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Keywords

Cuticular lipids, genetic differentiation, *Glossina pallidipes*, hydrocarbons, population variation, tsetse flies

Disciplines

Biochemistry | Entomology | Genetics | Structural Biology

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Cuticular lipid mass and desiccation rates in *Glossina pallidipes*: interpopulation variation

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Abstract

Tsetse flies, *Glossina pallidipes* (Diptera: Glossinidae) are said to have strong dispersal tendencies. Gene flow among these populations is estimated to be the theoretical equivalent of no more than one or two reproducing flies per generation, thereby raising the hypothesis of local regimes of natural selection. Flies were sampled from four environmentally diverse locations in Kenya to determine whether populations are homogeneous in desiccation tolerance and cuticular lipids. Cuticular hydrocarbon fractions known to act as sex pheromones do not differ among populations, thereby eliminating sexual selection as an isolating mechanism. Cuticular lipid quantities vary among populations and are not correlated with prevailing temperatures, humidities, and normalized density vegetation indices. Females demonstrate a stronger correlation than males between cuticular lipid mass and body weight. Desiccation rates also vary among populations, but are not correlated with the amounts of cuticular lipid. Chemical analysis of cuticular hydrocarbons by gas chromatography–mass spectroscopy shows that one of the four populations has more 11,15- and 11,21-dimethyl-31 hydrocarbon on females. These results are discussed in the context of population differences and estimates of gene flow.

Keywords

Cuticular lipids; genetic differentiation; *Glossina pallidipes*; hydrocarbons; population variation; tsetse flies

Introduction

Tsetse flies (Diptera: Glossinidae) constitute a small and unusual taxon of approximately 32 species and subspecies that reproduce by adenotrophic viviparity. Sexual maturity is reached in approximately 3 days for females and 5 days in males. Pregnant females deposit their first larva at approximately 15 days and maximally at 9-day intervals thereafter. Tsetse fly populations of any species are discontinuously distributed in their ranges. All species are exclusively haematophagous. They are the only vectors in Africa of the trypanosomes that cause sleeping sickness in people and ‘nagana’ in cattle and, as such, have profound medical and economic importance.

Studies have shown that *Glossina pallidipes* populations, like other tsetse flies, are strongly differentiated genetically (Krafsur, 2002, 2003; Gooding & Krafsur, 2005). The causes of genetic differentiation include genetic drift (changes in allele frequencies by chance, inversely proportional to population size) but the role of natural selection, if any, remains to be demonstrated. Long standing theory states that the exchange among populations of even small numbers of reproductive flies prevents genetic differentiation at selectively neutral genetic loci (Wright, 1969; Crow & Kimura, 1970). Ecological research has demonstrated that *G. pallidipes* in particular is highly vagile (Vale *et al.*, 1984; Williams *et al.*, 1992), suggesting that populations should be more homogeneous genetically than the genetic data indicate. What might the putative isolating mechanism(s) be? Mate recognition is a key component of reproductive isolating mechanisms in speciation (Butlin & Ritchie, 1994). Cuticular hydrocarbons are required for mating behaviours in tsetse (Carlson *et al.*, 1984) and have been shown to respond rapidly to natural selection in reinforcement of mate recognition in sympatric *Drosophila* species (Higgie *et al.*, 2000; Rundle *et al.*, 2005). Loci coding for the hydrocarbons shows much geographical variation in *Drosophila melanogaster* (Takahashi *et al.*, 2001). In theory, variation in cuticular hydrocarbons, especially the sex pheromone, could contribute to population isolating mechanisms. Alternatively, spatially distinct tsetse populations might encounter and adapt to markedly different environmental conditions, making individuals less fit in novel environments, thus reducing the chances of gene flow between them. Spatial variation in physiological traits such as desiccation resistance and thermal tolerance is certainly common in insects (Addo-Bediako *et al.*, 2000, 2001; Chown, 2001), and some evidence from cage experiments (similar to transplants) indicates that individuals perform poorly in novel environments to which they are not accustomed (Jenkins & Hoffmann, 1999; Griffiths *et al.*, 2005). Therefore, differential natural selection on spatially distinct tsetse populations could rationalize the contrast between ecological prediction and genetic inference. Few studies, however, have sought to quantify the strength and direction of selection in tsetse, possibly because of the constraints presented by the life history of these species and the areas in which they occur. Indeed, although investigations of natural selection in the wild are not uncommon (Endler, 1986), they are nonetheless relatively rare for insects (Hoekstra *et al.*, 2001). Thus, in the present study, a post-hoc test of the hypothesis is applied by investigating whether spatially separated populations respond differently to environmental stress and, if so, whether such differences correlate with cuticular lipid amounts and cuticular hydrocarbon profiles.

