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ANOPHELES GAMBIAE LARVAL HABITATS IN AN ARID SAVANNA VILLAGE OF BURKINA FASO: CHARACTERIZATION OF BIONOMICAL PARAMETERS AND POTENTIAL MARKERS OF ECOLOGICAL NICHE PARTITIONING AMONG THREE SYMPATRIC TAXA OF THE COMPLEX.

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INTRODUCTION

MALARIA AND PUBLIC HEALTH

Malaria, together with HIV/AIDS and tuberculosis, is one of the major public health challenges undermining the development of the poorest countries in the world. Malaria is a life-threatening parasitic disease caused in the man by 4 species of protozoan Apicomplexa belonging to the genus *Plasmodium* (*P. malariae*, *P. ovale*, *P. vivax* and *P. falciparum*). The latter species is the only that can lead to the death of the infected host. The parasite has a dixenic cycle (Fig. 1), with a mosquito of the genus *Anopheles* as definitive host.

Recent studies report that the exposed population at risk from malaria is around 48% of the global population, distributed in 88 countries; most of them in endemic areas, localized in the tropical belt (see Fig. 2 and Hay *et al*., 2004). The number of deaths caused by malaria is about 1.200.000 of people each year, mainly children less than five years of age living in sub-Saharan Africa (WHO, 2003). Typical consequences of malaria infection are acute febrile illness, chronic debilitation, complications of pregnancy, impairment of physical development and learning ability of children, which have a huge negative social impact in high burden areas (Holding & Snow, 2001).

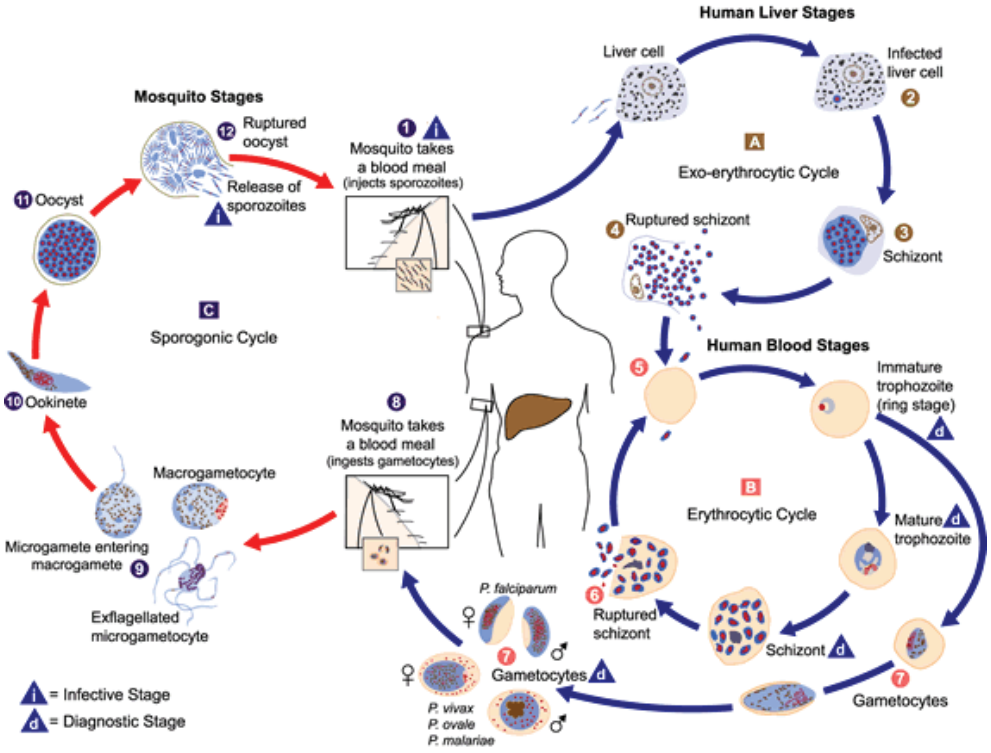

Figure 1: Malaria life cycle. (http://www.med.sc.edu:85/parasitology/blood-proto.htm)

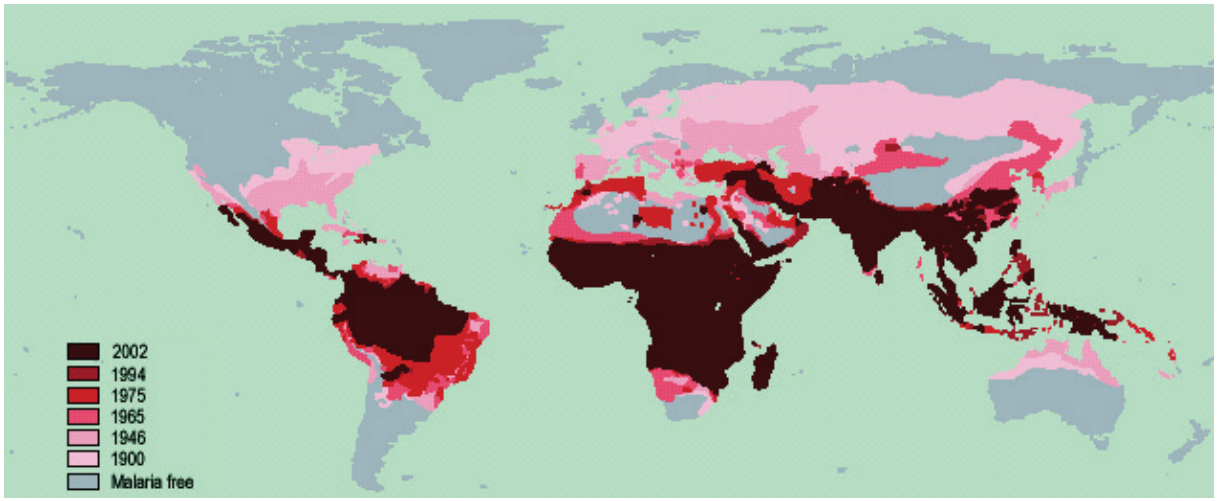

Figure 2: Global distribution of malaria since year 1900 (Hay et al.*, 2004).*

This disease may cost more than 1% of economic growth per year, particularly in sub-Saharan Africa, with a very important impact on these countries (Sachs & Malaney, 2002).

At present, the strategies against malaria in Africa are supported by multilateral research and control programs, funded by public and private international partnerships (Alilio *et al.*, 2004). Research programs are inserted in the context of the Global Malaria Control Strategy, developed since 1990 and discussed in several meetings during these years, that includes four technical elements: provision of early diagnosis and prompt treatment; planning and implementation of selective and sustainable preventive measures, including vector control; early detection and containment or prevention of epidemics; strengthening of local capacities in basic and applied research to permit and promote the regular assessment of a country's malaria situation, in particular the ecological, social and economic determinants of the disease (Teklehaimanot & Bosman, 1999).

THE MAJOR MALARIA VECTOR IN AFRICA: *ANOPHELES GAMBIAE*

THE ANOPHELES GAMBIAE *COMPLEX*

Malaria transmission in Africa is mainly due to two vector mosquitoes: *Anopheles gambiae* Giles and *An. funestus* Giles. *Anopheles gambiae* is by far the most important vector of malaria in Africa, and therefore the most important vector in the world. This species belongs to a complex of seven isomorphic sibling species (Davidson, 1964; White, 1974): *An. quadriannulatus* species A and B, *An. merus*, *An. melas*, *An. bwambae*, *An. gambiae sensu stricto* and *An. arabiensis*. They are morphologically indistinguishable but can be distinguished based on fixed differences on the banding pattern of their polytene chromosomes. Ten fixed paracentric chromosomal inversions can be used to identify individual specimens, but more than 120 polymorphic inversions have been detected in natural populations *(*Coluzzi *et al.*, 1979, 1984, 1985, 2002).

Not all the species of the *An. gambiae* complex are good malaria vectors, because of differences in their distribution, ecology, and behaviour. In fact, *Anopheles gambiae s.s.* and *An. arabiensis* are the major malaria vectors, because of their greater anthropophily and wide distribution. On a continental basis, they show dissimilar distributional limits and asynchronous seasonal prevalence, apparently due to divergent responses to climatic factors (White, 1974 and Touré *et al.*, 1998). Both species of *An.*

quadriannulatus are strictly zoophilic, hence they have no medical importance. *Anopheles gambiae s.s.*, *An. arabiensis* and *An. quadriannulatus* are typically "freshwater species", with a similar larval ecology. They breed in shallow, freshwater pools, exposed to sunlight. Conversely, *An. merus* and *An. melas* develop in saltwater pools and are distributed along the East and West African coastlines, respectively. The last species of the complex, *An. bwambae* has an extremely limited distribution, having been observed so far only in mineral spring pools of the Semliki forest in Uganda. The last three species show a more catholic feeding behaviour, biting man particularly in the absence of alternative hosts (White, 1974).

