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SHORT COMMUNICATION

Enhanced blood feeding of *Anopheles* mosquitoes (Diptera: Culicidae) through membranes with applied host odour

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Introduction

The development of a membrane feeding system, now marketed as Hemotek™, in which the blood is electrically heated to a pre-set temperature and sustained at a chosen level (Cosgrove *et al*., 1994), has made possible the routine blood feeding of colonized *Anopheles* mosquitoes (Diptera: Culicidae) without animals, the use of which is now strictly regulated in some countries. However, in order to utilize the full reproductive potential of mosquitoes taking blood from the Hemotek™ system, it is important that the device is as similar as possible to a live animal host in attractiveness. During a build up of stocks of *Anopheles stephensi* Liston and *Anopheles gambiae* Giles *sensu stricto* for use in genetic experiments, it was observed that the output of eggs was low, and the cause was narrowed down to a reluctance to take full blood meals. De Jong & Knols (1995) investigated preferential biting sites of the highly anthropophilic *A. gambiae* s.s. and concluded that the strongest olfactory stimuli came from unwashed human feet. Therefore an initial small scale experiment was conducted in the insectaries of the London School of Hygiene and Tropical Medicine to establish what, if any, influence repeatedly-worn sports socks wrapped around the membrane feeding unit would have on blood feeding. The results indicated that the reproductive performance of membrane-fed, colonized mosquitoes could be markedly increased by using this stimulus (Andreasen, 1997). After the initial findings, expanded trials were carried out at the London School of Hygiene and Tropical Medicine which took into account the observation by Mboera *et al*. (1998) that moisture is an attractant to the mosquito *Culex quinquefasciatus* Say.

An independent set of experiments, using slightly different procedures, was carried out at Manchester University, School of Biological Sciences. The results of the two studies are reported here.

Materials and methods

At the London School of Hygiene and Tropical Medicine, defibrinated, sterilized, horse blood was offered in Hemotek™ membrane feeders, set at 35°C ± 1°C, using stretched Parafilm® as the membrane material. The ambient conditions for feeding and maintenance were 27°C and 60% RH. The widely applied procedure of sugar starvation for 24 h prior to the blood meal was omitted, as this was not standard local practice. The membrane feeders were presented initially to the mosquitoes at 27°C, then the heater was switched on, i.e. it was not 'pre-heated'. The mosquito strains used were either *A. stephensi* strains BEECH or DUBS or *A. gambiae* s.s. strains G3 or KIL. Females of each of the two species were tested in batches of 30, aged between 24 and 48 h, picked at random from their cages. A total of 900 females was tested, no males were present. Blood from membrane feeding units was offered under five different conditions: (i) surrounded by a 'sweaty sock', (ii) with a clean sock, (iii) with a moist sock, or (iv) with the membrane rubbed with a 'sweaty sock', (v) a control blank membrane without any additives to the normal blood feeding set up. The 'sweaty' and clean socks were adjusted for different moisture content by dripping water onto the clean sock until the weights were equal. All socks were of dark blue cotton. The clean and moist socks were new and had been washed once without soap. The 'sweaty' socks had been worn 6–8 times by the same male person during strenuous exercise,

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lasting 40–60 min. Between experiments the socks were kept in a closed plastic bag in the insectary. The socks were replaced with freshly used ones about every two to three weeks. At least two days passed between the 'sweaty' socks being worn and the first time they were used experimentally. No difference in their attractiveness was observed during the two to three weeks period after being worn.

Members of the same three batches of mosquitoes were used for the replicate tests. The membrane feeders were placed on the mosquito cages for 1 h. The numbers of mosquitoes in each group that probed or engorged were recorded after 5, 20, 40 and 60 min. Two days after a blood meal, the experimental mosquitoes were placed in universal tubes containing moist cotton wool and filter paper for egg laying with access to a 10% v:w glucose solution as food. After four days, the numbers of eggs laid by individual females were counted and the females dissected for retained mature eggs. In the case of females having taken blood but failing to produce any mature eggs, the stage of ovarian development was recorded according to the categories of Clements & Boocock (1984). The data were tested for normality by the Kolmogorov-Smirnov and Sharpiro-Wilks tests and the appropriate parametric or non-parametric tests applied.

At Manchester University, the same procedure was applied with the following modifications. The ambient conditions for rearing and testing were 28°C ± 1°C and 75% ± 5% RH. The cotton socks were black and had been worn during normal activity for 3 days only and pig blood was used for feeding through a stretched Nescofilm membrane. The temperature of the blood was raised to 38°C before the Hemotek units were placed on the cages and *A. gambiae* ZANU strain was used for the tests. The mosquitoes, which were 6–7 days old, and starved for 12 h, were tested in batches of 20 accompanied by 20 males. Five replicates of each test were carried out with (i) a 'sweaty sock', (ii) a clean sock and (iii) no sock. A total of 300 mosquitoes was tested. Fecundity was recorded but the females were not dissected for unlaid eggs at the end of the experiment.