Cuticular lipids could vary for adaptive reasons associated with restriction of water loss. Such adaptive variation has been suggested for and documented in some insect species, although not in others (Gibbs *et al.*, 1991; Gibbs, 1998, 2002). It is concluded that cuticular lipid amounts are not correlated with water loss rates in *G. pallidipes*. Some geographical variation, however, is found in both traits. Geographic differences in cuticular hydrocarbon profiles are also found. It remains to be determined whether these physiological parameters have adaptive significance.

Materials and methods

Sampling and study populations

Glossina pallidipes were sampled from four locations in Kenya, East Africa (Table 1). To determine seasonal variation in physiology, the Nguruman (warm, mid-altitude) and Lambwe (warm, high altitude) populations were sampled twice, at approximately 4-month intervals. The first sampling of these populations was from mid-July to mid-August 2003 (the end of the 'long rains') and the second occasion took place after 17 weeks, in mid-November to mid-December 2003 (the end of the 'short rains'). The November to December sampling also included physiological assessments in the Narok (cool, highest altitude) and

Kwale districts (warm, low altitude). The total duration of the sampling and experimental physiology work was 2 and 4 weeks for the long and short rains, respectively. For each of the field experiments, flies were collected from odour-baited, biconical traps (Challier & Laveissière, 1973). From the time of collection in the field, the flies were kept in a cooler box layered with moist paper towels, during transportation to the field laboratories. Upon arrival at the laboratories, flies were separated immediately into groups for each of the experimental procedures. Typically, flies were collected in the late afternoon (1700–18.00 h). Temperature and relative humidity was recorded using ThermoChron iButtons (0.5 °C accuracy; Dallas SemiConductors, Dallas, Texas) for the duration of research at each study site (approximately 6 days). Humidity was calculated from a simple psychrometer using two iButtons as wet and dry bulbs, respectively. Normalized density vegetation index values (NDVI; Table 1) are historical average annual values that correlate with long-term saturation deficits and precipitation. In principle, NDVI may vary from –1 to 1. The NDVI is an important predictor variable of tsetse distribution and abundance, particularly in East and southern Africa (Rogers, 2000; Rogers & Robinson, 2004). In most tsetse habitats, NDVI varies from 0.4 to 0.8. In Nguruman, the index was only 0.2 but varied from 0.4 to 0.5 in the other study sites.

Desiccation rates

Male flies, individually contained in 5 mL cuvettes, were subjected to flowing air desiccation (less than 2.5% RH) for 10 h at 25.0 ± 1.0 °C using a Peltier-controlled temperature cabinet (Sable Systems PTC-1, Henderson, Nevada). Cuvettes containing the flies were attached to a Sable Systems MF8 airflow manifold. This was in turn connected to an aquarium pump regulated by a mass flow-controller to produce a flow-rate of 100 mL min^{-1} through each cuvette. The experiment was replicated twice at each sampling location ($n = 16$ per replicate per experimental treatment) and was carried out during 21.00–07.00 h, a period of minimal activity in tsetse. Simultaneously, ambient desiccation rates were estimated for 16 male flies collected the same day on two consecutive days (total $n = 32$) from the same localities to determine rates of water loss under field conditions. The measuring apparatus was the same as in the desiccation experiments except ambient temperatures and humidities were utilized, which were measured for the duration of these experiments (Table 1). Mass was recorded before and after an experiment on an electronic microbalance (0.1 mg; Avery Berkel FA 304T, EU, Fairmont, Minnesota). Flies were dried to constant mass (50–60 °C for approximately 72 h) and re-weighed. Flies were stored in glass vials and shipped to Iowa State University for cuticular lipid analysis. Flies that died during an experiment were excluded from the analyses.

