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Comparative analysis of epicuticular lipid profiles of sympatric and allopatric field populations of *Anopheles gambiae* s.s. molecular forms and *An. arabiensis* from Burkina Faso (West Africa)

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Abstract

We analysed by gas chromatography-mass spectrometry (GC-MS) and Gas Chromatography-Flame Ionization Detector (GC-FID) the epicuticular lipid profiles of field females of the major Afro-tropical malaria vector, *Anopheles gambiae*. The samples were collected in three villages in Burkina Faso (West Africa), where *An. gambiae M* and *S* molecular forms and *An. arabiensis* live sympatrically. The aim was to compare the cuticular hydrocarbon (CHC) composition of individual field specimens of these three taxa, to highlight possible differences among them. All the samples analysed by GC-MS (55 individuals and eight pools) were characterized by the same 48 CHCs and 10 oxygenated compounds. The 19 most abundant CHCs were quantified in 174 specimens by GC-FID: quantitative intra-taxon differences were found between allopatric populations of both *An. arabiensis* and *S*-form. Inter-taxa quantitative differences in the relative abundances of some hydrocarbons between pairs of sympatric taxa were also found, which appear to be mainly linked to local situations, with the possible exception of $diMeC_{35}$ between *An. arabiensis* and *S*-form. Moreover, MeC_{29} shows some degree of differentiation between *S*- and *M*-form in all three villages. Possible causes of these differences are discussed.

Keywords: Cuticular hydrocarbons; Gas-Chromatography; *Anopheles*; Malaria; Africa; Semiochemicals; Mating recognition system.

1. Introduction

Anopheles gambiae s.s. is undergoing a speciation process which is increasing the diversity and ecological flexibility of this major vector and is expected to have serious consequences for malaria transmission in the Afro-tropical region (Touré et al., 1998). Two "molecular forms" (provisionally named *M*- and *S*-forms) have been recently defined on the basis of SNPs in the IGS and ITS rDNA regions (della Torre et al., 2001; Gentile et al., 2001) and later shown to be differentiated in other restricted regions of their genomes (Barnes et al., 2005; Stump et al., 2005; Turner et al., 2005). Even though *An. gambiae* molecular forms exist in sympatry throughout much of West Africa, they show very different frequencies in relation to their ecology (della Torre et al., 2005). Gene flow between the two forms is strongly reduced, probably due to premating isolation mechanisms (Tripet et al., 2001, 2003; della Torre et al., 2005). Although the understanding of these mechanisms would be instrumental in clarifying the process

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of incipient speciation within *An. gambiae*, little is currently known on this subject. Mating takes place predominantly during swarming (Charlwood and Jones, 1980) and mixed swarms have been observed between *An. gambiae s.s.* and its sibling *An. arabiensis* (Marchand, 1984) and between *M*- and *S*-forms, although at a frequency lower than that expected by chance (Diabate et al., 2006). This raises the question of how individuals recognize partners of their own species/form within swarms. In a recent paper, Tripet et al. (2004) did not support the hypothesis that acoustic stimuli are involved in premating isolation between *An. arabiensis* and the two molecular forms of *An. gambiae*, and suggested that contact pheromones may be the cues at the basis of premating isolation, as well as of female–male interactions. In fact, contact pheromones have been associated with the sexual behaviour of several biting flies (Yuval, 2006).

Cuticular hydrocarbons (CHCs) generally represent the most abundant lipids on insect cuticle and their semiochemical functions have been widely demonstrated (Howard, 1993; Ferveur, 2005). Among the several insects where these compounds have been studied, *Drosophila* is the genus where the most complete picture on their semiochemical functions, their biosynthesis and their endocrine biosynthesis regulation has been achieved (see Jallon and Wicker-Thomas, 2003). In particular, the publication of the *D. melanogaster* genome, facilitated the elucidation of fatty acid desaturase genes, whose products are involved in the biosynthesis of the cuticular alkenes acting as sex pheromones (see Jallon and Wicker-Thomas, 2003). Knowledge of CHCs is currently much more limited for medically important Diptera. However also in these, experimental bioassays have suggested that CHCs may also act as contact pheromones during mating, as previously demonstrated in *Drosophila* (Ferveur, 1997; Cobb and Jallon, 1990; Kim et al., 2004; Ferveur, 2005; Mas and Jallon, 2005). CHCs have been hypothesized to mediate sexual attractiveness during courtship in *Culex* species (Gjullin et al., 1967), co-specific mate recognition in different *Stegomyia* species (Nijhout and Craig, 1971) and female sexual receptivity in *An. gambiae* and *Aedes aegypti* (Polerstock et al., 2002). However, a deep knowledge of the possible role of epicuticular pheromones as cues mediating courtship and mating behaviour in mosquitoes is difficult to achieve, particularly in the case of Anophelines, whose mating behaviour is both altered and very difficult to analyse under laboratory conditions.

Studies on medically important Diptera, particularly mosquitoes, have shown various levels of variation in the relative abundance of CHCs correlated to geographic and physiological parameters. Geographic variations have been shown among several North American and Asian populations of *Ae. albopictus* (Kruger et al., 1991; Kruger and Pappas, 1993), among three *An. darlingi* populations from Brazil (Rosa-Freitas et al., 1992) and among laboratory strains of *An. stephensi* of different geographic origin (Anyanwu et al., 1993). Age-related variations have been shown in females of *Ae. aegypti* (Desena et al., 1999a, b, Horne and Priestman, 2002; Polerstock et al., 2002; Gerade et al., 2004, Hugo et al, 2006), *Culex quinquefasciatus* (Chen et al., 1990), *An. gambiae* (Polerstock et al., 2002; Caputo et al., 2005), *An. stephensi* (Brei et al., 2004) and *An. farauti* (Hugo et al., 2006). Epicuticular lipids have also been investigated as taxonomic characters in several insect taxa, including mosquito species complexes. Although no inter-specific qualitative differences in the composition of the CHC profile have been ever found, the relative abundance of some compounds has been found to differ among closely related Anopheline species: (i) *An. gambiae* and *An. arabiensis* (Carlson and Service, 1979, 1980); (ii) some species of the *An. maculipennis* complex (Phillips et al., 1990); (iii) malaria vector and non-vector forms of the *An. maculatus* complex (Kittayapong et al., 1990, 1993). CHC patterns were also used to provide a key to discriminate the five members of the *An. quadrimaculatus* complex (Carlson et al., 1997).