ANOPHELES GAMBIAE SENSU STRICTO*: CHROMOSOMAL AND MOLECULAR FORMS*

Polytene chromosome studies on *Anopheles gambiae s.s.*, carried out on population samples collected from various African sub-Saharan localities, have revealed paracentric inversion polymorphisms on the second chromosome associated with ecotypic differentiation, e.g. with the frequency of some arrangements correlated with the degree of aridity (Coluzzi *et al.*, 1979, 1984, 1985, 2002; della Torre *et al.*, 2001, 2002; Touré *et al.*, 1994, 1998). As the significant deviations from Hardy-Weinberg equilibrium of many samples for all inversions of the right arm of chromosome 2, and

the persistence of heterozygote deficiencies despite variations in the frequency of the alternative arrangements, the species *An. gambiae s.s.* was tentatively splitted into five taxa. These taxonomic units were named chromosomal forms Mopti, Savanna, Bamako, Forest and Bissau (Coluzzi *et al.*, 1985). Despite their interfertility in laboratory condition, these taxa showed little or no intergradations between certain of them, as demonstrated by cytogenetical analysis (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985; Touré *et al.*, 1983a, 1994, 1998). In particular, in Sudan savannah areas of Mali and Burkina Faso the chromosomal forms Mopti, Savanna and Bamako have a strongly reproductive isolation, despite their simpatry (Touré *et al.*, 1998).

Subsequent to the discovery of *An. gambiae s.s.* chromosomal forms, the genotyping of X-linked rDNA in this species has led to the characterization of two taxa, named molecular forms M and S. Between these two taxa the gene flow is highly reduced, as shown from the rare heterogamous mating (less than 1%; Tripet *et al.,* 2001). The molecular forms M and S differ in both the transcribed and non transcribed spacers in the rDNA repeat unit (della Torre *et al.*, 2001 and Gentile *et al.*, 2001). The relationship between the molecular forms and chromosomal forms varies according to their ecological and geographic distribution. In some areas of West Africa, such as Mali and Burkina Faso, there is a complete correspondence between the M molecular form and Mopti chromosomal form. Similarly, the S molecular form always corresponds to the Savanna or Bamako chromosomal form (Favia *et al.*, 1997). This correspondence

breaks down in other regions of West Africa, as shown in Figure 3 (della Torre *et al.*, 2001, 2002). The importance of chromosomal inversions in ecological adaptation is well known (Powell, 1999). Then, probably the different chromosomal forms are indicators of adaptation to different ecological habitats. In contrast, the molecular forms M and S reflect barriers to gene flow indicating a process of incipient speciation (della Torre *et al.*, 2001, 2002)

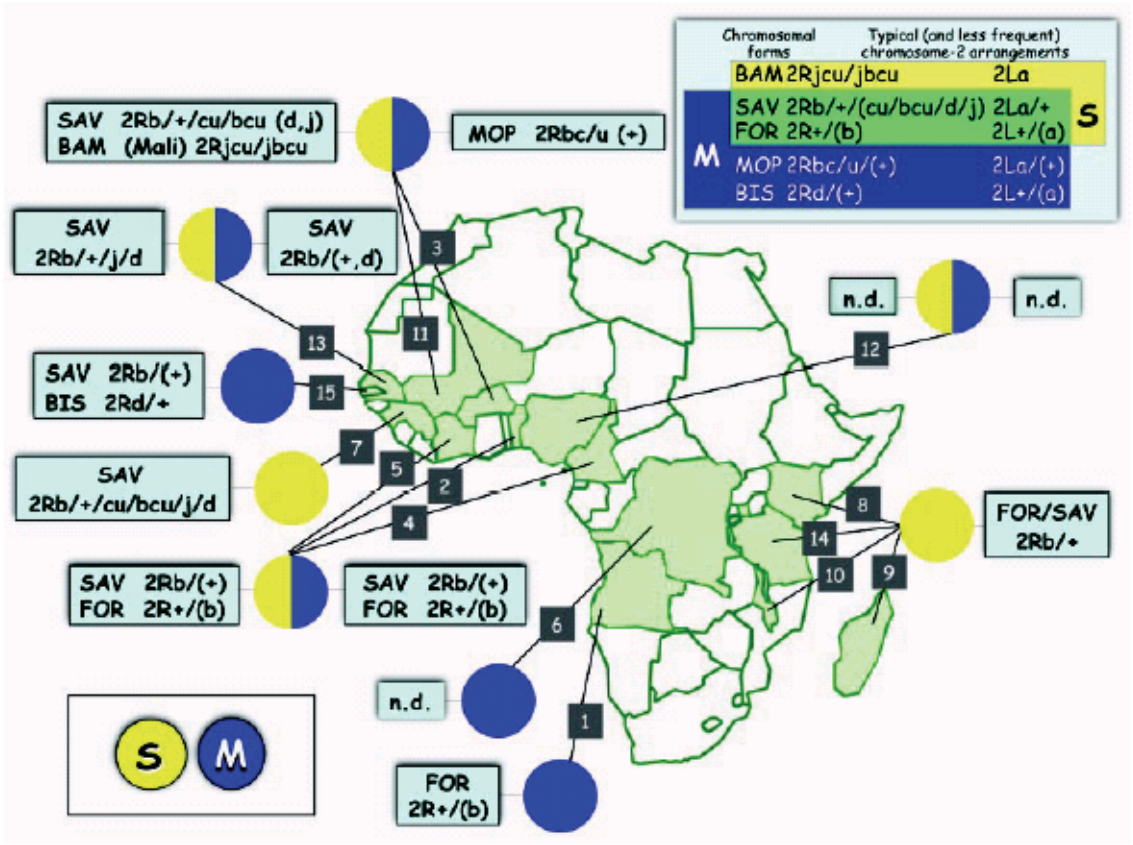

Figure 3: Geographical distribution of the molecular forms (S and M) of Anopheles gambiae sensu stricto *and their relation to chromosomal forms (see della Torre* et al.*, 2002).*

LARVAL ECOLOGY AND OVIPOSITION BEHAVIOUR OF MOSQUITOES.

The distribution of larvae among natural larval habitats is usually determined by the choice of the oviposition site by conspecific gravid females (Clements, 1999). This means that, in the case of many species, adult females lay their eggs only in particular biotopes (Macan, 1961) and larval densities depend on such behaviour (Herms, 1921; Rudolfs, 1929; Beattie, 1932; Bates, 1940; Kennedy, 1942; Wallis, 1954). There are some mosquito species that are completely opportunistic with respect to the site where to oviposit, e.g. *Culex nigripalpus*, that lay eggs in almost any kind of water collection (Day& Edman, 1988). The various oviposition strategies of mosquitoes have been reviewed by Mattingly (1971).

Bentley & Day (1989) summarized four broad ovipositional strategies in mosquitoes. Oviposition of single eggs on the water surface, usually hovering above it and often making no contact with the water. The genera that show this behaviour include *Anopheles*, *Sabethes*, *Toxorhynchites* and *Wyeomyia*. Instead, genera *Coquillettidia*, *Culex* and *Culiseta* deposit egg rafts directly on the water surface. *Aedes* and *Phosophora* species generally oviposit single eggs on moist soil before the flooding of the potential breeding site. Finally, most *Mansonioides*, *Mansonia* and *Aedeomyia*,

and some *Culex* and *Anopheles* use a complicate strategy that involves the attachment of an egg raft to vegetation, usually below the water surface.

Mosquitoes that oviposit single eggs above the water line, and those that oviposit in permanent sites, can lay their eggs as soon as egg development has been completed. Conversely, females that lay eggs on the water surface of temporary pools (some *Anopheles*, for instance) need to locate a site that is immediately suitable for larval development. In the absence of a suitable site, these mosquitoes can retain their eggs until they find one. These species are generally long-lived, and this life history trait is important in relation to their ability of disease transmission, because longevity increases their vectorial capacity (Bellamy *et al.*, 1968; Day & Edman, 1988; Shroyer & Sanders, 1977).

Mosquito egg laying is conveniently distinguished in two stages (Clements, 1999): pre-oviposition behaviour and oviposition. Pre-oviposition behaviour has two phases: behavioural responses of gravid females that result in arrival at potential oviposition sites, and behavioural responses that indicate the suitability or acceptability of such sites. Oviposition is the act of depositing eggs. Ovulation, the emission of mature oocytes from the ovarian follicles into the calyces and oviducts, is considered part of oviposition.