Cuticular lipids

Flies were weighed individually, soaked in 400 μL of hexane for 10 min, removed, dried, and re-weighed to estimate cuticular lipid mass. Weights were recorded on an electronic microbalance (0.01 mg; Mettler Toledo AG245, Greifensee, Switzerland). For analysis of cuticular hydrocarbons, the hexane extract was applied to a silica gel column and hydrocarbons purified as described previously (Jurenka *et al.*, 1998). Hydrocarbons were analysed using a nonpolar capillary column (DB-5 0.25 μm i.d. \times 30 m, J & W Scientific) in a Hewlett Packard 5890 GC (Hewlett Packard, Palo Alto, California). The oven was programmed to increase from 80 to 330 °C at 10 °C min^{-1} . Inlet and detector temperatures were set at 330 °C. Mass spectra were obtained with a Hewlett Packard 5890 II mass selective detector.

Gene flow

Estimates of gene flow were based on analysis of spatial variation in mitochondrial DNA using procedures set forth in Krafur (2003) and Ouma *et al.* (2005). Mitochondrial variation

is single copy and inherited matrilineally. Variation was estimated by sequencing cytochrome oxidase (*COI*) and 16S2 ribosomal RNA (*16S2*) genes. The theoretical relationship between genetic differentiation (G_{ij}) between populations i and j and gene flow Nm is $G_{ij} = (2Nm + 1)^{-1}$ and is based on Wright's island model of gene flow (Avice, 2004). The chief assumptions include the infinite allele mutation model and mutation-drift equilibrium.

Results and Discussion

Cuticular lipid mass

Cuticular lipid quantities were measured in individual flies from four populations. Females had greater amounts of cuticular lipids than male flies (Table 2). Although cuticular lipids were not systematically identified, most are saponifiable lipids, tri-, di- and monoacylglycerols (Carlson *et al.*, 1998; Jurenka, unpublished observations). Hydrocarbons in *G. pallidipes* make up approximately 10% of the total cuticular lipids by weight (R. Jurenka, unpublished observations), which is consistent with other tsetse species (Carlson *et al.*, 1998). Females had greater cuticular lipid mass and dry body weight than males (Table 2). Correlation between lipid mass and dry weight was examined by sex using flies pooled from the four populations. The results showed that males had a lower Pearson correlation coefficient than females (male: $r^2 = 0.30$, $n = 453$; female: $r^2 = 0.60$, $n = 127$). Females from Narok showed the strongest correlation between lipid mass and body weight ($r^2 = 0.83$, $n = 22$). These results indicate that female flies produce more cuticular lipids as they increase in weight and size. Female weight gains are most likely due to growth of developing larvae and increased cuticular lipids would presumably help in restricting water loss.

No significant differences were found in cuticular lipid mass in females between the July and December samples at Lambwe and Nguruman (Table 2). Lambwe males had more cuticular lipids in December than July but the opposite was found in Nguruman. Dry weights and cuticular lipids were lowest in the Nguruman flies, but the body water contents of males was not (Table 3). There is no obvious adaptive significance to the foregoing population differences. Body size in tsetse is largely a function of the mother's quality of nutrition and therefore the mother's environment (Hargrove, 2004).

Desiccation rates and cuticular lipids

There was no correlation between cuticular lipid mass and water loss in the flowing air desiccation experiment ($r^2 = 0.01$, $n = 81$) (Fig. 1a and Table 3). The greater water loss rates found in Kwale flies could not be attributed to lower cuticular lipids or body water contents when compared with the other populations. The ambient desiccation experiment also indicated higher rates of water loss in Kwale but this also could not be attributed to cuticular lipid mass or to body water content. Terblanche *et al.* (2006) showed that interpopulation variation in water loss was not likely to have been caused by differences in activity levels. A stronger correlation between cuticular lipid mass and water loss rates was observed in the ambient desiccation flies pooled over populations, but the correlation was weak ($r = -0.34$, $n = 83$) and negative, indicating that lesser rates of water loss are found in flies with greater amounts of cuticular lipid. It appears that variation in water loss among populations cannot be attributed to cuticular lipid mass, body water content, or activity patterns (Terblanche *et al.*, 2006).