Milligan et al. (1993) analysed the CHC profiles of F1 progenies of a limited number of wild-caught *An. gambiae s.s.* and *An. arabiensis* females originating from different villages in Mali (West Africa). At the time of that study, *An. gambiae* was known to include in Mali 3 chromosomal forms (named SAVANNA, BAMAKO and MOPTI) defined on the basis of polymorphic inversions on chromosome-2 (Coluzzi et al., 1985). Today, we know that in dry savannah areas of Burkina Faso and Mali, *M*- and *S*-molecular forms correspond to MOPTI and SAVANNA/BAMAKO chromosomal forms, respectively (Favia et al., 1997). Milligan et al. (1993) analysed and compared the CHC profiles of the three chromosomal forms and of *An. arabiensis*: in one village, they found no differences in the CHC profiles of *An. arabiensis* and BAMAKO, which was the only chromosomal form present. However, in a different village (located about 30 km apart) where the three chromosomal forms occurred sympatrically with *An. arabiensis*, discriminant function analysis (DFA) based on the relative abundance of four (not identified) compounds, correctly attributed 75% of specimens to their group. A lower rate of correct attributions was found when specimens of different allopatric species/forms were considered.

We recently reported a complete list of 58 compounds characterizing the epicuticular profile of a laboratory population of *An. gambiae M*-form (Caputo et al., 2005). Here we report the results of analyses carried out on sympatric *An. arabiensis* and *An. gambiae* molecular forms directly collected in the field. Our aim was to highlight possible differences in the specific CHC composition between these taxa and support a possible role of these compounds as cues favouring premating isolation, thus encouraging further studies on this subject. The analysed samples were collected in three different localities in Burkina Faso to check the consistency of possible inter-taxa differences in populations from different ecological/geographical settings.

2. Materials and methods

2.1. Field sampling and molecular characterization

More than 1,000 adult female mosquitoes were collected by mouth aspirators (Coluzzi and Petrarca, 1973) in two villages of Burkina Faso: Goundry (12°03′N 01°29′W) and Balonguen (12°30′N 01°20′W), situated at about 80 km from each other and close to the capital city Ouagadougou (October 2003). A small sample ($N = 16$) was collected at Bama-VK7, located at the periphery of the rice cultivation area of Vallée du Kou (11°24′N 4°25′W), about 400 km SW from the former villages (October–November 2000). Mosquitoes were maintained directly in the paper-cups covered with a wet towel and kept in a dark and cool place, until frozen at −20 °C for 5 min. Individual carcasses were stored in Eppendorf tubes with a desiccant until molecular and chemical analyses. *An. gambiae* s.l. specimens were identified following the protocol of Fanello et al. (2002), performed by dipping a single wing directly in the PCR mix.

2.2. Chemical analyses

2.2.1. Samples preparation

CHC qualitative differences by gas chromatography-mass spectrometry (GC-MS) were assessed by carrying out analysis on pools of 10 females of each taxon collected in the three villages. Quantitative study of CHCs by Gas Chromatography-Flame Ionization Detector (GC-FID) analysis was carried out on the extracts of single females of the three taxa.

Pools of 10 females or individual females were plunged into Reactivials™ containing 1 and 0.1 ml of *n*-hexane, respectively, and gently agitated for 5 min. The *n*-hexane extract was removed from the vial taking extreme care not to damage the mosquitoes. A superficial wash with a further 0.5 ml of *n*-hexane for the pools and 0.1 ml for the single specimens was performed to retrieve any remaining extract from the wall of the vial; this was added to the original extract. CHCs were separated from other constituents by adsorption chromatography on activated gel minicolumns 6 × 0.5 and 4 × 0.3 cm i.d. for pool extracts and for single specimens, respectively (slurry packed with 0.7 and 0.4 g dry weight of SIL-A-200 silica gel, Sigma). CHCs were eluted with *n*-hexane (2.25 ml for the pools and 1.25 ml for individual specimens; in both cases the first 0.25 ml were discarded) and collected into a Reactivial™. The hydrocarbon fraction was evaporated to dryness overnight at room temperature. Analysis was performed within 24 h. The CHC residues were redissolved for 5 min in a *n*-hexane solution containing 10 ppm of *n*-pentadecane as internal standard (20 µl for the pooled samples and 5 µl for the individual samples) and 1 µl was injected into the GC-FID or GC-MS. The use of the internal standard allows minimization of any differences in injection volume and in the daily response of the equipment (Horne and Priestman, 2002; Kittayapong et al., 1990).

In order to analyse non-hydrocarbon components of cuticular waxes, we also prepared the crude extracts (without purification) of single specimens from Goundry (*An. arabiensis*, $N = 9$; *S*-form, $N = 10$; *M*-form, $N = 8$) and Balonguen (*An. arabiensis* $N = 10$; *S*-form, $N = 10$; *M*-form *M*, $N = 8$). The samples were extracted in 10 µl heptane for 10 min in an ultrasonic bath and 2 µl of the extract was then directly injected into the GC-MS.

2.2.2. Gas chromatography-mass spectrometry analyses

Purified extracts (1 µl each) from pools were analysed at Staffordshire University (UK) on a Carlo-Erba Mega Gas Chromatograph fitted with a cold on-column injector operating at room temperature and a DB-5 capillary column (30 m, 0.32 mm I.D., 0.25 µm phase thickness, J. & W. Scientific) linked to a VG Trio mass spectrometer operating at −70 eV. The source and the analyser temperatures were set at 200 °C and room temperature, respectively. Helium was the carrier gas (column head pressure 40 kPa, flow rate of 1 ml/min, operating at constant pressure). The oven temperature rose from 75 to 150 °C at 25 °C/min, then from 150 to 320 °*C* at 8 °C/min with a final hold at 320 °C for 45 min. Peaks were scanned for m/z from 50 to 700. Data were collected on-line with the use of Mass Lab version 1.2 software. Crude extracts from each individual field specimen (2 µl) were analysed at Florence University (Italy) on a HP 6890 GC coupled to a 5973 Mass Selective Detector (operating in electronic impact mode at −70 eV and acquiring m/z values from 40 to 550; the source and the analyser temperature were set at 200 and 150 °C, respectively). The GC was installed with a HP 5-MS column (30 m, 0.25 mm I.D., 0.25 µm phase thickness). Helium was used as carrier gas (column head pressure 82 kPa, flow rate of 1 ml/m, operating at constant flow). After 2 min oven temperature rose from 80 to 120 °C at 30 °C/min, and from 120 to 310 °C at 5 °C/min with a final hold at 310°C for 20 min. Injector temperature was kept at 280°C and transfer line at 290°C.