Pre-oviposition behaviour has many similarities with host-seeking behaviour for blood feeding. In fact, both require a complex integration of physical and chemical cues

by questing mosquitoes (Bentley & Day, 1989). Finding blood sources is the consequence of a series of a complex behavioural program, influenced both by internal and external stimuli, leading eventually the mosquito female close to and onto the host (Takken, 1991 and Costantini *et al.*, 1999). Host-seeking usually occurs when a responsive female perceives a series of exogenous host stimuli, some of which are graphically sketched in Figure 4 (Clements, 1999 and Costantini *et al.*, 1999); after engorgement of a sufficient quantity of blood, host-seeking behaviour is generally inhibited by endogenous factors until eggs are laid (Klowden, 1990; Clements, 1999; Takken *et al.*, 2000).

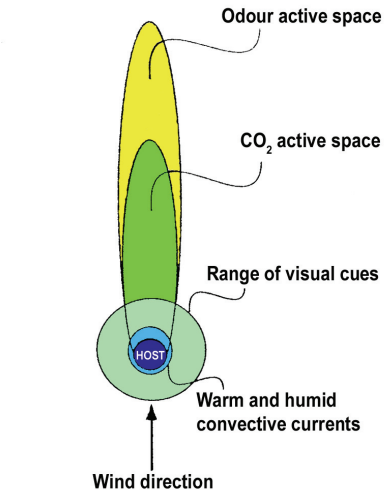

Figure 4: Sketch of how host cues are presented to an approaching mosquito (modified from Costantini et al.*, 1999).*

As for host-finding, the identification of suitable larval habitats is initiated by long-range cues, both chemical and visual (Bidlingmayer, 1975). Then, other stimuli,

mainly volatile factors, become informative for the approach to the site. When the site is identified, short-range cues, such as water temperature and chemicals perceived after contact with the water, become important to elicit oviposition (Bentley & Day, 1989 and Clements, 1999). The identification of volatile oviposition-site stimuli involves the antennal sensilla (Mourdue *et al.* 1992; Blackwell *et al.*, 1993; Collins & Blackwell, 1998; Blackwell & Johnson, 2000), whereas non-volatile molecules present in the water are identified by sensilla on the antennae and tarsi, as well as on the labella or labrum (Ikeshoji, 1966c, d).

Dethier and others (1960) categorized chemical compounds in terms of the responses given to them by gravid females:

- *Oviposition attractant*. A substance that induces gravid females to make oriented movements towards its source.
- *Oviposition arrestant*. A substance that induces gravid females to remain in its presence through their own undirected kinetic responses, either by stopping their locomotion or changing their rate of turning.
- *Oviposition repellent*. A volatile substance that causes gravid females to make oriented movements away from its source.
- *Oviposition deterrent*. A non-volatile substance that inhibits oviposition when present in a place where insects, in its absence, would oviposit.

– *Oviposition stimulant*. A substance that elicits oviposition.

It is difficult to characterize larval habitats in terms of the parameters to which gravid females respond to. For one thing, many habitat variables are interrelated, and some characteristics of larval habitats that provide directional cues for gravid females have little or no survival value for the larvae (Clements, 1999). Despite that, many studies have been made to understand the influence of chemical and physical parameters on the pre-oviposition behaviour of mosquitoes. Hereafter, I will provide an overview of the main results obtained so far in this field. For more comprehensive reviews, see chapter 40 of Clements (1999) and Takken & Knols (1999).

Inorganic salts – Most rock-hole species deposit their eggs on the rock surface, and for coastal species both surface roughness and salinity are significant cues for ovipositing females (Clements, 1999). For freshwater species the tolerance range to inorganic salt is generally narrow. For instance, a series of NaCl solutions up to 0.15M were presented with distilled water to *Cx. pipiens*. The majority of egg rafts was deposited on the most dilute NaCl solutions. The same response was shown to solutions of a number of other salts, such as Na_2SO_4, $NaHCO_3$, KCl and KNO_3. The acceptability of most solutions declined with increasing concentration above 0.04M. Females of both *Cx. pipiens* and *Ae. aegypti* laid very few eggs in salt concentrations greater than 0.17 M, and none in concentrations greater than 0.34M (Wallis, 1954b and Hudson, 1956). *Culex quinquefasciatus* tolerate depositing their eggs in 0.05, 0.1, and 0.15M solutions

of $NaHCO_3$ and Na_2CO_3. When distilled water and salt concentrations in the range 0.3-1.2M were available, even the lowest concentrations of $NaHCO_3$ and Na_2CO_3 were strongly deterrent to *Ps. confinnis*, *Ae. sollicitans* and *Ae. taeniorhynchus,* possibly due to their high pH. *Aedes solicitans* and *Ae. taeniorhynchus* showed some tolerance of 0.3M NaCl, $MgCl_2$ and $CaCl_2$, but laid many more eggs on distilled water (Petersen, 1969; Petersen & Willis, 1970). Fresh water normally is not distilled water; hence it is not anomalous that more eggs were laid by freshwater species in dilute sale solutions than in distilled water. Fresh water is defined as having a salinity of <0.5 parts per thousand (0.0085M NaCl), or alternatively of <2 parts per thousand (0.034M NaCl) (Lincoln *et al.,* 1982).

Organic substances of environmental origin – Many authors demonstrated that when gravid females of a variety of species were presented with both distilled water and water from a natural larval habitat, few eggs were laid on or near distilled water (Gjullin *et al.*, 1965; Wilton, 1968; Ahmadi & McClelland, 1983). As an example, females of *Culex quinquefasciatus* laid significantly more often on samples of water from ditches, pit latrines and septic tanks in which the larvae occurred than on samples from ponds where no *Cx. quinquefasciatus* larvae were found (Ikeshoji, 1966b). Water that contains human or animal faeces is acceptable to gravid females of some species of *Culex* and *Armigeres*, but the extent of pollution affects its activity (Ikeshoji, 1966a; lkeshoji and Mulla, 1970; Mian and Mulla, 1986). It has long been known that mosquitoes will

oviposit on or near infusions of vegetable matter (Buxton & Hopkins, 1927). Such infusions mimic some natural waters containing decomposing plant materials, and have been used to bait ovitraps. Infusions are complex mixtures of organic substances, and their composition is variable. In laboratory assays, fermented grass and hay infusions usually proved more acceptable than tap water to gravid females of *Cx quinquefasciatus* and *Ae. aegypti* (Hazard *et al.,* 1967; Reiter *et al.,* 1991; Millar *et al.,* 1992; Chadee *et al.,* 1993b). The acceptability of infusions of plant material to gravid females increases and eventually declines with age, presumably because microbial fermentation changes the content of active substances (Murphey & Burbutis, 1967; Hazard *et al.*, 1967; Bentley *et al.*, 1979; Millar *et al.*, 1992). Volatile factors can act as oviposition stimulants. Before being exposed to ovipots containing tap water, one-half of a batch of gravid females of *Cx. quinquefasciatus* was briefly exposed to the vapour of a volatile fraction from water from the larval habitat; the remaining females were exposed to the open air. The control and treatment groups differed significantly in the proportion of females ovipositing within 30 min after exposure to the ovipots, and in the median times to oviposition (Ikeshoji, 1966b). Non-volatile constituents of infusions also affect egg laying, presumably by acting as arrestants or oviposition stimulants. When Bermuda grass infusion was fractionated by centrifugation and dialysis the non-dialyzable fraction containing large molecules (>12 kDa) significantly enhanced egg deposition by *Cx. tarsalis*. Also, that fraction retained its activity after filter sterilization (Isoe & Millar, 1995).