In field populations of *G. pallidipes* males, gross cuticular lipid mass may not play a critical role in restricting rates of water loss. In ambient conditions, however, the opposite may be true, where rates of water loss may be negatively correlated with cuticular lipid mass (Table 2). This can be seen in the Lambwe population, which had the least rate of water loss but the most cuticular lipids. Rates of water loss, however, were almost equal between flies in

Nguruman and Kwale, although cuticular lipids varied significantly. Using laboratory-reared flies, only a weak correlation between desiccation rates and cuticular lipid quantities was obtained (J. S. Terblanche, unpublished data). Overall, these results suggest only a small role for cuticular lipid mass in modifying water loss rates.

Cuticular lipids are said to inhibit transpiration across the cuticle and therefore play a critical role in water balance (Gibbs, 1998; Gibbs, 2002). In *Drosophila melanogaster*, however, no differences are found in cuticular lipid mass between desiccation-selected and unselected populations (Gibbs *et al.*, 1997). Multiple physiological factors contribute to desiccation resistance in the selected strains. Other studies indicate that cuticular lipid mass is important in preventing desiccation but that increased amounts do not provide additional waterproofing properties (Gibbs, 2002).

Data from the ambient air desiccation experiments (Fig. 1b and Table 3) indicate statistically significant differences among populations. Tsetse flies in Nguruman and Kwale had higher desiccation rates with relatively low cuticular lipid quantities. Lambwe and Narok flies had low desiccation rates and Lambwe flies had the most cuticular lipids. As already indicated, however, a strong correlation is not observed between desiccation rates and cuticular lipid quantities. Differences in desiccation rates among populations may have been due to the ambient conditions prevailing during the experiments. Ambient temperatures were highest at Nguruman and Kwale and lowest at Narok and Lambwe (Table 1). Higher ambient temperatures could have caused the higher desiccation rates observed in these experiments. In controlled laboratory studies, most geographical variation in water loss rates is explained by phenotypic plasticity induced by acclimation (Terblanche & Chown, 2006; Terblanche *et al.*, 2006).

Cuticular hydrocarbons

The composition of the major tsetse cuticular hydrocarbons among sampling sites is shown in Figure 2. These hydrocarbon profiles are similar to those reported by Carlson *et al.* (1984, 2000, 1993). A major difference between populations was that Lambwe females had approximately 3.5-times the level of 11,15- and 11,21-dime31 as the other populations. This is illustrated in Figure 3(a), showing partial gas chromatography–mass spectroscopy chromatograms from Lambwe and Nguruman flies. The peak in Figure 3(b) contains both dime31 and 2me31. In Lambwe flies, dime31 was the major component in the peak whereas, in Nguruman and the other populations, dime31 occurred at a much lower level. Figure 3(c) shows the mass spectra for 11,15- and 11,21-dime31 and 2me31. Males generally exhibited similar hydrocarbon profiles among populations but Nguruman flies had a lower level of 11,15- and 11,21-dime31.

The biological significance of the hydrocarbon profile difference is unclear. The sex pheromone, required for courtship behaviour and copulation in *G. pallidipes*, was identified by Carlson *et al.* (1984) as 13,23-dimethylpentatriacontane (hereafter, 13,23-dime35). This is the major cuticular hydrocarbon found in female *G. pallidipes* and does not differ in quantity among the sampled populations. Carlson *et al.* (2000) show that 13,23-dime35 quantities were almost the same among flies from geographically diverse populations, ranging from Ethiopia to Zimbabwe and including five independently derived laboratory colonies. It is most unlikely therefore that the greater fraction of dime31 in Lambwe flies contributes to reproductive isolation. The difference in 11,15- and 11,21-dime31, however, does indicate variation in hydrocarbon biosynthesis among conspecific tsetse populations, possibly suggestive of allozyme variation.