2.2.3. Gas chromatography of individual purified extract

Gas chromatography was performed using a Hewlett-Packard 5890 gas chromatograph equipped with an on-column injector and flame ionization detector (340 °C). A DB-1 capillary column (15 m, 0.32 mm I.D., phase thickness 0.1 µm J & W Scientific) was used with helium as the carrier gas (4 ml/min) and temperature programming from 75 to 150 at 25 °C/min then from 150 to 310 °C at 8 °C/min with a final hold at 310 °C for 15 min. The internal standard, used for quantification, was *n*-pentadecane (10 ppm). A PC-based data station (HP-CHEM), was used for data recording and quantification, with manual adjustment of baselines for some small peaks to provide consistent integration.

2.2.4. Epicuticular lipid identification

Hydrocarbon identification was based on their mass spectra and on the comparison of their retention times with

those of linear alkanes (Carlson et al., 1998). Determination of the branching position of the methyl alkanes was based on the fragmentation patterns reported in literature (Nelson, 1978). Retention times of the analytes were compared with those of several straight chain synthetic hydrocarbons (*n*-C13, *n*-C15, *n*-C17, *n*-C19, *n*-C20, *n*-C22, *n*-C23, *n*-C24, *n*-C25, *n*-C26, *n*-C27, *n*-C28, *n*-C29, *n*-C32, *n*-C34, *n*-C36, *n*-C38, *n*-C40, Sigma) and of 9-methyl C31 (kindly provided by Dr. Graeme R. Jones, Keele University, UK).

2.2.5. Quantitative analysis of CHCs

Hydrocarbon concentrations of the purified extracts were calculated relative to the pentadecane used as internal standard and then expressed as relative abundances with reference to the total hydrocarbon concentration. Only peaks with relative abundances above the threshold of 0.3% were selected for statistical analysis, to avoid inaccuracies due to the integration of low intensity peaks.

2.3. Statistical analysis

Intra and inter-taxa differences in the relative concentration of each compound were assessed using the Mann–Whitney test (with Dunn-Sīdak correction in inter-taxa comparisons) and exact probabilities (two tailed) were considered; DFA was performed for interpreting the group differences, based on the whole CHC profiles. DFA is designed to maximize the inter-group and to minimize the intra-group differences, and to generate $(n-1)$ discriminant functions, where (n) is the number of groups (i.e. taxa and populations). The functions are each in the form $Y = A_1 \times {}_1 + A_2 \times {}_2 + \ldots A_m X_m$, where $A_1 \ldots A_m$ are standardized coefficients and $X_1 \ldots X_m$ are the standardized values of the m^{th} variable (hydrocarbon concentration); the standardized coefficients are useful in ascertaining which variables contribute most in determining scores on the functions: the larger the absolute value, the greater is the contribution of that variable. We used instead the total structure coefficients, which are the product-moment correlations between each single variable and each discriminant function. Such structure coefficients describe how closely a variable and a function are related; when the absolute magnitude of the coefficient comes near to 1.0 or to -1.0, the function is carrying almost the same information as the variable (Klecka, 1989). The Wilks' Lambda (and its associated *P*-value) and the percentage of correct assignments were considered to assess the statistical robustness of the discriminant functions; values of Wilks' Lambda approaching zero indicate high discrimination among groups, while its maximum value of 1 indicate that there is no discrimination among groups. Since we wanted to compare the entire CHC profile, DFA was performed on the whole set of variables, using the automatically integrated areas of each hydrocarbon peak obtained by GC. DFA for samples of Balonguen and Goundry was performed on the whole set of variables. Tabachnick & Fidell (1996, page 512) suggest that in DFA "the sample size of the smallest groups should exceed the number of predictor variables" and since the sample size of M- and S-forms of *An. gambiae* from Bama-VK7 ($N = 8$ each) did not exceed the predictor variables, we used a forward stepwise method of variable selection that gives an unbiased measure of the discrimination between groups. Although of a preliminary nature, Bama-VK7 data will be shown to be compared with those obtained from villages geographically distant from Balonguen and Goundry. Statistics were performed by SPSS-for-Windows, ver.11.5.

3. Results

We here report the first data on the CHC composition of single *An. gambiae* s.l. specimens collected in the field, i.e. three sampling sites in Burkina Faso. At Balonguen and Goundry both the molecular forms of *An. gambiae* and *An. arabiensis* were sympatric, the latter species representing about 50% of the samples (grand total, $N = 889$; $M+S$ vs. Ar; χ^2, 1 degree of freedom (df) $= 0.16$, $P = 0.69$). Interestingly, the relative frequencies of the molecular forms differed remarkably between the two villages: the *S*-form was predominant at Balonguen ($M+S$, $N = 173$; 83%), whereas it was less frequent at Goundry ($M+S$, $N = 291$; 23%; χ^2, 1 df $= 153.3$, $P<0.0001$). Eight *M*-form and eight *S*-form collected in Bama-VK7 were also analysed.

3.1. Qualitative analysis

The analysis of the mass spectra and of the retention times allowed the identification of 48 CHCs in the pooled mosquito samples ($N = 8$), as well as in the 55 individual mosquito crude extracts; all were already characterized and described in Caputo et al. (2005). No qualitative differences were found between populations of different geographic origins (intra-taxon) and between taxa of the same geographic origin (inter-taxa).

Ten oxygenated compounds were also identified in the crude extracts of single specimens, as in Caputo et al. (2005); no qualitative differences were found between the species in crude extracts, while quantitative differences were not investigated.