Emanations from larval stages – In many laboratory studies, gravid mosquitoes oviposited preferentially on water that had previously contained larvae, pupae, exuviae, or emerging adults, and this was ascribed to chemical factors produced by them (Hudson & McLintock, 1967; Soman & Reuben, 1970; Bentley *et al.,* 1976; Reisen & Siddiqui, 1978; McDaniel *et al.,* 1979). These putative factors, tentatively classified as pheromones by some authors, were thought to enhance the oviposition rate on bodies of water already successfully colonized by the same species. Most early studies of putative larval and pupal factors did not take account of the effects of bacteria, which can modify the acceptability of water to gravid females, as mentioned before. The enhanced acceptability to gravid females of *Aedes togoi* of pupal "holding water" was lost after passage through a filter of 0.2µm-pore. When "holding water" was prepared with larvae of *Cx. pipiens* and *Ae. togoi* that had previously been glutted with kaolin, and was passed through filters excluding bacteria before the bioassay, it remained significantly more active than the control (Dadd & Kleinjan, 1974; Trimble & Wellington, 1980). In the work of Benzon and Apperson (1988) with *Ae. aegypti*, in which the effects of both bacteria and bacterial metabolites were examined, the results indicated that enhanced acceptability of larval holding water was due to the presence of bacterial metabolites (see below) produced during the holding period, and not to factors produced by the larvae themselves. In the absence of bacterial metabolites, larval factors could have repellent or deterrent effects. Despite these data, the effect of a demonstrated oviposition pheromone, *erythro*-6-acetoxy-5-hexadecanolide (Fig. 5), was described by

Laurence & Pickett (1982, 1985). This molecule was extracted from the apical droplet left at the tip of the eggs by ovipositing females of *Cx. quinquefasciatu*s. Gravid conspecifics as well as *Culex tarsalis* are highly attracted to the pheromone (Otieno *et al.*, 1988; Pile *et al.*, 1991, 1993; Millar *et al.*, 1994). Mordue *et al.* (1992) demonstrated the presence of electrophysiological activity in *Cx. quinquefasciatus* in response to the pheromone. Other oviposition pheromones have not been described, although Osgood (1971) reported a pheromone-like substance associated with the apical droplets of egg rafts of *Cx. tarsali*s. The chemical nature of this substance has not been elucidated, but available data suggest that it is related to *erythr*o-6-acetoxy-5-hexadecanolide.

Effects of organic compounds – Fatty acids, i.e. saturated and unsaturated monobasic carboxylic acids, when active with gravid females, tended to induce negative responses. Saturated fatty acids from C_2 (ethanoic) to C_{11} (undecanoic) had significant negative oviposition responses with *Cx. quinquefasciatus*. In general, *Ae. aegypti* was somewhat less, and *Cx. tarsalis* substantially less, sensitive to these compounds than *Cx. quinquefasciatus.* Activity was greatest in the C_8 to C_{10} acids, with nonanoic acid (C_9) eliciting significant negative responses in *Ae. aegypti* at $3x10^{-5}$M (Hwang *et al.,* 1980, 1982). With *Culiseta incidens,* the oviposition activity was dose-dependent, being negative at $6x10^{-1}$ and $6x10^{-2}$M, and positive at $6x10^{-3}$ and $6x10^{-4}$M. Four other species did not respond positively at lower concentrations (Kramer *et al.,* 1980). Saturated fatty

acids between C_{15} and C_{22} were inactive against *Cx. quinquefasciatus* when presented in aqueous suspension at concentrations 10^{-3}M and less, but had negative effects at 10^{-4}M (Hwang *et al.*, 1984). The avoidance of potential biotopes that contain high concentrations of some carboxylic acids may have a selective advantage. In the laboratory, $6x10^{-2}$M butanoic acid caused 60% mortality among first instar larvae of *Cx. quinquefasciatus* (Hwang *et al.,* 1980). The addition of decanoic (capric) acid to artificial ponds at 150 p.p.m. kills *Cx. restuans* larvae within 3 days at 26°C and within 14 days at 10°C, during which period the sites were avoided for oviposition. After this period the pools gradually became suitable and some, which were found to contain bacteria of the family Pseudomonadaceae, became highly attractive to gravid females (Maw, 1970). That the females responded to metabolic products of the decanoic acid was suggested by laboratory experiments with females of *Cx. pipiens* exposed to test solutions containing decanoic acid laid 14 times more rafts on solutions that had been incubated with *Pseudomonas aeruginosa* (Ikeshoji *et al.,* 1967). Esters derived by short chain carboxylic acids (ethanoic, propanoic and butanoic) yielded similar responses when essayed with *Ae. aegypti*. Weak positive responses was induced by methyl-, ethyl- and propyl- esters, whereas butyl- and amyl- esters were inactive or leaded to negative responses (Perry & Fay, 1967). The methyl- and some dimethyl- derivatives of phenol produced positive responses with some *Aedes* and *Toxorhynchites* and *Cx. quinquefasciatus*, as the *p*-cresol (Ikeshoji, 1975; Bentley *et al.*, 1981; Linley, 1989; McCall & Eaton, 2001; Collins & Blackwell, 2002; see Fig. 5). However, the most

active molecules bioassayed were indole and skatole (3-methylindole, see Fig. 5), the latter active at nanomolar concentrations (Millar *et al.,* 1992, 1994; Mordue *et al.*, 1992; McCall & Eaton, 2001; Collins & Blackwell, 2002). Indole is present in the faeces of some animal species, whereas 3-methylindole is present in human faeces (Stoddart, 1990). Conversely, Trexler and co-workers (2003) did not find any effect of skatole, *p*-cresol, indole, dimethyl-disulfide and trimethylamine as oviposition attractants for *Ae. albopictus*. In conclusion, all these compounds, when effective, are more active as complex blends rather than as single molecules, as showed by the experiments of Du and Millar (1999) with *Cx. tarsalis* and *Cx. quinquefasciatus*.

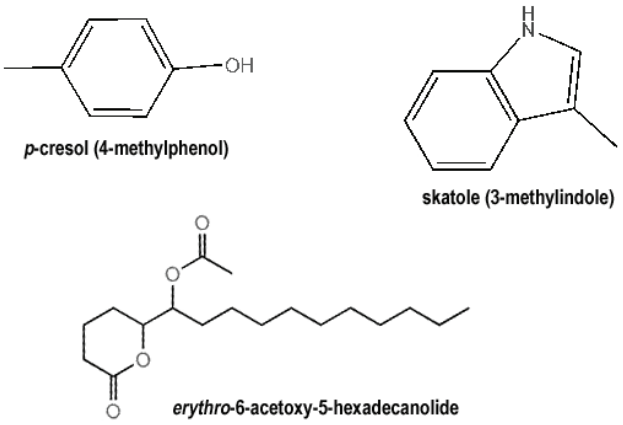

Figure 5: Molecular structure of certain compounds that affect the pre-oviposition behaviour of gravid mosquitoes (see text for details).

Role of microbial flora – Some authors reported that exposure to bacteria alone enhanced the oviposition rate of mosquitoes. In fact, semiochemicals produced in infusions are often microbial metabolites due to the decomposition of organic matter (Millar *et al.*, 1992). Bacteria are abundant in the tree hole habitats of *Aedes triseriatus* (Walker *et al.*, 1991) and the marsh habitats of *Anopheles quadrimaculatus* (Walker & Merritt, 1993; Smith *et al.*, 1998). The analysis of larval gut content has shown that bacteria are common in the food bolus of both species (Wallace & Merritt, 1999); feeding by *Ae. triseriatus* has been shown to reduce bacterial density (Walker *et al.*, 1991), and alter the microbial community structure (Kaufman *et al.*, 1999).These observations indicate that bacteria serve as the primary food source for larvae, converting carbon and inorganic nutrients into forms that can be assimilated by larvae. Mosquitoes oviposit differently in relation to the composition of microbial species present in the media. We reported in a previous paragraph the role of bacteria of the family Pseudomonaceae in the production of attractive molecules for *Culex restuans* (Maw 1970), *Aedes aegypti* and *Cx. pipiens molestus* (Ikeshoji *et al.*, 1975). Also, Hazard and co-workers (1967) reported isolating bacterial species from an infusion of alfalfa hay that produced oviposition stimulants in *Ae. aegypti* and *Cx. quinquefasciatus*. Rockett (1987) tested various bacterial species against *Cx. quinquefasciatus*, which laid significantly more egg rafts in solutions containing agar washes of *Enterobacter agglomerans*, *Pseudomonas maltophilia* and *Bacillus cereus* than in control containing water only. Therefore, Benzon and Apperson (1988) isolated

from larval-rearing water *Acinetobacter calcoaceticus* and *Enterobacter cloacae*, which lead positive oviposition responses for gravid *Ae. aegypti*. Vythilingam et al.(1999) also observed that *Ae. aegypti* preferred to oviposit in well water that contained *Acinetobacter anitratus*. Other authors observed differential oviposition responses by *Ae. aegypti* and *Ae. albopictus* to several bacterial species (Hasselschwert & Rockett, 1988 and Pavlovich & Rockett, 2000). In particular, Trexler and co-workers (2003) observed that water containing *Psychrobacter immobilis*, *Sphingobacterium multivorum*, and an undetermined *Bacillus* species elicited in *Ae. albopictus* significantly higher oviposition rate than control water without bacteria.