Gene flow

Pairwise estimates of genetic differentiation (Table 4) were converted to estimates of gene flow, expressed in terms of theoretical numbers of reproducing flies (Nm) exchanged among populations given the simplifying assumptions of the island model of dispersion. Gene flow varied from 1–2 females per generation (Table 4), which is a small amount considering the accepted view that *G. pallidipes* is the most vagile tsetse, capable of massive invasions; for example, seasonally in Nguruman (Brightwell *et al.*, 1997). The genetic evidence, however, indicates Nguruman populations are long-standing and genetically isolated from other *G. pallidipes* population centres (Ouma *et al.*, 2006) and this conclusion is firmly supported by new mitochondrial data (E. S. Krafur, unpublished data). Populations would rapidly become genetically homogeneous if theoretical exchange rates in the order of 5–10 reproducing flies per generation prevailed. The generally accepted estimate of the mean displacement rate of *G. pallidipes* is 0.25 km day⁻¹ (Vale *et al.*, 1984; Williams *et al.*, 1992; Hargrove, 2000). Such rates of dispersion predict much less genetic differentiation than has been recorded among numerous *G. pallidipes* populations (Krafur, 2003). The contrast between genetic and ecological views of dispersion remains to be resolved.

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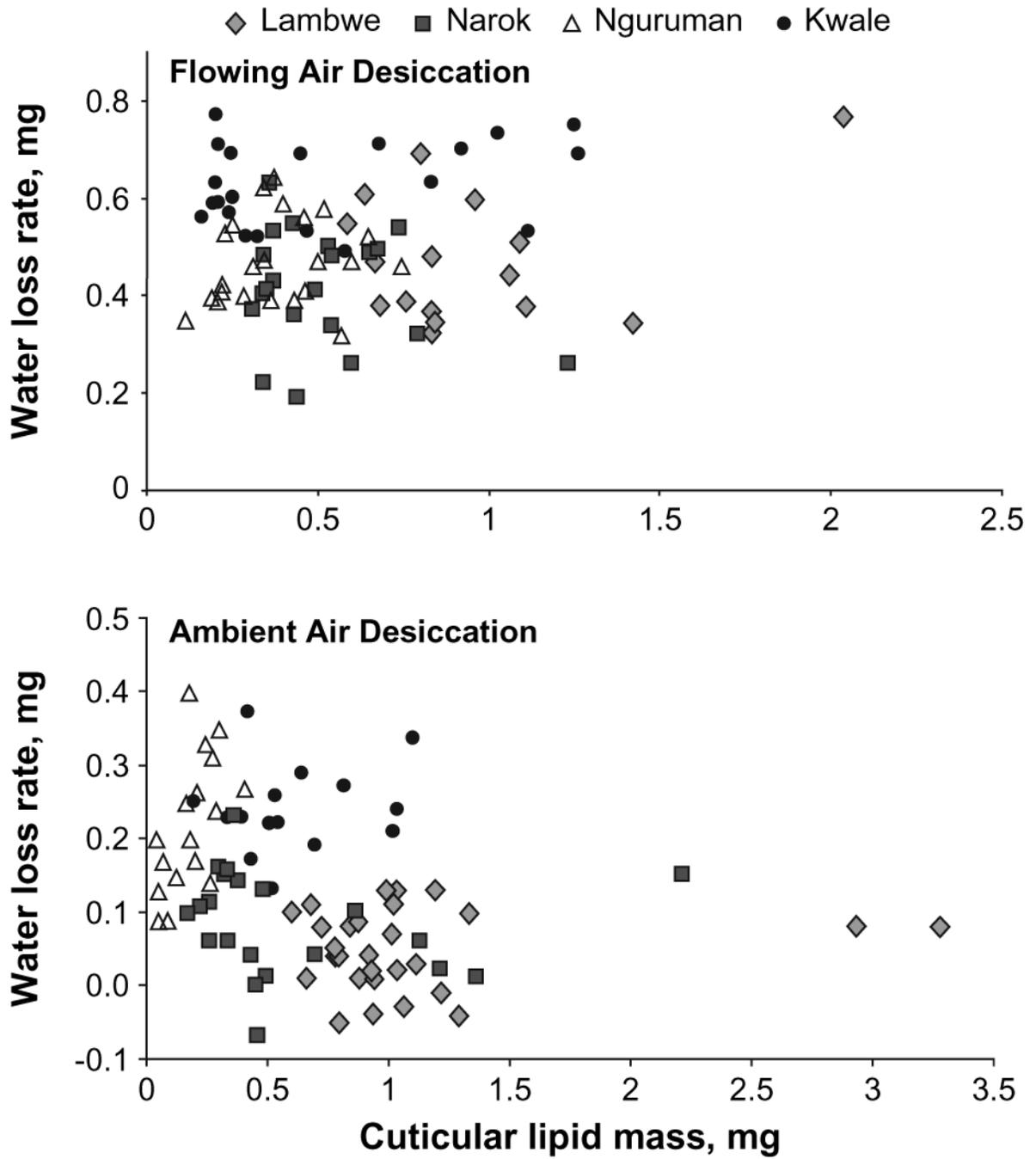