3.2. Quantitative analysis

A total of 174 specimens were analysed by GC. Table 1 shows the averages of the relative abundances of the 19 CHCs quantified in: (i) 32 *M*-form, 26 *S*-form and 30 *An. arabiensis* specimens from Goundry; (ii) 13 *M*-form, 30 *S*-form and 27 *An. arabiensis* from Balonguen and (iii) eight *M*- and eight *S*-form from Bama-VK7. The 19 CHCs included in the analyses all showed a relative abundance above 0.3% with reference to the total hydrocarbons (see Material and methods). Two additional CHCs showing a suitable abundance were not included in

Table 1
Means of the relative abundances and standard errors of 19 CHCs from field-collected females of *An. arabiensis* and *An. gambiae M*- and *S*-form from three villages of Burkina Faso

Cuticular hydrocarbons	Goundry						Balonguen						Bama-VK7			
	M-form ($N = 32$)		*S*-form ($N = 26$)		*An. arabiensis* ($N = 30$)		*M*-form ($N = 13$)		*S*-form ($N = 30$)		*An. arabiensis* ($N = 27$)		*M*-form ($N = 8$)		*S*-form ($N = 8$)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
n-C_{23}	0.384	0.049	0.362	0.093	0.438	0.090	0.590	0.223	0.301	0.052	0.325	0.062	0.325	0.116	0.692	0.214
n-C_{24}	0.582	0.079	0.661	0.178	0.879	0.164	0.676	0.260	0.604	0.090	0.578	0.084	0.487	0.179	0.332	0.132
n-C_{25}	4.051	0.313	4.004	0.392	4.293	0.627	4.458	0.789	5.036	0.531	3.625	0.287	2.327	0.208	3.637	0.499
n-C_{26}	0.905	0.217	1.025	0.351	1.228	0.292	0.915	0.418	0.616	0.108	0.761	0.153	1.029	0.200	1.069	0.232
n-C_{27}	5.269	0.322	5.677	0.353	5.931	0.396	4.398	0.386	5.114	0.346	5.106	0.313	11.304	1.978	10.649	1.565
n-C_{28}	0.881	0.202	1.044	0.336	1.288	0.280	0.850	0.255	0.696	0.093	0.787	0.196	2.101	0.245	3.150	0.550
n-C_{29}	9.488	0.427	10.302	0.547	10.272	0.451	10.319	0.821	11.677	0.685	8.737	0.462	15.057	1.002	20.801	1.811
15-; 13-; 11-; 9- MeC_{29}	7.965	0.392	9.260	0.447	8.515	0.311	7.505	0.471	9.379	0.510	10.148	0.486	5.433	0.345	7.595	0.561
n-C_{30}	0.522	0.083	0.503	0.108	0.711	0.147	0.440	0.106	0.371	0.038	0.454	0.127	1.008	0.157	1.083	0.171
15-; 14-; 13-; 12-; 11-MeC_{30}	0.537	0.044	0.574	0.086	0.523	0.058	0.715	0.057	0.707	0.055	0.834	0.054	1.297	0.135	1.488	0.125
n-C_{31}	4.486	0.309	4.651	0.363	4.725	0.243	3.748	0.575	4.617	0.384	3.350	0.249	4.026	0.552	5.621	0.766
15-; 13-; 11-; 9-MeC_{31}	20.544	0.717	20.747	0.725	19.275	0.677	20.599	0.879	22.775	0.748	22.548	0.768	12.512	1.323	12.850	1.402
17-; 15-; 13-; 11-MeC_{33}	3.214	0.147	2.876	0.145	3.186	0.103	3.405	0.539	3.172	0.130	3.253	0.179	3.189	0.365	2.509	0.490
11,21-; 13,19-diMeC_{33}	5.879	0.333	5.409	0.282	4.980	0.225	5.664	0.395	4.929	0.207	5.182	0.306	2.897	0.567	2.389	0.668
11,23-diMeC_{35}	2.876	0.182	2.588	0.169	3.455	0.235	3.018	0.326	2.700	0.175	4.040	0.264	1.750	0.387	2.028	0.512
13,25-; 13,23-diMeC_{39}	0.748	0.210	0.712	0.160	1.848	0.517	0.602	0.274	0.382	0.110	1.395	0.261	1.886	0.637	0.304	0.243
13,23-diMeC_{41}	8.421	0.526	8.690	0.556	8.297	0.400	8.539	0.799	7.579	0.474	8.564	0.406	11.333	0.988	6.163	1.251
13,23-diMeC_{43}	15.233	0.499	15.010	0.608	14.063	0.526	15.648	0.857	13.627	0.512	13.892	0.435	14.838	1.741	12.211	1.991
X,y-diMeC_{45}	8.017	1.213	5.907	0.419	6.092	0.321	7.912	0.828	5.717	0.446	6.420	0.405	7.202	1.063	5.428	0.976

N = sample size; SE = standard error.

the statistical analysis because we suspected them to co-elute with contaminants. We compared the relative abundances of selected peaks to evaluate intra-taxon and inter-taxa differences.

3.2.1. Intra-taxon comparisons

The comparison of the relative abundance of each CHC between the *An. arabiensis* populations of Balonguen and Goundry by Mann–Whitney test, showed average ranks of the seven compounds that differed significantly (Table 2). No univariate differences were detected between allopatric populations of each molecular form of *An. gambiae* s.s. (data not shown). The DFA of the above-described 19 CHCs gave the following results: (i) 85.2% ($N = 27$) and 93.3% ($N = 30$) of the *An. arabiensis* specimens were assigned to the correct population in Balonguen and Goundry, respectively (Wilk's lambda = 0.37, $\chi^2 = 45.9$; df = 18; $P = 0.0003$); (ii) 96.7% ($N = 26$) and 88.5% ($N = 30$) of the *An. gambiae S*-form specimens were assigned to the correct population in Balonguen and Goundry, respectively (Wilk's lambda = 0.35, $\chi^2 = 47.0$; df = 19; $P = 0.0004$); (iii) the *M*-form populations turned out not to be different in the two villages (Wilk's lambda = 0.44, $\chi^2 = 28.3$; df = 18; $P = 0.06$). The inspection of the structure coefficients (Table 3) shows that the variables that were more important in influencing the separation between the two populations of *An. arabiensis* were *n*-C_{31}, 15-; 14-; 13-; 12-; 11-MeC_{30}, 15-; 13-; 11-; 9-MeC_{31}, and 15-; 13-; 11-; 9-MeC_{29}, whereas for the *S*-form populations several compounds had similar absolute values, such in a way that the whole CHC profile seems to contribute to distinguish the populations from different geographical locations.

3.2.2. Inter-taxa comparisons

Table 4 reports the average ranks of the pairwise comparisons between sympatric species and forms from Balonguen, Goundry and Bama-VK7 analysed by Mann–Whitney test. Differences in the relative abundance of some CHCs between pairs of taxa are as follows: (i) 13,25- and 13,23-diMeC_{39} were significantly less abundant in *M*-form than in *An. arabiensis* from Goundry; (ii) 15-; 13-; 11-; 9-MeC_{29} was significantly less abundant in *M*-form than *An. arabiensis* from Balonguen; (iii) 11,23-diMeC_{35} was significantly more abundant in *S*-form than in *An. arabiensis* in both Goundry and Balonguen; (iv) *n*-C_{29} and 13,25-; 13,23-diMeC_{39} were significantly less and more abundant in *S*-form than in *An. arabiensis* from Balonguen, respectively; (v) 15-; 13-; 11-; 9-MeC_{29} was significantly more abundant in *S*- than in *M*-form in Goundry, and had a higher, although not significant, abundance in *S*-form in Balonguen as well; (vi) several CHCs (among which 15-; 13-; 11-; 9-MeC_{29}) were significantly different between the two molecular forms at Bama-VK7.