However, the effect on oviposition behaviour of each factor varied according to what other factors were presented simultaneously, and it's impossible to define their order of importance. Also, no factor has been shown to be more attractive than all others when tested against them singly (Clements, 1999).

LARVAL ECOLOGY AND OVIPOSITION BEHAVIOUR IN ANOPHELES GAMBIAE.

Larvae of the freshwater species of malaria vectors of *Anopheles gambiae* complex are most common in pools and puddles exposed to sunlight, devoid of marginal vegetation and often with turbid water. However, they are found also in other

water collections, often resulting from human activities (Muirhead-Thomson 1951; Holstein, 1954; Gillies & De Meillon, 1968; White *et al.*, 1972; Charlwood & Edoh, 1996; Minakawa *et al.*, 1999; Gimnig *et al.*, 2001).

The in Sub-Saharan areas, some species of the complex show different population dynamics. In West Africa, *An. gambiae* chromosomal form Mopti and *An. arabiensis* are present all-year round exploiting large water collections, not dependent on rainfall, e.g. edges of man-made lakes, pools formed as river levels subside, irrigated gardens, wells and flooded borrow pits (White & Rosen, 1973; Touré et al., 1983b; Coluzzi et al. 1985). In some areas, during the dry season, *An. arabiensis* and *A. gambiae* S-form densities are very low, and virtually no specimens can be found during this period. They reappear after the onset of rains, when rainy-dependent breeding sites become available, with population densities that increase in abundance very rapidly (Holstein 1954; Gillies and De Meillon 1968; Taylor et al. 1993; Fontenille et al. 1997). There are locations where *An. gambiae* S-form, despite its large presence during rainy season, becomes impossible to find during dry season, even when breeding sites are made available (Touré, 1985; Petrarca *et al.*, 1986). Omer and Cloudsley-Thomson (1968, 1970) found evidence for gonotrophic dissociation in *An. arabiensis* females that survived the 8-month dry season, taking only partial blood meals and experiencing a single gonotrophic cycle during the entire period. The mosquitoes apparently lived in dwelling huts, cracks down dry wells, and in rodent burrows. Taylor and others (1993) calculated

the effective size (N_e) of *An. arabiensis* populations for several locations in West Africa. Their findings support the hypothesis that populations of this taxon are continuous trough the year in this region, as the same way of *An. gambiae* M-form, with many individuals surviving through the dry season, probably in an altered physiological state. Despite these evidences for *An. arabiensis*, it is still unknown how *An. gambiae* S-form populations are maintained through the dry season.

Recent studies (Minakawa *et al.*, 1999; Gimnig *et al.*, 2001) showed that small size, the presence of turbid water and algae, and the absence of emergent vegetation were associated with the presence of *An. gambiae* larvae. However, these studies were descriptive surveys that did not determine the adult productivity of the habitats observed. In a study made in the western Kenyan highland, *An. gambiae s.s.* larvae were present mostly in small pools (mainly animal hoof prints), in swamp margins, and roadside ditches, and in habitats characterized by a prevalence of short plants, whereas they were absent in habitats with floating vegetation (Minakawa *et al.*, 2004). The same study did not find any association between the presence of *An. gambiae s.s.* larvae and pH or conductivity. Also, the occurrence of this species was not significantly associated with the distance of each breeding site to the nearest house, where resting adults were sampled using the pyrethrum spray catch method. Up to now, no field studies found a significant association between the chemical parameters of breeding sites and larval occurrence of freshwater malaria vectors of *An. gambiae* complex.

The importance of the presence of algae and bacteria as larval food on *An. gambiae* breeding sites was demonstrated by a semi-field experiment in western Kenya (Gimnig et al., 2002). In artificial larval habitats the most likely food source for *An. gambiae* larvae was algal growth, which was significantly reduced by the presence of larvae. Bacterial densities were not significantly affected by the presence of larvae, although they could provide a secondary food source when algal resources are minimal.

A detailed study of *Anopheles gambiae* oviposition behaviour was carried out by McCrae (1983, 1984). This author measured the temporal pattern of oviposition, and the role of different kinds of water, in conjunction with visual stimuli, that may mediate oviposition behaviour in this mosquito. Despite a common feature of *An. gambiae* breeding sites is the paleness of turbid water, McCrae found that this species preferred a dark over a light background as oviposition substrate. Also, water from a natural breeding site attracted more ovipositing females than tap or distilled water. In the presence of white oviposition targets, *An. gambiae* females laid their eggs from a settled posture, whereas over black targets they oviposited in flight, thereby showing two laying strategies dependent upon visual stimuli from the substrate. This author suggested that the settled posture might be a response to sub-optimal stimuli, whereas oviposition in flight is the normal behavioural repertoire of *An. gambiae* under natural conditions.

As in the case of other mosquito species, *An. gambiae s.s.* gives electro-antennogram responses to 3-methylindole, indole, and *p*-cresol, suggesting that it is receptive to these volatiles (Blackwell and Johnson, 2000). This study did not investigate the behavioural role of these molecules, but their effect as oviposition attractants observed in some species of *Culex*, *Aedes*, *Toxorhynchites* (see previous paragraph) may suggest a similar function in *An.gambiae*.

BASELINE DATA AND RATIONALE

ECOLOGICAL STUDIES

Burkina Faso, and in particular our study area, the village of Goundry, are ideal environment for studies on behavioural and ecological differences between members of on the vector *An. gambiae s.l.*, given the presence in simpatry of the most anthropophilic species of the complex: *An. arabiensis* and *An. gambiae s.s*. The latter taxon shows a great intra-specific diversity due to the presence of chromosomal forms Mopti and Savanna. These forms, as previously shown, are overlapped in this area respectively with the molecular forms M and S (Petrarca et al., 1986b; della Torre et al., 2001). Moreover, in this village are present permanent water collections that increase the availability of breeding sites, as during the dry season as during the rainy season, influencing indirectly the malaria transmission.

In this zone, at the end of rainy season 1996, preliminary entomological surveys have been carried out to better understand the biology of the taxa of *An. gambiae* complex by larval samplings and capture of indoor-resting adults (Sagnon, 1999). This analysis confirmed the simpatry in this area of the three taxa *An. arabiensis*, *An. gambiae* molecular form M and *An. gambiae* molecular form S, as already evidenced in previous studies (Petrarca *et al*., 1986; Merzagora, 1993; Costantini *et al*., 1996a). Therefore, the study suggested some hypotheses in order to explain the strong difference found between the relative frequencies of the three taxa in the larval sample in relation to the adult sample.

Therefore, the studies made on 1996 and 2000 (Sagnon, 1999; Costantini *et al.*, unpublished data) hypothesize competition phenomena and/or ecological niche partitioning between the larval populations of the two *An. gambiae s.s.* molecular forms. These studies found differences of larval niche between the taxa in relation to the origin of breeding sites. The S form was relatively more frequent in breeding sites originated by accumulation of rainfall onto impermeable clay soil, creating small temporary puddles (hereafter, "residual breeding sites"). On the contrary, M was more frequent in breeding sites characterized by emergence of the water table through seepage and originated by human activities (hereafter, "filtration breeding sites"). The last type of breeding site is not directly rainy-dependent from the precipitations, then is generally less ephemeral than puddles of residual origin. The same studies found also the greater

frequency of the taxon M in the area around to the artificial lake, suggesting that this larval habitat can constitute the source of major productivity for *An. gambiae s.s.* molecular form M respect to the S-form. However, these studies were completed in a short period of time, "photographing" the entomological situation of the area, without verify if this situation represents a stable equilibrium rather than a dynamic one.

As part of these studies, this thesis attempts to verify the observations on *An. gambiae* complex carried out from Sagnon, Costantini and collaborators during a longer period, specifically all the rainy season (from July to October). We extended the larval samplings to a wider range of larval habitats, to verify the hypothesis of a possible larval niche partitioning between the two molecular forms of *An. gambiae s.s*.

Moreover, we will try to characterize more accurately the biotic and abiotic characteristics of the larval habitats of *An. gambiae* complex, in the attempt to characterize eventual ecological markers associated to the larval presence of the three sympatric taxa in different habitats. We measured a broad range of physico-chemical parameters and was carried out a preliminary characterization of the water surface bacterial flora present in the breeding habitats sampled. The aim of this last aspect of the study is not only to define ecologically the *An. gambiae* larval biotope, but also to verify if there is some parameter associated to the presence of a taxon, allowing therefore obtaining new knowledge for the study of the ecology and behaviour of the taxa of *An. gambiae s.s*.