Fig. 1. Scatterplots of individual *Glossina pallidipes* comparing water loss rates as determined in flowing and ambient air desiccation experiments and cuticular lipid mass from four locations in Kenya. The desiccation experiments are described in Materials and Methods.

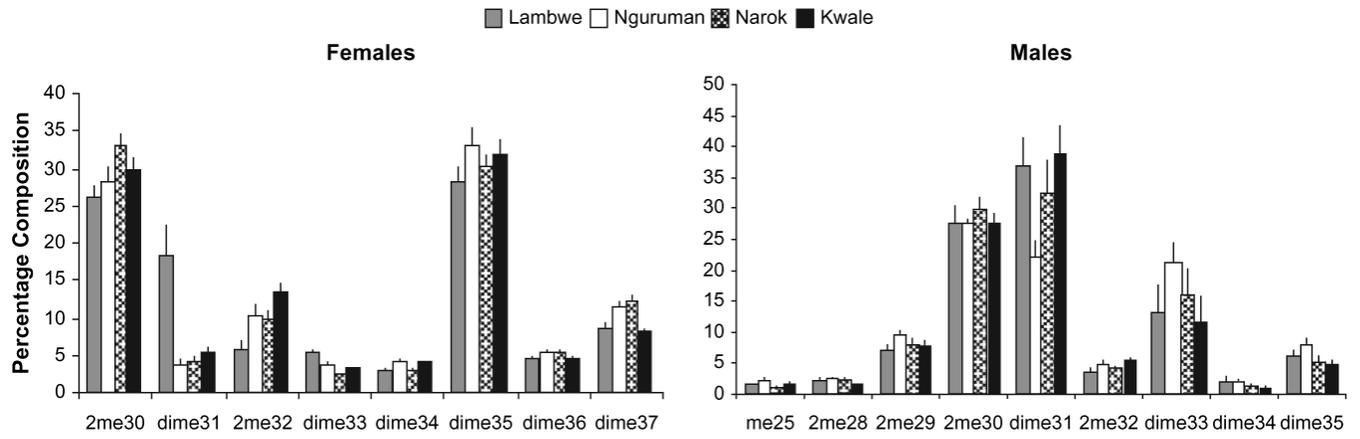


Fig. 2. Percentage composition of the major cuticular hydrocarbons from male and female *Glossina pallidipes* collected and pooled from four locations in Kenya. Bars represent the mean \pm SE of at least seven individuals. 2me, Methyl group on carbon 2; dime, two methyl groups, where the last number indicates the number of carbons in the hydrocarbon chain.