Table 2
Average ranks and associated *P* values resulting from Mann–Whitney test for CHCs whose relative abundance differed significantly between *An. arabiensis* populations from Goundry and Balonguen (*P* from Mann–Whitney test)

Cuticular hydrocarbons	Goundry ($N = 30$)	Balonguen ($N = 27$)	*P*
n-C_{28}	33.367	24.148	0.036
n-C_{29}	34.267	23.148	0.0012
15-; 13-; 11-; 9-MeC_{29}	22.667	36.037	0.002
n-C_{30}	33.833	23.630	0.02
15-; 14-; 13-; 12-; 11-MeC_{30}	21.483	37.352	$\ll 0.001$
n-C_{31}	36.600	20.556	$\ll 0.001$
15-; 13-; 11-; 9-MeC_{31}	21.933	36.852	0.001

Table 3
Structure coefficients of the discriminant functions derived from the intra-taxon comparison between populations from Goundry and Balonguen

Cuticular Hydrocarbons	*An. arabiensis*	*S*-form
n-C_{23}	0.110	0.059
n-C_{24}	0.161	0.029
n-C_{25}	0.093	−0.166
n-C_{26}	0.153	0.116
n-C_{27}	0.171	0.117
n-C_{28}	0.155	0.106
n-C_{29}	0.247	−0.163
15-; 13-; 11-; 9-MeC_{29}	−0.306	−0.019
n-C_{30}	0.138	0.121
15-; 14-; 13-; 12-; 11-MeC_{30}	−0.395	−0.102
n-C_{31}	0.413	0.006
15-; 13-; 11-; 9-MeC_{31}	−0.324	−0.165
17-; 15-; 13-; 11-MeC_{33}	−0.044	−0.143
11,21-; 13,19-diMeC_{33}	−0.051	0.117
11,23-diMeC_{35}	−0.167	−0.048
13,25-; 13,23-diMeC_{39}	0.008	0.175
13,23-diMeC_{41}	−0.020	0.174
13,23-diMeC_{43}	0.040	0.181
x,y-diMeC_{45}	−0.066	0.050

At Goundry and Balonguen, discriminant analysis using the relative abundances of the 19 CHCs correctly ascribed about 74% of the individuals to their proper taxon (see Table 5—Goundry: Wilks' Lambda = 0.45, $\chi^2 = 60.3$, df = 38, $P = 0.01$; Balonguen: Wilks' Lambda = 0.32, $\chi^2 = 66.3$, df = 36, $P = 0.002$). It is noteworthy that *An. arabiensis* represented the taxon with the highest proportion of correctly classified individuals (>80%; Table 5). An inspection of Table 6 shows that the same CHCs contributed to the weighting of the discriminant functions, as follows. At Goundry, the largest absolute correlations between each variable and the first discriminant function were shown by 13,25-; 13,23-diMeC_{39} and 11,23-diMeC_{35}; this function is responsible for separation of the *An. arabiensis* specimens from both the molecular forms (not shown). The second discriminant function is mostly influenced by 15-; 13-; 11-; 9-MeC_{29}, a compound which, as already seen (Table 4), differed between the two *An. gambiae* forms at Goundry. At Balonguen, the coefficients of 11,23-diMeC_{35}, 13,25-; 13,23-diMeC_{39},15-; 13-; 11-; 9-MeC_{29} showed the strongest correlation with the first discriminant function, which was responsible for the

Table 4
Average ranks and associated *P* values resulting from Mann–Whitney test for CHCs whose relative abundances differed significantly among sympatric species or forms, at least in one of the villages of Goundry, Balonguen and Bama-VK7

Cuticular hydrocarbons	Goundry			Balonguen			Bama-VK7			
	M	**S**	***P***	**M**	**S**	***P***		**M**	**S**	***P***
15-; 13-; 11-; 9- MeC_{29}	24.44	35.73	0.011*	15.23	24.93	0.019	*n*-C_{25}	5.38	11.63	0.038*
	M	**Ar**	***P***	**M**	**Ar**	***P***	*n*-C_{29}	6.00	11.00	0.038*
15-; 13-; 11-; 9-MeC_{29}	28.31	34.90	0.154	11.77	24.70	0.001*	15-; 13-; 11-; 9- MeC_{29}	5.38	11.63	0.007*
13,25-; 13,23-diMeC_{39}	25.69	37.70	0.009*	14.62	23.33	0.027	13,25-; 13,23-diMeC_{39}	11.25	5.75	0.022*
	Ar	**S**	***P***	**Ar**	**S**	***P***	13,23-diMeC_{41}	11.50	5.50	0.010*
n-C_{29}	28.15	28.80	0.890	36.00	21.22	0.001*				
11,23-diMeC_{35}	22.23	33.93	0.007*	20.87	38.04	$<<0.001$*				
13,25-; 13,23-diMeC_{39}	23.88	32.50	0.049	22.32	36.43	0.001*				

For Goundry and Balonguen, *P*-values were considered as statistically significant only if $P<0.017$, which is the significance threshold obtained through the Dunn-Sïdak correction for multiple comparisons. Ar = *An. arabiensis*, *M* = *An. gambiae* M-form; S = *An. gambiae S*-form; * = significant *P*-value.

