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Mechanical transmission of *Trypanosoma evansi* and *T. congolense* by *Stomoxys niger* and *S. taeniatus* in a laboratory mouse model

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Abstract. Mechanical transmission of *Trypanosoma evansi* (South American origin) and *T. congolense* of Kilifi DNA type (Kenyan origin) was studied in laboratory mice using the African stable flies *Stomoxys niger niger* and *S. taeniatus*. Altogether, 355 flies were interrupted after feeding on infected blood and then transferred immediately to an uninfected mouse to complete feeding. Microscopy and subinoculation of triturated flies into uninfected mice demonstrated the survival of *T. congolense* in *Stomoxys* for up to 210 min and *T. evansi* for up to 480 min. Parasites survived for much longer periods in the digestive tract than inside or on the mouthparts. *Trypanosoma congolense* was transmitted only by *S. n. niger*, and only at low rates of 3, 8 and 10% using flies of different feeding histories: fed on blood the previous day, freshly caught, and teneral. *Trypanosoma evansi* was transmitted by both *Stomoxys* species at higher rates: *S. taeniatus* range 13–18%; *S. n. niger* range 17–35%. The highest transmission rate occurred with the combination of teneral *S. n. niger* and *T. evansi*.

Key words. *Stomoxys*, *Trypanosoma*, mechanical transmission, mouse model, Kenya.

Introduction

Since the reviews of Wells (1972) and Foil (1989), few researchers have investigated the ability of either tabanids (Otte & Abuabara, 1991) or stable flies (Diptera: Muscidae: Stomoxyinae) (Mihok *et al*., 1995a; D'Amico *et al*., 1996) to transmit pathogenic trypanosomes mechanically. In the case of stable flies, mechanical transmission has been confirmed on many occasions in laboratory rodent models, most often with the cosmopolitan species *Stomoxys calcitrans* (L.) (Wells, 1972). This mode of transmission appears to occur through either contamination of mouthparts or regurgitation of gut contents (Straif *et al*., 1990; Kloft, 1992), but there are few data on the relative importance of these mechanisms and any intrinsic factors in flies that may facilitate transmission.

In addition to questions about the mechanisms involved, information is lacking on the capacity of many relevant wild species to transmit trypanosomes mechanically. The main fly used in past laboratory studies, *S. calcitrans*, is not the most typical experimental model for the natural situation in Africa, as it is usually only a minor component of the fly community (Mihok *et al*., 1996). This study was therefore conducted to complement our previous work (Mihok *et al*., 1995a), which demonstrated an overall potential for mechanical transmission by many African stable flies. In particular, we sought to interpret the causes of variation in transmission rates associated with fly–parasite combinations by examining trypanosome survival in the fly mouthparts and midgut. To do so, observations were focused on two widespread and often dominant species in East Africa: *Stomoxys niger niger* Macquart and *S. taeniatus* Bigot. Both species failed to transmit stocks of *Trypanosoma congolense* and *T. evansi* in previous work, and hence it was thought to be informative to investigate the causes of these failures. Lastly, experiments were conducted with both wild and laboratory-reared flies, so that feeding histories could be manipulated before attempting transmission. This allowed investigation of the consequences of the natural situation in which flies often feed on floral nectars rather than blood.

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Materials and Methods

Trapping

Wild stable flies were collected from the forest in Nairobi National Park, about 25 km from the International Centre of

Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya. Limited collections were also made from the thickets and woodlands of Nguruman in South-west Kenya, and from the large animal facility at ICIPE. Flies were captured in Vavoua traps (Laveissière & Grébaut, 1990; Mihok *et al*., 1995b) and Nzi traps (Mihok, unpublished), both baited with 1-octen-3-ol. Flies were identified using the keys of Zumpt (1973). The Nzi trap is similar to the many triangular blue and black traps used for catching savannah tsetse flies, but it has numerous modifications in the positioning and amount of netting material relative to the amounts of blue and black material. Some wild flies were also retained in captivity to collect eggs. These eggs were reared to provide flies of various feeding histories. Rearing was done using the protocols for *S. calcitrans* (Bailey *et al*., 1975).