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MATERIALS AND METHODS

STUDY AREA: BURKINA FASO AND THE GOUNDRY VILLAGE

Burkina Faso (13°N - 2°W, Fig.6) is surrounded by six countries: Mali to the north, Niger to the east, Benin to the southeast, Togo and Ghana to the south, and the Côte d'Ivoire to the southwest.

Figure 6: Map of Burkina Faso.

The larger part of the country is covered by lowland, which forms a gently undulating landscape with, in some areas, a few isolated hills, and the last vestiges of a Precambrian massif. The south-west of the country forms a sandstone massif, the Banfora, where the highest peak is found: Ténakourou (749m). The average altitude is 400m and the difference between the highest and lowest terrain is no greater than 600m.

The country owed its former name of Upper Volta to three rivers which cross it: le Mouhoun (formerly Black Volta), le Nakambé (the White Volta) and le Nazinon (the Red Volta). Le Mouhoun, along with la Comoé which flows to the south west, is the country's only river which flows year-round. The basin of the Niger River also drains a little part of the country's surface. The country also contains numerous lakes.

Burkina Faso has a tropical climate with two very distinct seasons: the rainy season with between 600 and 900mm of rainfall, and the dry season during which the harmattan blows, a hot dry wind from the Sahara. The rainy season lasts approximately 4 months, May/June to September, and is shorter in the north of the country.

Three large climatic zones are present in the country, from the north to the south: the Sahel zone, the Sudanese savannah and the Guinean savannah.

The sampling activity in the field was carried out in the Goundry village (12°30’ N 1°20' W, Fig. 7), 35 km N-E from the capital Ouagadougou, in the Oubritenga province. About 800 people live in Goundry, distributed in c. 400 huts grouped in c. 80 compounds, dispersed over an area of c. 4 km2.

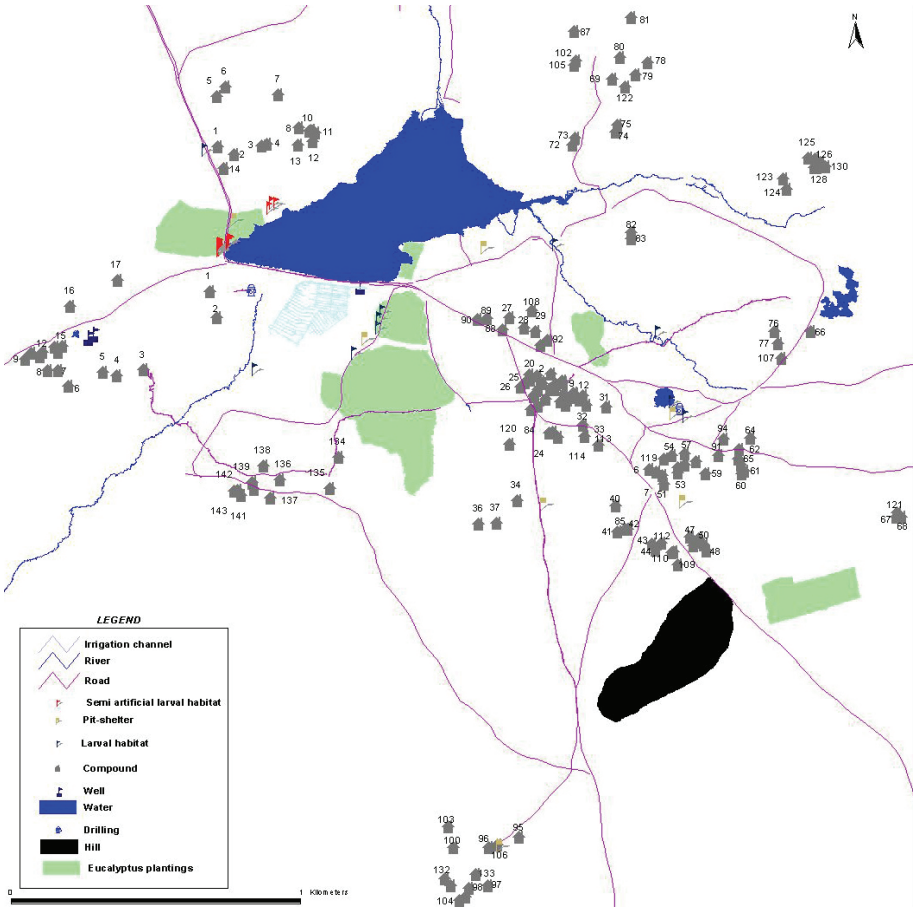


Figure 7: Map of the Goundry village.

The village lie in the West African Sudan savannah vegetation belt. The original vegetation in this area has been substantially modified by human activities. The landscape is therefore characterised by patches of tree-savannah or bush-savannah, according to the degree of degradation of the primary vegetation and the nature of the soil. Domestic animals are diffused in the village, with a presence of sheep, goats, poultry, dogs and donkeys.

SAMPLING OF LARVAL HABITATS

In the Goundry village, during the rainy season, there are a variety of water collections, different for origin, persistence in and number: a series of puddles, located mainly along the roads; two borrow pits, sites of clay extraction for the fabrication of huts; some pools, result of the activity of sand extraction for the same reason; an artificial lake, the only permanent water collection of the village; three irrigation streams, with several associated puddles, that collect the rainwater flowing into the lake or originating from it; various artificial pits at the margins of the lake, used for the irrigation of the small cultivations of vegetables; finally, a great amount of hoof prints of cattle in proximity of the lake, that are filled up of water by filtration from the ground.

These water collections can qualitatively classified based on their origin and temporality. In Figure 8 there is a schematic illustration of the eight categories of water collections present in the village, all potential breeding sites of *Anopheles gambiae*. This classification, purely qualitative, is presented just to show the variability of larval biotopes presents in the area. In the figure, the two orthogonal axes represent the gradients of temporality (vertical axis) and origin (horizontal axis) of larval habitat water. As an example, it can be experiential as the lake is the most permanent water collection, with mixed water, originated as from rainwater as from water originated from the seepage. To the other end we find more ephemeral water collections, like puddles and hoof prints, which are distinguished themselves for the nature of the water: in fact first they contain exclusively rain water, while the second ones have a strong filtration component, being constituted from water that permeate from the mud soil.

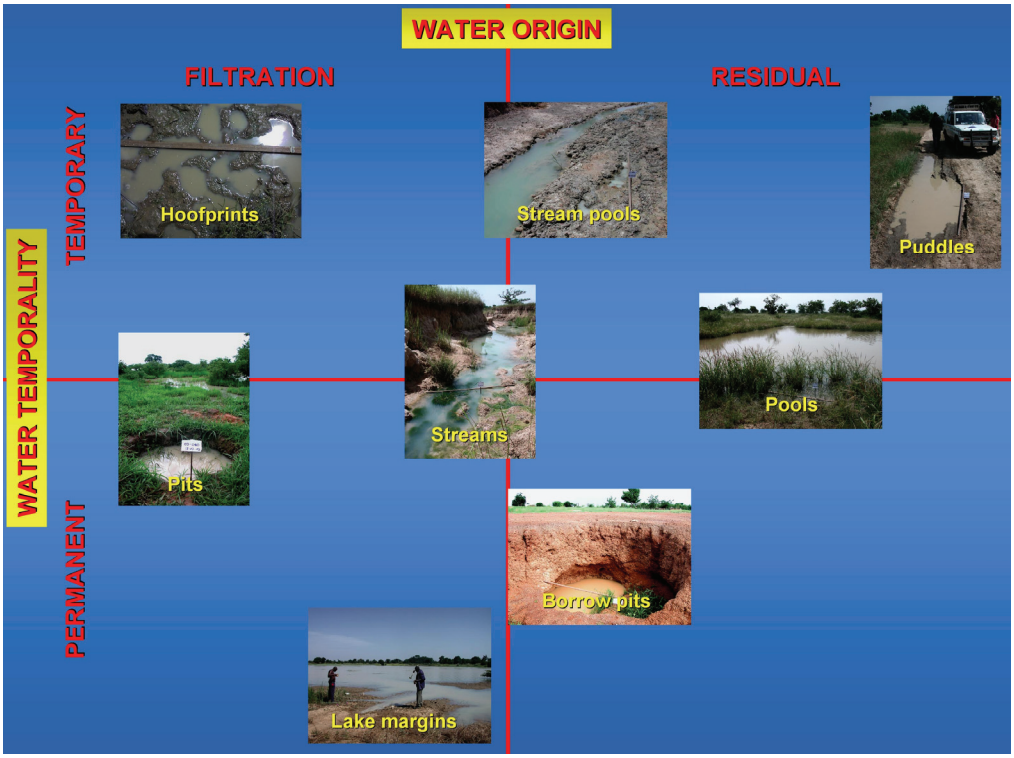


Figure 8: Classification of Anopheles gambiae *larval habitats observed in the Goundry village, based on water origin and temporality.*

During the rainy season 2003, in the 4 months between July and October, an extended sampling of the breeding sites of *An. gambiae* has been carried out in the village of Goundry. In total 113 larval habitats have been sampled. All the kind of habitats are sampled so that for every month there was an equal number of habitats sampled for each category, assuring therefore that each category was equally represented during the time.