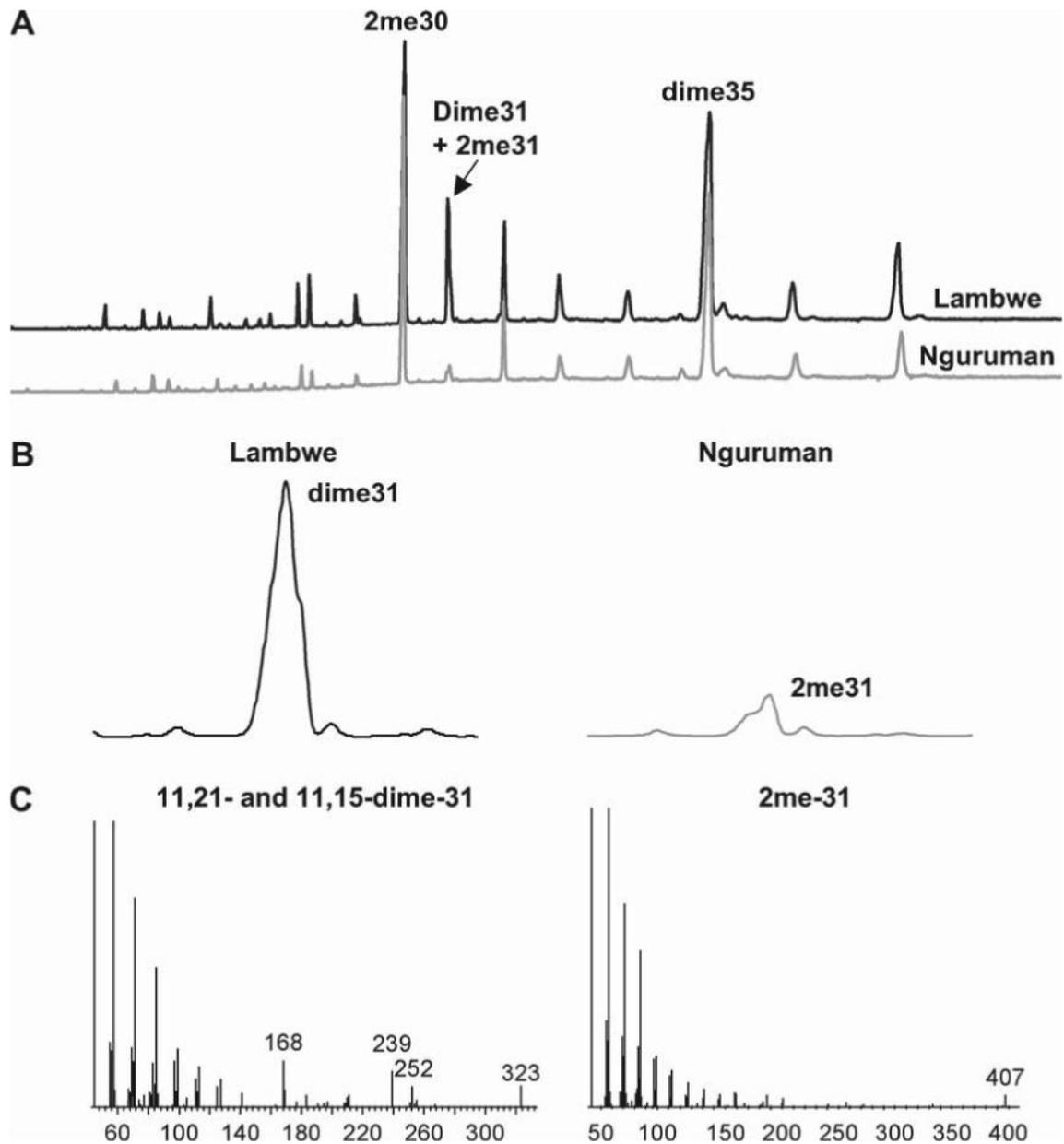


Fig. 3. (A) Partial chromatogram of cuticular hydrocarbons obtained from female *Glossina pallidipes* from Lambwe and Nguruman, Kenya. (B) Expanded portion of chromatogram from (a) showing the dime31 and 2me31 peaks. (C) Mass spectra of dime31 and 2me31.

Table 1

Temperatures, relative humidities, global positioning co-ordinates, altitudes, and normalized density vegetation indices (NDVI) at the field laboratories where *Glossina pallidipes* were sampled.

Population	Temperature (°C)	RH (%)	Latitude (°)	Longitude (°)	Altitude (m)	NDVI
Narok	19.6 ± 5.6	71.1 ± 25.0	1.12N	35.20E	1691	0.5
Lambwe	23.2 ± 5.2	76.4 ± 17.4	0.64N	34.31E	1353	0.5
Nguruman	29.0 ± 5.2	76.4 ± 10.9	1.85S	36.10E	670	0.2
Kwale	25.7 ± 1.2	80.3 ± 5.3	4.18S	39.46E	388	0.4

Table 2

Geographic differences in cuticular lipid mass and dry mass in male and female *Glossina pallidipes*.