Results and Discussion

Number of females blood feeding

The presence of a sweaty sock was attractive and significantly increased blood feeding through membranes of *A. stephensi* and *A. gambiae* relative to other treatment (table 1). Moist socks, although less effective than sweaty socks, significantly increased feeding when compared with the effects of either a clean sock or no sock at all. Rubbing the feeding membrane briefly with a sweaty sock did not make it significantly more attractive to mosquitoes than an untreated membrane (table 1).

Number of eggs developed or laid by individual females

A total of 19 out of 169 *A. stephensi* and 21 out of 177 *A. gambiae* s.s., took a blood meal but did not develop eggs. Upon dissection they were all found to be in stages IIIa–IIIb, indicating that they were in a pre-gravid condition (Clements & Boocock, 1984). The proportions of blood-fed female *A. stephensi* that developed eggs did not differ significantly among the various treatments, but a slightly positive effect was observed with *A. gambiae* exposed to a membrane surrounded by a sweaty sock

Overall there were highly significant differences between the numbers of eggs developed by females that fed on the modified membranes ($F_{9, 296} = 35.50$; $P < 0.0001$). Pairwise comparisons showed that the number of eggs developed by each species that had fed in the presence of a sweaty sock was significantly greater than after feeding in the presence of a clean or moist sock or a rubbed membrane or with a blank membrane (fig. 1).

In the tests at Manchester University, a total of 174 females took a blood meal and, of these, 101 oviposited (fig. 1). The proportion of blood-fed females laying eggs differed according to the modification of the feeding membrane, the highest proportion of blood-fed being those exposed to a membrane surrounded by a sweaty sock ($\chi^2 = 11.83$, $P = 0.0027$).

The number of eggs laid by different individual females did not follow a normal distribution so a Kruskal-Wallis analysis was used. This showed significant differences in the numbers of eggs laid by females blood-fed on the various membranes ($KW_3 = 34.84$; $P < 0.0001$). A significantly larger number of eggs was laid by females that fed through the membranes surrounded by a sweaty sock than those surrounded with a clean sock or no sock ($z = 44.83, 43.34$; $P < 0.001$) but the latter two categories did not differ significantly.

Female probing response

As shown in fig. 2, there was a significant overall difference in the mean numbers of both species that were

Table 1. Proportion and mean number ± SE of female *Anopheles stephensi* and *A. gambiae* feeding over 60 min on a modified Hemotek™ membrane feeder unit.

Treatment added	*A. stephensi* LSHTM		*A. gambiae* LSHTM		*A. gambiae* MU	
	Proportion	Mean±SE	Proportion	Mean±SE	Proportion	Mean±SE
Blank	0.16	4.67±1.20a	0.27	7.00±1.15a	0.49	9.80±1.74a
Clean sock	0.24	7.33±0.88a	0.21	6.33±0.88a	0.54	10.8±2.42a
Membrane rubbed with sweaty sock	0.26	7.67±0.67a	0.32	9.67±0.88a		
Moist sock	0.40	12.0±1.15b	0.39	11.7±0.33b		
Sweaty sock	0.62	18.7±0.88c	0.58	17.3±1.20c	0.71	14.2±1.96b

In the LSHTM study there were three replicates of 30 females; in the MU study, there were five replicates of 20 females. There was no significant difference between the two species ($F_{4, 1} = 30.25$, $P = 0.003$), but a significant difference between treatments ($F_{4, 8} = 9.62$, $P = 0.004$). Note: For each column of data, treatments sharing a letter did not differ significantly. Those indicated as differing significantly did so at least at $P < 0.05$.