Mechanical transmission trials

Trypanosomes used were *T. congolense* (K/60), a cloned Kilifi DNA type from a cow in Kenya (Masake *et al*., 1987), and *T. evansi* (IL 1934), a derivative of an isolate from a South American capybara (Magnus *et al*., 1982). These stocks were selected to provide a contrast with previously used parasites: a cloned savannah DNA type *T. congolense* of tsetse origin, and a stock of *T. evansi* of camel origin, both from Kenya (Mihok *et al*., 1995a). The parasites were grown and passaged in Balb/c mice from stabilates obtained from the International Livestock Research Institute (ILRI) in Nairobi.

Experiments were conducted when parasitaemia in a donor mouse was high, about 10^7/ml (estimated using a haemocytometer beforehand), using whatever stable flies were available from trap captures or rearing. Trials were carried out over 2 years in order to obtain a comprehensive sample of wild flies from different areas and seasons. When freshly-caught wild flies were used, trials were carried out in the afternoon with flies collected the morning of the same day.

Mechanical transmission was attempted with individual flies following the same protocols used in Mihok *et al*. (1995a). Briefly, each fly was held in a small plastic vial with one end consisting of netting. The fly was allowed to take a partial meal from a piece of cotton soaked in freshly prepared, infected mouse blood. Feeding was interrupted when the observer was sure that blood had been taken. From previous measurements of meal sizes using this method ($n = 53$, no statistically significant heterogeneity among fly species or sex; Mihok *et al*., 1995a), this should have resulted in a mean partial blood meal of 2.2 ± 0.2 mg, which at 10^7 parasites/ml, would have translated to a mean of 22 000 parasites ingested. The fly was then transferred to an uninfected mouse to complete the meal. The trial was recorded only if the fly was observed to feed or probe on the mouse. Parasitaemia was monitored for 1 month by microscopic examination of wet smears of blood drawn from a vein in the tail in this and similar experiments.

At the time of transmission, stable flies were classified into three types according to their origin and feeding history: (a) freshly obtained from the field (unknown feeding history), (b) wild-caught, but fed on citrated cow blood the previous day (blood-fed), (c) laboratory-reared and taking a first blood meal (tenerals).

To aid in interpretation of feeding histories for wild-caught flies, random samples of trapped flies (Mihok *et al*., 1996; this study) were processed at capture with the cold anthrone reagent to detect the presence of a sugar meal (Van Handel, 1972). Prior to each trial with wild flies, mouthparts and guts were dissected and examined from a random sample of twenty stable flies to ensure the absence of infection. No trypanosomes were ever detected.

Interpretative experiments

To determine how long trypanosomes could be detected microscopically in the fly, teneral and wild *S. n. niger*, and wild *S. taeniatus* were fed on blood infected with each parasite. To examine persistence in mouthparts, five flies in each treatment were killed at eight timed intervals (0, 1, 2, 4, 5, 7, 9 and 10 min), dissected, and their mouthparts examined for trypanosomes ($n = 40$ for each fly–parasite combination). To examine persistence in the midgut, five flies were killed at ten intervals (0, 15, 30, 60, 75, 90, 105, 125, 140 and 1440 min), dissected, and their midguts examined for trypanosomes ($n = 50$ for each fly–parasite combination). A final experiment was then conducted to confirm infectivity of parasites to mice after ingestion by the fly. In this case, fourteen wild flies in each fly parasite combination were fed on infected blood and killed at fourteen intervals (0, 5, 10, 20, 30, 45, 60, 120, 150, 180, 210, 300, 480 and 1440 min). Individual flies at each interval were homogenised in 0.5 ml normal saline. The resulting mixture was then injected into two mice to monitor parasite infectivity.

To simulate simple contaminative transmission, a 25 gauge syringe needle was inserted in a piece of cotton wool soaked in infected blood. This needle was then used immediately to prick the skin of an uninfected mouse. Infection by each parasite was attempted twenty times.