Table 5
Percentages of individuals of *An. arabiensis* and *An. gambiae M*- and *S*-forms correctly classified using predicted group membership from discriminant analysis at two villages of Burkina Faso

	% Correctly classified			
	Predicted group membership			
	M-form	*S*-form	*An. arabiensis*	Tot.
Goundry				
M-form	65.62	21.87	12.5	73.90
S-form	19.23	73.08	7.69	
An. arabiensis	10	6.67	83.33	
Balonguen				
M-form	76.92	7.69	15.38	74.30
S-form	13.33	66.67	20.00	
An. arabiensis	7.41	11.11	81.48	

Table 6
Structure coefficients of the discriminant functions derived from the inter-taxa comparison among *An. gambiae* molecular forms and *An. arabiensis* in Goundry and BalonguenGoundry

Cuticular hydrocarbons	Goundry		Balonguen	
	Function 1	Function 2	Function 1	Function 2
n-C_{23}	−0.069	−0.056	−0.161	−0.191
n-C_{24}	−0.199	0.063	−0.044	−0.012
n-C_{25}	−0.061	−0.035	−0.142	0.306
n-C_{26}	−0.105	0.050	−0.026	−0.143
n-C_{27}	−0.158	0.157	0.152	0.148
n-C_{28}	−0.140	0.083	−0.017	−0.091
n-C_{29}	−0.122	0.240	−0.215	0.496
15-; 13-; 11-; 9-MeC_{29}	−0.025	0.509	0.383	0.135
n-C_{30}	−0.174	−0.050	0.027	−0.106
15-; 14-; 13-; 12-; 11-MeC_{30}	0.038	0.092	0.237	−0.269
n-C_{31}	−0.063	0.070	−0.122	0.402
15-; 13-; 11-; 9-MeC_{31}	0.212	0.177	0.207	0.280
17-; 15-; 13-; 11-MeC_{33}	−0.075	−0.366	0.087	−0.001
11,21-; 13,19-diMeC_{33}	0.232	−0.249	0.014	−0.141
11,23-diMeC_{35}	−0.355	−0.275	0.366	−0.499
13,25-; 13,23-diMeC_{39}	−0.308	0.070	0.369	−0.447
13,23-diMeC_{41}	0.055	0.051	−0.164	−0.277
13,23-diMeC_{43}	0.190	−0.025	−0.086	−0.168
x,y-diMeC_{45}	0.135	−0.353	−0.117	−0.371

separation of the three taxa. These observations suggest that these three CHCs consistently separate the three taxa in both villages. Interestingly, the averages of relative abundances of these three compounds (Table 1) were partially consistent with the above results: (i) the concentration of $diMeC_{35}$ was always more abundant in *An. arabiensis*, had an intermediate value in *M*-form, and was less concentrated in *S*-form; (ii) the concentration of $diMeC_{39}$ was always more abundant in *An. arabiensis* than in *An. gambiae* molecular forms; (iii) the concentration of the centrally branched MeC_{29} was higher in *S*- than in *M*-form in the three villages; (iv) MeC_{29} was even more concentrated in *An. arabiensis* than in *S*-form at Balonguen, whereas in *An. arabiensis* had a mean value which was intermediate between *M*- and *S*-forms at Goundry.

The small samples of *An. gambiae* molecular forms from the village of Bama-VK7 were analysed by stepwise discriminant analysis (see Material and methods). The resulting function correctly assigned 100% *M*-form and 87.5% *S*-form specimens to the proper group (Wilks' Lambda = 0.38, $\chi^2 = 12.5$, df = 2, $P = 0.002$), suggesting a good differentiation between the two forms. It is worth noting that the stepwise method selected 15-; 13-; 11-; 9 MeC_{29} and *n*-C_{29}.

4. Discussion

We here present the first data on the CHC composition of field collected *An. gambiae* s.l. In all single specimens analysed by GC-MS for each of the three taxonomic units (i.e. *M* and *S An. gambiae* molecular forms and their sibling species *An. arabiensis*) we confirmed the presence of all the 48 CHCs previously characterized in pools of laboratory *An. gambiae* s.s. by Caputo et al. (2005). CHC profiles of each specimen described on the basis of the 19 most abundant compounds found in GC-analyses and compared among populations of the same taxon and among different taxa.

The intra-taxon analysis shows that, although the CHC profiles of all samples analysed consisted of the same compounds as expected, *An. arabiensis* populations from the villages of Goundry and Balonguen have significant quantitative differences, mainly due to the concentration of a few compounds. On the contrary, no specific CHCs differentiate the two populations of *An. gambiae S*- and *M*-form, although the two allopatric *S*-form populations could be separated by the whole CHC profile.

As a consequence, the inter-taxa analyses were carried out separately by comparing the three taxa within each village. In fact, the differences found in the relative abundances of some hydrocarbons between pairs of taxa appear to be linked to local situations, with the exception of $diMeC_{35}$ that significantly differentiates *An. arabiensis* from *S*-form in both Goundry and Balonguen. However, the discriminant analysis shows that the relative abundance of $diMeC_{35}$, $diMeC_{39}$ and MeC_{29} contributes consistently to the differentiation among taxa in both villages and allows to assign an average of about 80% of *An. arabiensis* specimens to the correct taxon, and a lower percentage of *An. gambiae* specimens to the correct molecular forms. An incomplete separation among *An. arabiensis* and *An. gambiae* chromosomal forms was also observed by Milligan et al. (1993); however, they did not identify the discriminant compounds. Our data show also that MeC_{29} is the compound presenting the highest differentiation between *S*- and *M*-form not only in Goundry and Balonguen, but also in the more distant village of Bama-VK7.

It could be hypothesized that the above-mentioned CHCs play a role in species/form recognition during mating. Interestingly, MeC_{29} shows a significant quantitative sexual dimorphisms in *Ae. aegypti* and has been already proposed as a candidate pheromone involved in mosquito mating behaviour (Horne and Priestman, 2002). Reproductive isolation between *An. gambiae* species and forms is believed to be maintained by strong assortative mating, presumably through premating barriers (Coluzzi et al., 2002). Since the frequency of mixed swarms seems too high to explain the low frequencies hybrids between *An. gambiae* and *An. arabiensis* (Marchand, 1984) and between *S*- and *M*-forms (Diabate et al., 2006), it seems reasonable to hypothesize that short distance specie species-specific or form-specific signals, such as contact and/or short-range volatile pheromones, may play a role in the final recognition of co-specific or co-form partners in the swarms. However, it should be stressed that the inter-taxa differences we found could be unrelated to mating behaviour and be the by-product of differential population dynamics of the three taxa in different ecological set-ups, as supported by the intra-taxon differences we observed. In particular, age-differences may have a crucial role in determining the CHC pattern differences both at the inter-taxa and intra-taxon levels (Caputo et al., 2005). Moreover, non-parallel ecological adaptations may also play a role: for instance, microgeographical factors, such as taxon-specific preferences for breeding and/or resting sites, could have influenced the CHC profiles we found.

Our experimental design does not allow further speculations: additional analyses are therefore ongoing in our laboratories on adults of the same age derived from field-collected larvae reared in semi-natural controlled conditions. Males will be also analysed, since male CHCs may play a very important role in partner recognition during mating. This kind of sample will allow us to minimize the effect of physiological and ecological factors on the CHC composition and to investigate more in depth the possible role of CHCs as pheromones in the mate recognition system of the *An. gambiae* complex. Moreover, the availability of the *A. gambiae* genome (Holt et al., 2002) allows the analysis of genes whose products are involved in the biosynthesis of CHCs and of genes encoding for proteins possibly involved in CHC reception. Interestingly, Turner et al. (2005) reported the presence of two Olfactory Receptor genes within one genomic area of differentiation (i.e. "speciation island") between *M*- and *S*-forms.