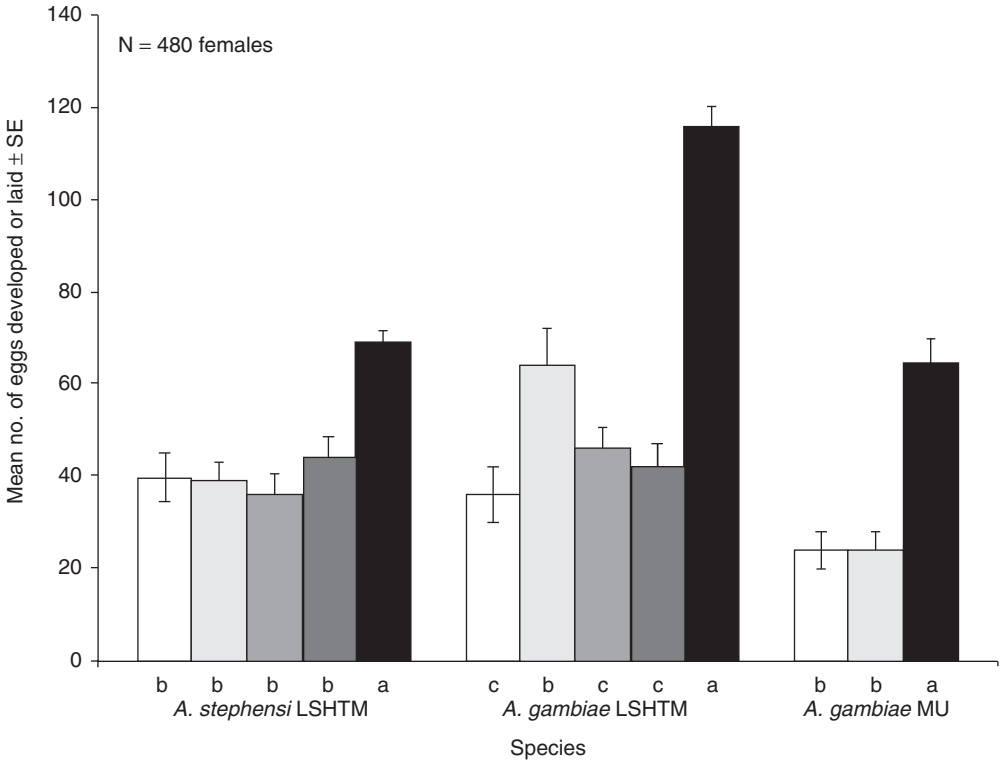

Fig. 1. Mean number of eggs developed (LSHTM) or laid (MU) by mosquitoes after a blood meal on membranes with different characteristics. For each mosquito species and laboratory, categories sharing a significance indicator letter did not differ significantly. Otherwise there was a significant difference at $P < 0.001$ for *Anopheles gambiae* and $P < 0.01$ for *A. stephensi*. □, Blank, ▫, clean sock; ▒, rubbed membrane; ■, moist sock; ■, sweaty sock.

probing or had fed on the different membranes when observed at four set times over a 1 h period (from repeated measures ANOVA $F_{9,3} = 42.24$, $P < 0.0001$). For both species the presence of a sweaty sock significantly accelerated the feeding process (fig. 2). The presence of a moist sock had an effect similar to that of the sweaty sock on *A. stephensi*. For *A. gambiae*, moist socks were intermediate in their effect compared with sweaty socks (fig. 2).

For both *Anopheles* species it was demonstrated that socks contaminated with foot odour ('sweaty socks') were attractive when applied to Hemotek™ feeding membranes, and that wet (moist) socks also had an effect. The attractancy to moisture in the two anopheline species resembled that found by Mboera *et al*. (1998) for *C. quinquefasciatus*. A membrane rubbed with a sweaty sock that was subsequently removed did not prove to be a good attractant. This may have been because, as the membrane warmed up, there was rapid evaporation of the probably small amount of attractant transferred to the membrane. Sweaty socks have been observed to quickly lose their attractancy if left in the open (B.G.J. Knols, personal communication). In the present study, the socks were stored in plastic bags until immediately before use. Braks *et al*. (2000) found that sweat incubated at 37°C was more attractive than fresh, unincubated sweat, presumably because of microbial activity generating volatile attractants. It seems likely that the 28°C temperature used in the present tests encouraged microbial activity to some extent. Furthermore, it is often recommended that mosquitoes should be starved for 24 h before feeding by removing the sugar solution. This, and pre-heating of the membrane feeders, may bring about additional improvement in feeding.

Preliminary tests using moistened or steamed sweaty socks seemed to induce an attractancy even greater than that reported above. However, these apparent effects of combined treatments have not yet been quantified.

Socks with the sweat from two different human males in London and Manchester, respectively, were evaluated in the current tests and yielded remarkably consistent results. However, in view of the results of Braks *et al*. (2000) it might be expected that a larger sample of individuals would reveal differences in the attractiveness of their socks to mosquitoes. It may be that choosing a person emitting the optimal kairomones from the feet will be necessary if maximal feeding rates are to be achieved.

The method may be applicable to membrane feeding of other species, including *C. quinquefasciatus*. The latter species is significantly attracted to socks that have been worn under laboratory conditions as well as strongly attracted to traps baited with human foot odour in the field (Mboera *et al*., 1998, 2000).