Results

Mechanical transmission trials

In total, 150 transmission trials were completed with *S. taeniatus* and 205 trials with *S. n. niger*. As *S. taeniatus* could not be reared in sufficient numbers, it was possible to compare only wild *S. taeniatus of* unknown feeding history with similar flies kept in captivity and fed on blood 1 day earlier. Cold anthrone tests ($n = 243$) of freshly-caught stable flies indicated that 78% of *S. taeniatus* had probably not fed recently (no colour reaction for fructose, no discolouring of the reagent for blood). As the anthrone test detects as little as 6 μg of fructose, these flies had probably not fed on sugar within the last 2 days (including ≈ 16 h spent in the trap before it was collected in early morning). Of the remaining 22%, 19% had fed on sugar. Grossly visible reddish-brown discolouring of the reagent further indicated that at least 3% had fed on blood. Mixed

Table 1. Frequency of mechanical transmission of *Trypanosoma* spp. to Balb/c mice following interrupted feeding by two *Stomoxys* spp. Biochemical tests indicated that most flies trapped in the wild, and hence of unknown feeding history, had not fed recently or had fed on sugar rather than blood.

	T. evansi		*T. congolense*	
Stable fly species and feeding history	Trials	% successful transmissions	Trials	% successful transmissions
S. taeniatus				
Blood-fed	30	13.3	40	0
Unknown	40	17.5	40	0
S. n. niger				
Blood-fed	30	16.7	30	3.3
Unknown	35	20.0	40	7.5
Lab-reared tenerals	40	35.0	230	10.0

sugar/blood meals were difficult to quantify due to the strong colour reactions resulting from even minute quantities of fructose.

In a total of eighty attempts, *T. congolense* could not be transmitted with *S. taeniatus*. In seventy attempts, *T. evansi* was transmitted 16% of the time by *S. taeniatus* (Table 1), with no difference in transmission rate between flies of different feeding histories (Fisher's exact test, $P = 0.75$).

Transmission trials were conducted with all three types of *S. n. niger* (unknown feeding history, blood-fed and laboratory-reared tenerals). Of 502 wild *S. n. niger*, 40% were positive for a sugar meal by the anthrone test, but only 2% showed evidence of a recent blood meal. The highest transmission rates were achieved with teneral flies (Table 1), but differences were not significant among the flies of different feeding histories (*T. evansi:* $\chi^2 = 3.73$, $P = 0.16$; *T. congolense:* $\chi^2 = 1.05$, $P = 0.59$). Overall, *T. evansi* was transmitted by *S. n. niger* at a significantly much higher frequency than was *T. congolense* (25 vs. 7%, respectively, $\chi^2 = 10.67$, $P = 0.001$).

Interpretative experiments

In total, 240 flies were dissected to assess the ability of trypanosomes to survive in or on the proboscis (Table 2). In many flies, small numbers of motile parasites were seen for up to 5–7 min inside the labrum (mostly at the base) or in the saline used to mount the proboscis. No trypanosomes (alive or dead) were seen at 9 and 10 min. There were no obvious differences in the timing of disappearance related to the parasite or fly species, or the feeding history of the fly.

A similar experiment was conducted with a total of 300 flies to assess the ability of trypanosomes to survive in the midgut. It was difficult to detect parasite mortality for the first hour due to the presence of very large numbers of motile trypanosomes. Parasite numbers and motility began to decrease noticeably after about 60–90 min. Motile trypanosomes mostly disappeared between 90 and 105 min (presumably lysed) and only a few dead or sluggish trypanosomes remained at about 2 h. There were no clear trends related to the parasite or fly species, or to the feeding histories of the flies. The longest surviving motile trypanosome was observed at 140 min in the combination of *T. evansi* and teneral *S. n. niger.*

Table 2. Numbers of flies with trypanosomes in or on the proboscis of *Stomoxys* spp. at various times following a blood meal (by microscopy, five flies dissected in each cell). *Snn = Stomoxys niger niger, St = Stomoxys taeniatus.*

	T. congolense			*T. evansi*		
Time (min)	*Snn* wild	*Snn* teneral	*St* wild	*Snn* wild	*Snn* teneral	*St* wild
0	5	5	5	5	5	5
1	1	3	4	4	3	4
2	5	3	2	5	4	5
4	4	3	5	3	1	1
5	5	5	2	2	2	1
7	0	1	0	1	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0