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References

Anyanwu, G.I., Davies, D.H., Molyneux, D.H., Phillips, A., Milligan, P.J., 1993. Cuticular hydrocarbon discrimination/variation among strains of the mosquito, *Anopheles* (Cellia) *stephensi* Liston. Ann. Trop. Med. Parasitol. 87, 269–275.

Barnes, M.J., Lobo, N.F., Coulibaly, M.B., Sagnon, N.F., Costantini, C., Besansky, N.J., 2005. SINE insertion polymorphism on the *X* chromosome differentiates *Anopheles gambiae* molecular forms. Insect Mol. Biol. 14, 353–363.

Brei, B., Edman, J.D., Gerade, B., Clark, J.M., 2004. Relative abundance of two cuticular hydrocarbons indicates whether a mosquito is old enough to transmit malaria parasites. J. Med. Entomol. 41, 807–809.

Caputo, B., Dani, F.R., Horne, G.L., Petrarca, V., Turillazzi, S., Coluzzi, M., Priestman, A.A., della Torre, A., 2005. Identification and composition of Cuticular Hydrocarbons of the major Afrotropical malaria vector *Anopheles gambiae* s.s. (Diptera: Culicidae): analysis of sexual dimorphism and age-related changes. J. Mass Spectrom. 40, 1595–1604.

Carlson, D.A., Service, M.W., 1979. Differentiation between species of the *Anopheles gambiae* Giles complex (Diptera: Culicidae) by analysis of cuticular hydrocarbons. Ann. Trop. Med. Parasitol. 73, 589–592.

Carlson, D.A., Service, M.W., 1980. Identification of mosquitoes of *Anopheles gambiae* species complex A and B by analysis of cuticular components. Science 207, 1089–1091.

Carlson, D.A., Reinert, J.F., Bernier, U.R., Sutton, B.D., Seawright, J.A., 1997. Analysis of the cuticular hydrocarbons among species of the *Anopheles quadrimaculatus* complex (Diptera: Culicidae). J. Am. Mosq. Control. Assoc. 13, 103–111.

Carlson, D.A., Bernier, U.R., Sutton, B.D., 1998. Elution patterns from capillary GC for methyl-branched alkanes. J. Chem. Ecol. 24, 1845–1865.

Charlwood, J.D., Jones, M.D.R., 1980. Mating in the mosquito *Anopheles gambiae* s.l. II. Swarming behaviour. Physiol. Entomol. 5, 315–320.

Chen, C.S., Mulla., M.S., March, R.B., Chaney, J.D., 1990. Cuticular hydrocarbon patterns in *Culex quinquefasciatus* as influenced by age, sex and geography. Bull. Soc. Vector Ecol. 15, 129–139.

Cobb, M., Jallon, J.M., 1990. Pheromones, mate recognition and courtship stimulation in the *Drosophila melanogaster* species subgroup. Anim. Behav. 39, 1058–1067.

Coluzzi, M., Petrarca, V., 1973. Aspirator with paper cup for collecting mosquitoes and other insects. Mosq. News 33, 249–250.

Coluzzi, M., Petrarca, V., Di Deco, M.A., 1985. Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae*. Boll. Zool. 52, 45–63.

Coluzzi, M., Sabatini, A., della Torre, A., Di Deco, M.A., Petrarca, V., 2002. A polytene chromosome analysis of the *Anopheles gambiae* species complex. Science 298, 1415–1418.

della Torre, A., Fanello, C., Akogbeto, M., Dossou-yovo, J., Favia, G., Petrarca, V., Coluzzi, M., 2001. Molecular evidence of incipient speciation within *Anopheles gambiae* s.s. in West Africa. Insect Mol. Biol. 10, 9–18.

della Torre, A., Tu, Z., Petrarca, V., 2005. On the distribution and genetic differentiation of *Anopheles gambiae* s.s. molecular forms. Insect Biochem. Mol. Biol. 35, 755–769.

Desena, M.L., Clark, J.M., Edman, J.D., Symington, S.B., Scott, T.W., Clark, G.G., Peters, T.M., 1999a. Potential for aging female *Aedes aegypti* (Diptera: Culicidae) by gas chromatographic analysis of cuticular hydrocarbons, including a field evaluation. J. Med. Entomol. 36, 811–823.

Desena, M.L., Edman, J.D., Clark, J.M., Symington, S.B., Scott, T.W., 1999b. *Aedes aegypti* (Ditera: Culicidae) age determination by cuticular hydrocarbon analysis of female legs. J. Med. Entomol. 36, 824–830.

Diabate, A., Dabire, R.K., Kengne, P., Brengues, C., Baldet, T., Ouari, A., Simard., F., Lehmann, T., 2006. Mixed swarms of the molecular M and S forms of *Anopheles gambiae* (Diptera: Culicidae) in sympatric area from Burkina Faso. J. Med. Entomol. 43, 480–483.

Fanello, C., Santolamazza, F., della Torre, A., 2002. Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. Med. Vet. Entomol. 16, 461–464.

Favia, G., della Torre, A., Bagayoko, M., Lanfrancotti, A., Sagnon, N.F., Tourè, Y.T., Coluzzi, M., 1997. Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. Insect Mol. Biol. 6, 377–383.

Ferveur, J.F., 1997. The pheromonal role of cuticular hydrocarbons in *Drosophila melanogaster*. Bioassays 19, 353–358.

Ferveur, J.F., 2005. Cuticular hydrocarbons: their evolution and roles in *Drosophila* pheromonal communication. Behav. Genet. 35, 279–295.

Gentile, G., Slotman, M., Ketmaier, V., Powell, J.R., Caccone, A., 2001. Attempts to molecularly distinguish cryptic taxa in *Anopheles gambiae* s.s. Insect Mol. Biol. 10, 25–32.

Gerade, B.B., Lee, S.H., Scott, T.W., Edman, J.D., Harrington, L.C., Kitthawee, S.J., Jones, W., Clark, J.M., 2004. Field validation of *Aedes aegypti* (Diptera: Culicidae) age estimation by analysis of cuticular hydrocarbons. J. Med. Entomol. 41, 231–238.