These observations demonstrate that the fecundity of anopheline mosquitoes can be increased by simple modifications to the Hemotek™ feeding system and the effect was approximately the same whether horse or pig blood was used. Marked improvements in egg yield by the simple addition of a sweaty sock to the membrane feeder should be of considerable practical use in the maintenance of

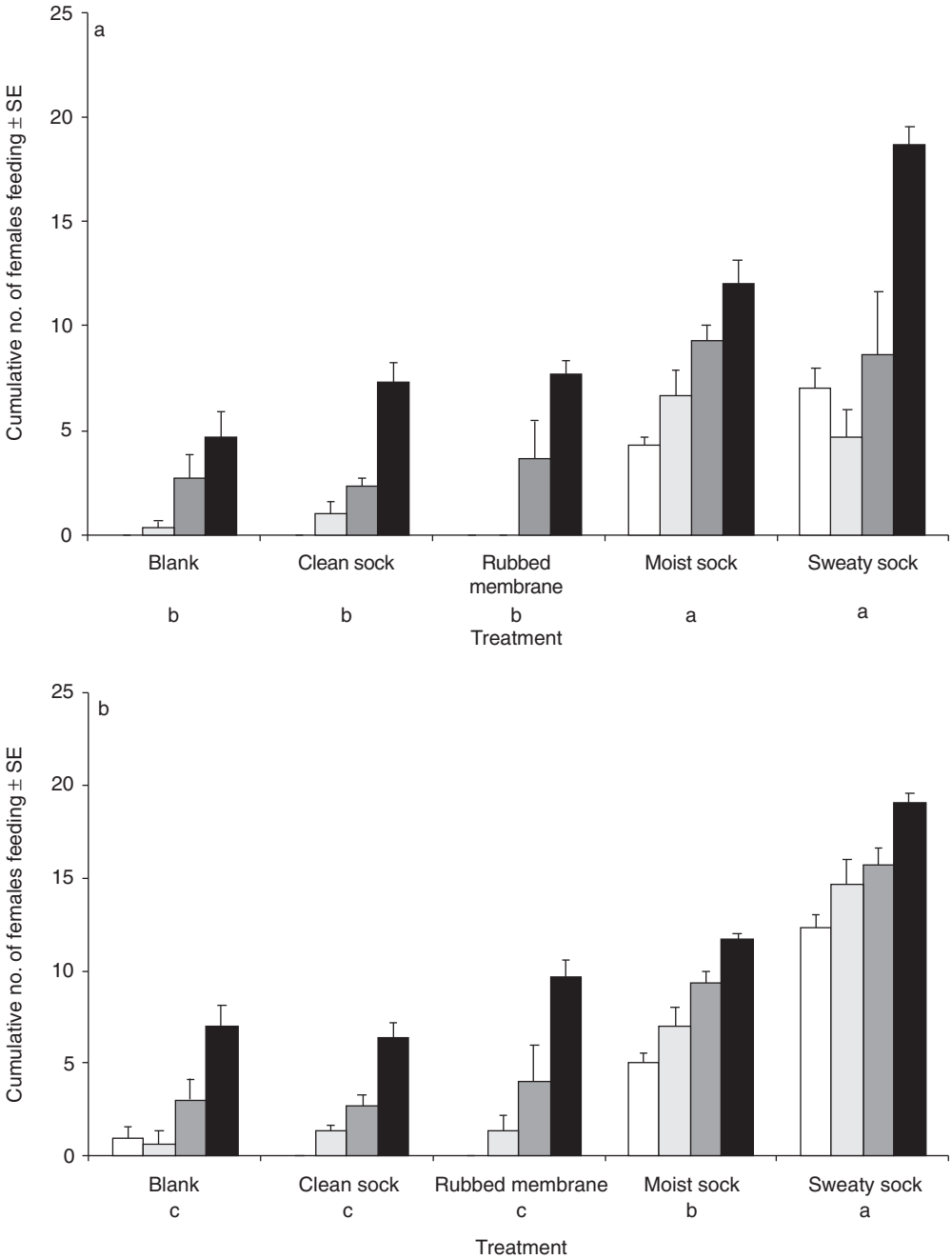

Fig. 2. Cumulative means (± SE.) at successive times of females of (a) *Anopheles stephensi* and (b) *A. gambiae* probing (□, 5 min) or having fed (▒, 20 min; ▒, 40 min; ■, 60 min) on modified Hemotek^{TM} membranes. Data are based on the three replicates of 30 females referred to in table 1. Note: For each species, treatments sharing a common letter did not differ significantly. Those that differed significantly did so at $P < 0.001$ level.

mosquito colonies and in obtaining larger progeny batches from individual females when searching for the rare female with a new induced mutation or transgenic construct of a desired type from which a valuable new stock would be reared, provided that sufficient male and female progeny of this female are obtained. This is especially topical in view of the current effort to develop transgenic mosquito strains, to be used for control of malaria vector populations (Gould & Schliekelman, 2004).

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