Microscopic observations of trypanosome survival in the insect were confirmed by subinoculation of whole fly homogenates from eighty-four flies into mice at various intervals after a blood meal (Table 3). Parasites retained their ability to infect mice in nearly all cases for the first hour, and in most cases for up to about 2–3 h. After 3 h, only one case of an infective parasite was detected at 480 min, again for the combination of *T. evansi* and teneral *S. n. niger.* Mimicking mechanical transmission with a syringe needle resulted in 100% transmission of *T. evansi* ($n = 20$) and 75% transmission of *T. congolense* ($n = 20$). Both trypanosomes grew well in mice with prepatent periods of 4 and 12 days, respectively. *Trypanosoma evansi* killed all mice by day 12; *T. congolense* did not kill any of the mice after 1 month.

Discussion

Based on comprehensive trials with ten species and subspecies of Stomoxyinae and four species of trypanosome, Mihok *et al.*

Table 3. Infectivity of trypanosomes in *Stomoxys* spp. following a blood meal (demonstrated by mouse subinoculation of homogenate from a single fly in each cell, total of eighty-four flies injected into 168 mice). + + = both mice infected; + – = one mouse infected; – = neither mouse infected. *Snn* = *Stomoxys niger niger, St* = *Stomoxys taeniatus.*

	T. congolense			*T. evansi*		
Time (min)	*Snn* wild	*Snn* teneral	*St* wild	*Snn* wild	*Snn* teneral	*St* wild
0	+ +	+ +	+ +	+ +	+ +	+ +
5	+ +	+ +	+ +	+ +	+ +	+ +
10	+ +	+ +	+ +	+ +	+ +	+ +
20	+ +	+ +	+ +	+ +	+ +	+ +
30	+ +	+ +	+ –	+ +	+ +	+ +
45	+ +	+ +	+ +	+ +	+ +	+ +
60	+ –	–	+ +	+ +	+ +	+ +
120	–	+ –	–	+ +	–	+ –
150	+ +	+ +	+ –	– +	+ –	–
180	–	–	–	+ +	+ –	+ –
210	–	+ –	+ –	–	–	–
300	–	–	–	–	–	–
480	–	–	–	–	+ –	–
1440	–	–	–	–	–	–

(1995a) proposed that most stable flies are capable of transmitting trypanosomes mechanically. Here, mechanical transmission has been further demonstrated for two species that failed to transmit previously in sixty-five attempts using other stocks of *T. congolense* and *T. evansi*. For unknown reasons, the occurrence of transmission seems to be specific to the vector–parasite combination, even when protocols are standardized carefully within one laboratory. Whether these results can be carried over to the field is difficult to say. For example, Mihok *et al*. (1995a) transmitted *T. evansi* from a camel in Africa only once in 110 attempts with ten stable fly species and subspecies. In this study, *T. evansi* from a capybara in South America was transmitted readily (16% by *S. taeniatus*, 25% by *S. n. niger*). Substitution of a Kilifi genotype for the savannah type of *T. congolense* resulted in transmission by *S. n. niger*, but not by *S. taeniatus*. Previous historical studies have rarely demonstrated transmission of unspecified genotypes *T. congolense* (Wells, 1972). The Kilifi genotype was chosen for investigation because it is distantly related to the other DNA types of *T. congolense* (Garside *et al*., 1995). It was originally isolated from the Kenya coast in an area with very low densities of tsetse flies. The transmission dynamics of this interesting genotype of *T. congolense* are not well known (Woolhouse *et al*., 1996).