Each breeding site sampled has been classified by a serial number and photographed. Afterwards was made a qualitative classification of the larval biotope considering of the category of the habitat, the composition of the soil and the presence of algae. Therefore, was carried out the

collection of water samples for the microbiological and physico-chemical analyses. Finally, it has been carried out the sampling of the larval population of Culicidae and has been measured the values of physico-chemical parameters that were possible to collect in the field (see following paragraphs).

ENTOMOLOGICAL SAMPLING

Mosquito larvae were collected using a 350ml dipper (Service, 1993), making a number of dipping necessary to collect at least 100 *Anopheles spp*. larvae for each breeding site. After the dipping, collected larvae were transferred on plastic tubes containing the water of the breeding site sampled. Thereafter, the larvae were killed in Carnoy solution and identified per species and instar (W.H.O., 1975; Gillies & Coetzee, 1987). Subsequently, larvae identified as *An. gambiae s.l.* were conserved in Carnoy and stored at -20°C.

COLLECTION OF WATER SAMPLES

From each breeding sites were collected two water samples. One sample were collected immerging a 50ml sterile tube until the edge and sampling the surface water of the breeding site, taking care to collect only the initial millimetres of water. These samples were preserved on ice for 2-3 hours and subsequently stored at -20°C, waiting for the microbiological analysis. The second water sample was collected immerging a 1tl plastic bottle. This sample was transported up to 2-3

hours to the laboratory of analysis for the measurement of the physico-chemical parameters (see below).

MEASUREMENT OF THE PHYSICO-CHEMICAL PARAMETERS

After the collection of water and larval samples we proceeded with the measurement of the parameters that could be measured directly on the field: pH, conductivity, CO_2 in solution. pH and conductivity were measured, respectively, with a portable pH-meter (mod. pHep®2, Hanna Instruments), and conduct meter (mod. HI 8633, Hanna Instruments), immerging the probes directly on the water. The concentration CO_2 in solution was calculated by colorimetric titolation with phenolphthalein. A standard volume of breeding site water (180 ml or 190 ml, depending on the acidity of the water) was collected, adjoined to a solution with NaOH in excess, and preserved on ice until the moment of titolation, made at the analysis laboratory 2-3 hours after the sampling.

The water samples collected on the village were transported during the morning to the laboratory of analysis AINA, at Ouagadougou, where were measured the following parameters: turbidity, CO_2, KH (hereafter expressed as TAC: *titre alcalimétrique complet*), total hardness (TH), Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, total Fe, NH_4^{+}, CO_3^{2-}, HCO_3^{-}, Cl^{-}, SO_4^{2-}, NO_2^{-}, NO_3^{-}, PO_4^{3-}, total P.

MOLECULAR IDENTIFICATION OF *ANOPHELES GAMBIAE S.L.* LARVAE

The molecular identification of the larvae was carried out at the CNRFP of Ouagadougou, with the support of the CNRFP technicians and under the supervision of the Dr. N'Fale Sagnon. The DNA extraction was made according to the protocol described in Favia *et al*. (1994). The quantity of reagents of this protocol was halved, because of the smaller dimensions of the larvae regarding to adult mosquitoes, for which the protocol was performed. The extracted DNA was therefore suspended in 300µl of water and preserved at -20°C until the successive step of the identification.

Molecular identification of the *Anopheles gambiae* complex species is based on the amplification of one portion of the 28S coding region and part of the IGS of the ribosomal DNA by PCR, as described in Scott *et al*. (1993). In the Anophelinae the rDNA is localized on the X chromosome and repeated in tandem hundreds of times (more than 500 copies per diploid genome in *An. gambiae*, see Collins *et al*. 1990). The dimension of the amplified DNA of *An. gambiae s.s.* and *An. arabiensis* is respectively 390 bp and 315 bp. This first amplification of DNA allows distinguishing between these two taxa, being the only species of the complex present on the study area.

The individuals identified as *An. gambiae s.s*. were subsequently identified as molecular form M or S using a second PCR, as proposed by Favia *et al*. (2001). In this protocol is amplified the same region of the rDNA, but for each molecular form results two DNA segments: one of 1.3 kb,

common to both forms and specific of *An. gambiae s.s.*, and one diagnostic band for the two forms, of 727 bp for M and 475 for S.

Finally, a smaller number of individuals were instead identified using the PCR-RFLP protocol described in Fanello *et al*. (2002). This protocol is currently the standard method for the identification of *An. gambiae s.s.* molecular forms, because more rapid of the previous one. In fact, it takes advantage of the same amplification described in Scott *et al*. (1993). Therefore, the amplified DNA is exposed to the action of the restriction endonuclease HhaI that, recognizing sequence GCGC, it cuts the DNA of the S form in two segments of 257 and 110 bp. The DNA of the M form does not possess such restriction site and it is not cutted by the enzyme. Therefore, in our specific case, with such protocol is possible to identify with one DNA amplification and one restriction, all the three taxa present in the study area, with a remarkable saving of time.

The identification of the amplified genetic material has been carried out through electrophoresis on agarose gel at 1%.

MICROBIOLOGICAL ANALYSIS

The microbiological activities were carried out in the Dipartimento di Scienze di Sanità Pubblica dell'Università di Roma "La Sapienza", under the supervision of the Prof. Lucilla Seganti.

The water samples collected was thawed at environmental temperature, vortexed in order to mix the sediment on the bottom and diluted 1:10 with distilled sterile water under sterility

conditions. Only occasionally were carried out dilutions 1:100 or 1:000, when the bacterial density was too much elevated to count the colonies and isolate bacterial strains. Subsequently, 10ml of the diluted sample were filtered on 0.45μm S-Pak cellulose filters using the Microfil filtration system (Millipore). Then, the filtered material containing the bacteria was posed on a Petri capsule containing the cultivation solid medium incubated at 37°C. These operations were repeated 6 times, one for each medium used. In fact, the bacteria were isolated using 6 selective media, specific for various groups:

- **TBX** (*Tryptone bile X-glucuronide*): selective medium specific for *Escherichia coli*, which allows also the growth of the other Enterobacteriaceae.
- **Aeromonas** (*Ryan*): selective medium for Vibrionaceae and Enterobacteriaceae.
- **Baird-Parker**: selective medium for *Staphylococcus*.
- **Columbia CNA**: selective medium for all Gram-positives.
- **Streptococcal KC**: selective medium for *Streptococcus*.
- **Cetrimide**: selective medium for Pseudomonas and the other Pseudomonadaceae**.**

The bacterial cultures were controlled for the presence of bacterial colonies after 24, 48 and 168 hours. At each verification the colonies presents were counted, distinguished for morphological characteristics such as colour, form and dimension.

Subsequently, the colonies have been purified through serial passages on the same solid medium from which they have been identified.

Oxidase and catalase testing of each isolate was performed with 1% tetramethyl *p*-phenylenediamine dihydrochloride and 3% hydrogen peroxide, respectively. For the first test, the presence of cytochrome C oxidase can be detected when colonies are exposed to tetramethyl *p*-phenylenediamine, which acts as an electron donor to the oxidase. If the bacteria have cytochrome C oxidase, they can oxidize the reagent, turning it from uncoloured to blue. For the second test, if hydrogen peroxide is dropped on a colony containing catalase, this enzyme converts it into water and oxygen gas, which causes the colony to bubble.

Subsequently, the strains were inoculated in 6ml of liquid medium (BHI, Brain Hearth Infusion) and incubated in rotation at 37°C. When the broth assumed an appreciable turbidity (approximately 12 hours in the majority of the cases, but in rare cases even 3-4 days) we estimated the colony motility at 37°C using the hanging drop wet mount test: a drop of the bacterial culture was placed in the middle of a cover slip, thereafter the cover slip was posed upside-down on a depression slide and the motility of bacteria was observed directly at the microscope.

Finally, the bacterial strains were Gram-stained, using "fresh" colonies (24 hours maximum) in order to observe, further than the type of coloration, also the morphology and the dimension of the bacteria. The stained bacteria were observed at the microscopy under 1000X magnification. The coloration has been repeated at a distance of some days on colonies "aged" for Gram-positive bacteria, with the aim to reveal the eventual presence of spores. This because the presence of adverse conditions, as the scarcity of nourishing resources due to the desiccating of the cultivation medium, induces in the case of the sporing Gram-positive bacteria the formation of spores, waiting for the return of favourable growth conditions.