Population and month collected	Male		Female				
	Cuticular lipid	Dry mass (mg)	Cuticular lipid	Dry mass (mg)			
Narok	Dec	0.55 ± 0.05 ^a	11.8 ± 0.3 ^a	60	1.22 ± 0.15 ^{ab,c}	21.0 ± 1.5 ^a	22
Lambwe	July	0.50 ± 0.02 ^{a,c}	11.3 ± 0.2 ^a	105	1.44 ± 0.16 ^a	20.8 ± 0.8 ^a	24
	Dec	0.87 ± 0.04 ^b	12.0 ± 0.4 ^a	66	1.39 ± 0.14 ^{ab}	17.8 ± 1.3 ^{b,c}	18
Nguruman	July	0.47 ± 0.02 ^c	10.6 ± 0.2 ^b	107	0.93 ± 0.15 ^c	15.8 ± 1.5 ^b	17
	Dec	0.38 ± 0.03 ^d	10.5 ± 0.3 ^b	65	1.02 ± 0.13 ^{b,c}	17.2 ± 0.8 ^b	16
Kwale	Dec	0.55 ± 0.05 ^a	11.8 ± 0.3 ^a	50	1.37 ± 0.11 ^{ab}	20.5 ± 0.7 ^{a,c}	30

Superscript letters after the mean ± SE in the same column indicate statistical differences (analysis of variance followed by Fisher's protected least significant difference test, $P < 0.05$).

Table 3

Geographic differences in rates of water loss, cuticular lipid mass, body size (dry mass) and initial body water content (BWC) in the experimental desiccation group (FAD: flowing air desiccation, 0% RH, 100 mL min⁻¹, 25 °C) and the ambient desiccation group (AD: ambient desiccation, ambient temperature and humidity, nonflowing air).

Population	Rate of water loss (mgH ₂ O h ⁻¹)	Cuticular lipid mass (mg)	Dry mass (mg)	BWC (mg H ₂ O)	N
FAD					
Narok	0.412 ± 0.026 ^a	0.52 ± 0.05 ^a	12.4 ± 0.5 ^a	23.5 ± 0.5 ^a	21
Lambwe	0.481 ± 0.033 ^a	0.95 ± 0.09 ^b	13.7 ± 0.7 ^a	21.3 ± 0.8 ^b	16
Nguruman	0.470 ± 0.019 ^a	0.38 ± 0.03 ^c	10.8 ± 0.4 ^b	25.0 ± 0.5 ^c	23
Kwale	0.629 ± 0.019 ^b	0.53 ± 0.08 ^a	12.2 ± 0.5 ^a	25.2 ± 0.5 ^c	21
AD					
Narok	0.085 ± 0.015 ^a	0.59 ± 0.11 ^a	13.3 ± 0.4 ^a	23.9 ± 0.6 ^a	22
Lambwe	0.043 ± 0.012 ^b	1.10 ± 0.09 ^b	12.3 ± 0.4 ^{a,b}	21.4 ± 0.4 ^{a,b}	29
Nguruman	0.221 ± 0.022 ^c	0.18 ± 0.03 ^c	9.7 ± 0.5 ^c	21.7 ± 1.0 ^{a,b}	17
Kwale	0.241 ± 0.016 ^c	0.61 ± 0.07 ^a	11.0 ± 0.5 ^{b,c}	17.5 ± 3.3 ^b	15

All flies were male *Glossina pallidipes*. Superscript letters after the mean ± SE in the same column indicate statistical differences (analysis of variance followed by Fisher's protected least significant difference test, $P < 0.05$).

Table 4

Pairwise estimates of gene flow in *Glossina pallidipes* among study sites.

Study site	Narok	Lambwe	Nguruman	Kwale
Narok	—	0.2	0.32	0.23
Lambwe	2.0	—	0.34	0.24
Nguruman	1.1	1.0	—	0.33
Kwale	1.7	1.6	1.0	—

Genetic differentiation G_{ST} (upper diagonal) and mean theoretical number of reproducing females (N_m) exchanged per generation in the lower diagonal, based on composite *r16S-COI* mitochondrial DNA sequences.