet comparisons. Some studies involving *An. gambiae* mosquitoes showed that nets treated with deltamethrin were better in preventing bites[25]. Though PermaNet and Supanet bednets had varying deltamethrin concentrations, 55 mg a.i. /m^2 and 25 mg a.i. /m^2 respectively, no difference was observed in the number of sandflies that were blood-fed. The reason for this observation could not be established. Perhaps, what could have mattered to sandflies was the mesh size which happened to be the same in both the bednets and not the insecticide concentration and distribution.

In the semi-field experiments, the numbers of unfed sandflies were not different across bednets tested when Supanet nets were not considered. This was in contrast with the findings of the laboratory experiments where Olyset nets had significantly higher number of unfed sandflies when compared with other bednets. The difference could have been a result of the distance that the sandflies had to cover to reach the net surfaces and pass through to the hosts. This distance was much greater in the green house enclosure than in tunnels used in the laboratory, hence, fewer sandflies managing to enter bednets. The other reason for lower number of sandflies captured in the nets could be due to the introduction of laboratory bred sandflies into environments they were not accustomed to host-seeking. The two reasons plus the fact that bednets are designed to eliminate host/insect contact, led to low sandfly recovery rate in this experiment.

Ordinary hand washing 20 times at home using a local bar soap of the two LLINs (washing was used also to simulate the bednet lifespan[23]) did not significantly reduce blood-feeding inhibition effect. This is in agreement with the manufacturer's claim that the efficacy of these nets lasts for up to 20 washes. These findings also agree in part with those of laboratory washing which reduced the efficacy of K-O Tab® conventionally treated nets but had least effect on the PermaNet 1.0 which retained significant biological activity after 20 washes[23]. A Tanzanian study involving mosquitoes showed that PermaNet 2.0 retained wash resistance[19]. Though Olyset could not significantly lose their biological activity in the current semi-field study, a field study in Western Kenya showed that Olyset nets lost their biological activity rapidly[15]. In the current study, however, bednets were used for a short time period.

Since deaths due to contact with Olyset net were higher than in other net treatments, the bednets were considered better products for controlling sandflies. This conclusion is also based on WHO laboratory bioassays which proposed insect death due to contact with insecticide as the criterion of choice for acceptability of efficacy[26]. The big mesh size may therefore not be a ground for down grading Olyset nets as shown by this study. An unexpected observation about PermaNet in the semi-field environment was that it was neither better than hand treated Supanet nor the untreated Supanet in terms of numbers of killed plus paralyzed sandflies. This is contrary to that of mosquitoes whose control (measured as mortality or knockdown) appears to decline from good to poor within very narrow ranges of surface concentration, with critical ranges for change evidently differing between and within products[27].

During an evaluation of Olyset nets in Tanzania against mosquitoes people were concerned about the big mesh size that "could allow entry of mosquitoes"[27]. Only results from laboratory bednet tests in the current study confirmed this concern in sandflies because Olyset nets permitted entry of the highest number of sandflies when compared with other treatments. However, this net also had the highest number of sandflies that had sluggish movement (either partial or complete), difficulty in probing and could not feed. Lack of probing attempts due to failure to recognize the host is an important quality of the bednet given that feeding attempts alone can lead to *Leishmania* transmission[28].

Other unequivocal differences between Olyset net and PermaNet bednets were observed in survival rates of sandflies that contacted the net barrier in the laboratory. In either case, the lowest proportion of all sandflies (sum of sandflies that entered the bednet and those did not) surviving was observed in Olyset net. Again, this implies that though Olyset nets allowed entry of many sandflies in comparison with other bednets in the study, few had survived, thus, further reducing biting chances. In the laboratory, highest sandfly mortality rates were observed in Olyset nets (53.4% within the bednet and 47.7% of both sandflies found inside and outside bednets). On the other hand mortality rates for sandflies that passed through PermaNet were quite low (6.2%) when compared with rate of flies that crossed the net barrier plus those did not (32.2%). This observation indicates that many sandflies in PermaNet were seemingly killed before they managed to enter the bednet and because of their smaller mesh size which increases contact with the insecticide.

Repellent effect known in permethrin-treated nets against mosquitoes[15] was not obvious in permethrin-treated Olyset nets in this study involving sandflies in the laboratory. Excito repellency is caused by permethrin's hypersensitivity to stimuli from sense organs through blockage of sodium ions movement from outside to the inside of the nerve cells[29] and probably this effect is much reduced in sandflies.

The findings of bednet efficacy tests in the greenhouse (semi-field environment) where some factors influencing sandfly ecology and behaviour in the field were controlled, revealed a good scenario for comparison with experiments under laboratory environments and also an indication of what the case would be under uncontrolled field environment. The scenario in the greenhouse also reflects a more practical case where sandflies in the field have to enter bednets during their host seeking exercise to take bloodmeals.

In conclusion, all bednets tested allowed entry and subsequent blood-feeding by *P. duboscqi* sandflies. Based on its associated high mortality rates, the use of Olyset nets in areas where sandflies are nuisance biters and/or disease vectors was recommended. However, authors recommend that mesh size of Olyset net be reduced to comfortable size especially for sandfly infested areas. Further investigations into appropriate mesh sizes, insecticide doses especially in the case of PermaNets[30] and field studies with LLINs are required to ensure better products for sandfly control.

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