A sufficient mechanism for mechanical transmission is the contamination of mouthparts. Many trypanosome stocks can be cloned in mice, and hence infection can be initiated by a single organism *in vivo*. A further necessary condition of relevance is the maintenance of parasite infectivity for times typical of interrupted feeding. Here, it was confirmed that motile and presumably viable trypanosomes remained in or on the proboscis for about 5–7 min after feeding. This process was also mimicked with a syringe needle. These detailed studies of persistence in stable fly mouthparts agree with more limited previous observations made with other stable fly and parasite combinations (Dixon *et al*., 1971; Wells, 1972). Contaminative transmission clearly provides a straightforward explanation for these laboratory observations, given conditions of high parasitaemia, brief time periods between feeds and highly infective stocks. In the natural situation, these conditions may be difficult to replicate. They are also be further influenced by the limited ability of the small, needle-like mouthparts of stable flies to retain parasites. These structures cannot facilitate parasite survival as well as the large, rasping mouthparts and sponging labella of tabanids (Foil, 1989).

Although contaminative transmission was supported in general by these experiments, it was surprising to find no correlations between the numbers or motility of trypanosomes that could have accounted for statistical differences in transmission rates among parasite–fly combinations. No trend was detected with respect to the contrast in proboscis size between the two fly species. *Stomoxys taeniatus* is about 50% larger than *S. n. niger*, presumably facilitating contaminative transmission. Differences in transmission rates to mice may therefore have been related to idiosyncrasies of fly feeding and probing behaviour that was not measured. These sorts of behavioural factors were held constant in the first step of this experimental model (initial feeding on a cotton swab), but could have varied in the final transmitting feed on the mouse. Better insights into the causes of variation in transmission rates among stable fly species can perhaps be gained only through further study of feeding and probing behaviour on appropriate hosts, such as cattle.

The main alternative to contaminative transmission is the regurgitation of viable parasites from the digestive tract. This process occurs when *S. calcitrans* is fed artificially on fluids in capillary tubes (Straif *et al*., 1990; Kloft, 1992), but its significance during normal feeding is unknown. If regurgitation is of significance as a mode of mechanical transmission, then a necessary condition is the existence of an environment in the fly gut supporting parasite survival. As with the interaction between the tsetse fly midgut and trypanosomes (Welburn & Maudlin, 1992), it can be hypothesized that the digestive environment of a teneral stable fly or one that has not fed recently should be more conducive to trypanosome survival than that of a non-teneral or recently-fed fly.

Most biting flies feed on both sugar and blood with direct transfer to the midgut during blood-feeding (Lee & Davies, 1979; Stoffolano, 1983; Friend, 1991). Previous meals of nectar, honeydew or plant sap may, however, influence both the processing of fluids and the ultimate survival of parasites, as in sand flies (Schlein & Jacobsen, 1994). The digestive tract of *S. calcitrans* contains many proteases and lysins, all presumably inimical to trypanosomes (Lehane, 1976; Deloach & Spates, 1979; Spates *et al*., 1982; Schneider *et al*., 1987). These compounds are induced following blood meals and return to low levels during starvation, hence providing scope for variation in levels of factors inimical to trypanosomes in the midgut, as in tsetse.

The above complex issues were addressed for the first time

in stable flies by comparing trypanosome survival in the digestive tract of two species of *Stomoxys* using flies of three prior feeding histories. In five combinations of parasite and fly, no correspondence was found between large differences in transmission rates and small differences in the survival times of trypanosomes in the midgut. In all cases, large numbers of parasites survived only for the first hour or two; this would have facilitated mechanical transmission by regurgitation for all experimental combinations. These results suggest that the stable fly midgut, regardless of species or prior feeding history, is generally hostile to trypanosomes, and therefore never provides the sort of permissive environment observed in teneral or starved tsetse flies (Welburn & Maudlin, 1992).

In conclusion, these experiments have confirmed that mechanical transmission of many trypanosomes is possible with various *Stomoxys* spp. Conditions under which contaminative transmission may occur are restrictive, with transmission possible for a few minutes only when small numbers of trypanosomes survive in or on the proboscis. Transmission by regurgitation has fewer restrictions, as trypanosomes remain infective in the midgut for at least a few hours, but this process remains unproven as a natural phenomenon. Altogether, it is puzzling why some trypanosome–fly combinations transmit parasites easily to mice, whereas others do not. Further research will only be useful in the context of the natural situation if it focuses on fly feeding and probing behaviour on appropriate live hosts. These studies would also benefit from the use of more sophisticated techniques for quantifying the presence and survival of trypanosomes in or on fly mouthparts.

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