Gjullin, C.M., Whitfield, T.L., Buckley, J.F., 1967. Male pheromones of *Culex quinquefasciatus*, C. *tarsalis* and *C. pipiens* that attract females of these species. Mosq. News 27, 382–387.

Holt, R.A., et al., 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298, 129–149.

Horne, G.L., Priestman, A.A., 2002. The chemical characterization of the epicuticular hydrocarbons of *Aedes aegypti* (Diptera: Culicidae). Bull. Entomol. Res. 92, 287–294.

Howard, R.W., 1993. Cuticular hydrocarbons and chemical communication. In: I.S.-S.D.W. a. N. D.R. (Ed.), Insect Lipids: Chemistry, Biochemisry and Biology. University of Nebraska Press, Lincoln, Nebraska, pp. 179–226.

Hugo, L.E., Kay, B.H., Eaglesham, G.K., Holling, N., Ryan, P.A., 2006. Investigation of cuticular hydrocarbons for determining the age and survivorship of australasian mosquitoes. Am. J. Trop. Med. Hyg. 74, 462–474.

Jallon, J.M., Wicker-Thomas, C., 2003. Genetic studies on pheromone production in *Drosophila*. In: Blomquist, G.J., Vogt, eR.G. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press.

Kim, Y.K., Phillips, D.R., Chao, T., Ehrman, L., 2004. Developmental isolation and subsequent adult behavior of *Drosophila paulistorum*. VI. Quantitative variation in cuticular hydrocarbons. Behav. Genet. 34, 385–394.

Kittayapong, P., Clark, J.M., Edman, J.D., Potter, T.L., Lavine, B.K., Marion, J.R., Brooks, M., 1990. Cuticular lipid differences between the malaria vector and non-vector forms of the *Anopheles maculatus* complex. Med. Vet. Entomol. 4, 405–413.

Kittayapong, P., Clark, J.M., Edman, J.D., Lavine, V.J., Marion, R., Brooks, M., 1993. Survey of the *Anopheles maculatus* complex (Diptera: Culicidae) in peninsular Malaysia by analysis of cuticular lipids. J. Med. Entomol. 30, 969–974.

Klecka, W.R., 1989. Discriminant Analysis. Sage Publications, Beverly Hills and London Sage University Paper Series on Quantitative Applications in the Social Sciences, No. 07-019.

Kruger, E.L., Pappas, C.D., Howard, R.W., 1991. Cuticular hydrocarbon geographic variation among seven North American populations of *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 28, 859–864.

Kruger, E.L., Pappas, C.D., 1993. Geographic variation of cuticular hydrocarbons among fourteen populations of *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 30, 544–548.

Marchand, R.P., 1984. Field observations on swarming and mating in *Anopheles gambiae* mosquitoes in Tanzania. Neth. J. Zool. 34, 367–387.

Mas, F., Jallon, J., 2005. Sexual isolation and cuticular hydrocarbon differences between *Drosophila santomea* and *Drosophila yakuba*. J. Chem. Ecol. 31, 2747–2752.

Milligan, P.J.M., Phillips, A., Broomfield, G., Molyneux, D.H., Toure, Y.T., Coluzzi, M., 1993. A study of the use of gas chromatography of cuticular hydrocarbons for identifying members of the *Anopheles gambiae* (Diptera: Culicidae) complex. Bull. Entomol. Res. 83, 613–624.

Nelson, D.R., 1978. Long chain methyl-branched hydrocarbons: ocurrance, biosynthesis and function. Adv. Insect Physiol. 13, 1–13.

Nijhout, H.F., Craig, J.G.B., 1971. Reproductive isolation in Stegomyia mosquitoes. III. Evidence for sexual pheromone. Entom. Exper. Applic. 14, 399–412.

Phillips, A., Sabatini, A., Milligan, P.J.M., Boccolini, D., Broomfield, G., Molynneux, D.H., 1990. The *Anopheles maculipennis* complex (Diptera: Culicidae): comparison of the cuticular hydrocarbon profiles determined in adults of five palaeartic species. Bull. Entomol. Res. 80, 459–464.

Polerstock, A.R., Eigenbrode, S.D., Klowden, M.J., 2002. Mating alters the cuticular hydrocarbons of female *Anopheles gambiae* sensu stricto and *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 39, 545–552.

Rosa-Freitas, M.G., Broomfield, V., Priestman, A., Milligan, P.J., Momen, H., Molyneux, D.H., 1992. Cuticular hydrocarbons, isoenzymes and behavior of three populations of *Anopheles darlingi* from Brazil. J. Am. Mosq. Control. Assoc. 8, 357–366.

Stump, A.D., Fitzpatric, M.C., Lobo, N.F., Traore, S., Sagnon, N., Costantini, C., Collins, F.H., Besansky, N.J., 2005. Centromere-proximal differentiation and speciation in *Anopheles gambiae*. Proc. Natl. Acad. Sci. USA 102, 15930–15935.

Tabachnick, B., Fidell, L.S., 1996. Using Multivariate Statistics III. Harper Collins College Publishers, New York.

Touré, Y.T., Petrarca, V., Traore, S.F., Coulibaly, A., Maiga, H.M., Sankare, O., Sow, M., Di Deco, M.A., Coluzzi, M., 1998. The distribution and inversion polymorphism of chromosomally recognized taxa of the *Anopheles gambiae* complex in Mali, West Africa. Parassitologia 40, 477–511.

Tripet, F., Toure, Y.T., Taylor, C.E., Norris, D.E., Dolo, G., Lanzaro, G.C., 2001. DNA analysis of transferred sperm reveals significant levels of gene flow between molecular forms of *Anopheles gambiae*. Mol. Ecol. 10, 1725–1732.

Tripet, F., Toure, Y.T., Dolo, G., Lanzaro, G.C., 2003. Frequency of multiple inseminations in field-collected *Anopheles gambiae* females revealed by DNA analysis of transferred sperm. Am. J. Trop. Med. Hyg. 68, 1–5.

Tripet, F., Dolo, G., Traore, S., Lanzaro, G.C., 2004. The "wingbeat hypothesis" of reproductive isolation between members of the *Anopheles gambiae* complex (Diptera: Culicidae) does not fly. J. Med. Entomol. 41, 375–384.

Turner, T.L., Hahn, M.W., Nuzhdin, S.V., 2005. Genomic islands of speciation in *Anopheles gambiae*. PLoS Biol 3, 285.

Yuval, B., 2006. Mating system of blood-feeding flies. Annual. Rev. Entomol. 51, 413–440.