Pure strains, once characterized, were conserved at -80°C in BHI with glycerol at 15%.

For the purposes of this thesis we thought sufficient a general characterization of the bacterial diversity in the breeding sites, without make a detailed identification of the single species. The principal aim of the microbiological collection was having stocks of isolates subsequently usable for behavioural laboratory test finalized to better understand the role of the bacteria in the oviposition behaviour of *Anopheles gambiae*, as we explained previously (page x). Is for that we have isolated the bacteria with the cultural technique rather than to use more sensitive systems of identification, like as example the 16S ribosomal DNA sequencing.

STATISTICAL ANALYSIS

All statistical analyses of the data sets were calculated as reported by Sokal & Rohlf (1995) using the statistical software Statistica (Statsoft, 2001). The breeding sites employed for statistical analyses are those where at least 10 larvae were molecularly identified, to reduce the sampling bias on the calculated entomological variables.

The relative frequencies of the taxa were calculated as the proportion of a taxon respect to the sum of all taxa. The specific larval densities are the product of the mean of larvae per dip per relative frequency of each taxon.

The raw data were tested for normal distribution fitting using the Kolmogorov-Smirnov test, and non-normal data were normalized. Continuous variables were normalized by a logarithmic transformation. Relative frequencies were normalized with the angular transformation $arcsen(\sqrt{p})$.

The ANOVA tests were made as by one-way method, as by factorial method, as reported in the results.

The Principal Component analysis was made based on correlations and the variances were computed as SS/(N-1).

The Pearson correlation and the multiple regression analysis were made with month-standardized data, to eliminate the confounding effect of seasonality as on the breeding site variables measured, as on the entomological variables calculated. The multiple regression analysis were made as forward stepwise, using an F-to-enter = 1.

RESULTS AND DISCUSSION

During the four months of the rainy season 2003 (July-October) a total number of 113 breeding sites was sampled, with a total number of 20.299 *Anopheles gambiae s.l.* larvae collected. The table reported below shows the number of larvae identified by molecular analysis for each larval habitat category.

	N. sites sampled	An. arabiensis		An. gambiae M-form		An. gambiae S-form		unid. An. gambiae s.s.		not readable		total
borrow pit	14	171	*22%*	118	*15%*	35	*4%*	7	*1%*	456	*58%*	787
hoofprint	11	161	*22%*	141	*19%*	92	*13%*	22	*3%*	316	*43%*	732
lake margin	15	328	*29%*	522	*47%*	156	*14%*	19	*2%*	88	*8%*	1113
pit	6	79	*25%*	97	*31%*	17	*5%*	9	*3%*	109	*35%*	311
pool	17	37	*7%*	11	*2%*	5	*1%*	10	*2%*	486	*89%*	549
puddle	22	587	*35%*	519	*31%*	223	*13%*	18	*1%*	319	*19%*	1666
stream	13	268	*35%*	225	*29%*	78	*10%*	13	*2%*	192	*25%*	776
stream pool	15	405	*45%*	255	*28%*	143	*16%*	10	*1%*	89	*10%*	902
total	113	2036	*30%*	1888	*28%*	749	*11%*	108	*2%*	2055	*30%*	6836

A total of 6.836 larvae were analyzed, but the 30% of these were not readable. This fraction is largely greater than the 5% reported in literature (Fanello *et al.*, 2002), and is mainly due to the larvae sampled from pools. Exceptionally, it was impossible to obtain the taxonomic identification from almost all these samples, principally the *An. gambiae s.s.* molecular forms, and actually investigations on the causes of this problem are in progress. Interestingly, no hybrids between the taxa were observed, supporting the evidences that the genetic flow between the *An. gambiae s.s.* molecular forms is very low. Then, these results are in accord to the hypothesis that *An. gambiae*

s.s. M-form and S-form not cross in nature and should be considered two different species (della Torre *et al.*, 2001 and 2002).

To obtain correct relative frequencies of *An. arabiensis*, we calculated the parameters of this taxon in relation to the sum of the two *An. gambiae* molecular forms, plus the unidentified *An. gambiae s.s.* larvae (that are the individuals identified as *An. gambiae s.s.* that was not possible to identify as molecular form). For the same motive, the frequencies of the two molecular forms were calculated the one in comparison to the other, without considering *An. arabiensis* and unidentified *An. gambiae* s.s. individuals.

The relative frequencies of the taxa in the total larval sample are (excluding unidentified *An. gambiae s.s.* and not readable): *An. arabiensis* 43.6%, *An. gambiae* M-form 40.4% and *An. gambiae* S-form 16%. The table below shows the relative frequencies of the taxa among the larval categories.

	An. arabiensis	*An. gambiae* M-form	*An. gambiae* S-form
borrow pit	*53%*	*36%*	*11%*
hoofprint	*41%*	*36%*	*23%*
lake margin	*33%*	*52%*	*16%*
pit	*41%*	*50%*	*9%*
pool	*70%*	*21%*	*9%*
puddle	*44%*	*39%*	*17%*
stream	*47%*	*39%*	*14%*
stream pool	*50%*	*32%*	*18%*

Pool and lake margin are the larval habitats with the major presence of *An. arabiensis* (70%) and *An. gambiae* M-form (52%), respectively. The S-form is more abundant in hoofprints (23%) respect to other larval habitats, even if at lower frequencies than the other two taxa. However, these

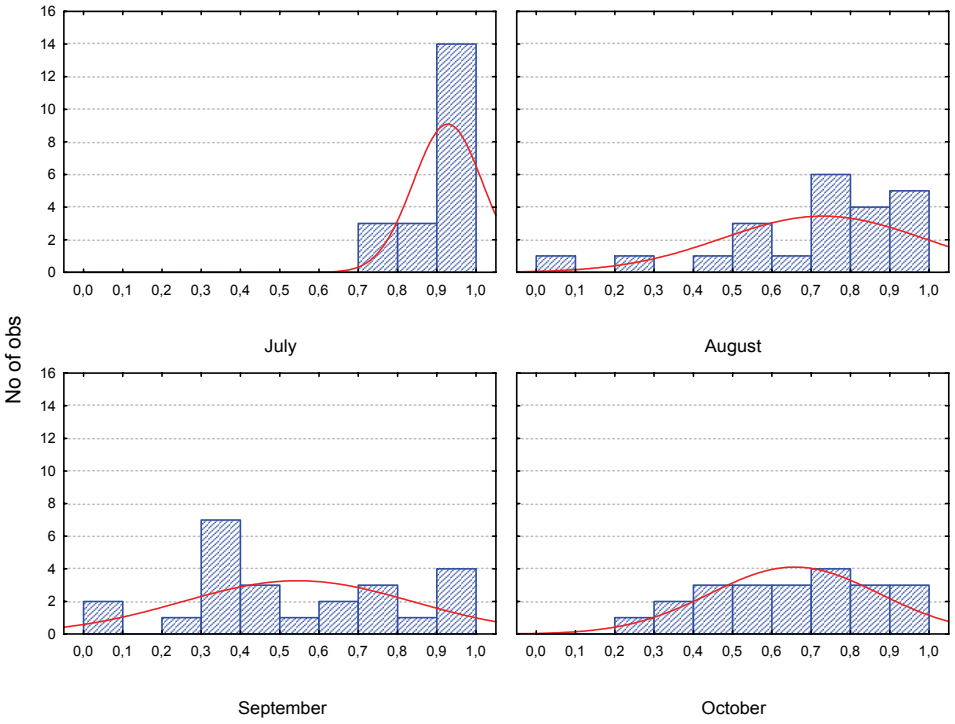
proportions underestimate the presence of *An. gambiae* S-form and *An. arabiensis*, because of the population dynamics of this taxon. In fact, significant temporal variation of *Anopheles gambiae* larval populations was observed during the four months (Figures 9 and 10), for both *An. arabiensis* ($F_{(3,40)}$=3.925; p=0.015) and *An. gambiae* molecular forms ($F_{(3,28)}$=4.879; p=0.007). The histograms of Figure 9 show that in July the relative frequency of *An. gambiae* M-form ranges from 70% to 100%. As the rainy season evolves, some breeding site with majority of *An. gambiae* S-form appears (frequency of M-form <0.5), with an abundance peak of this taxon in September. In October, when the rainy season is at the end, the breeding sites with majority of *An. gambiae* M-form becomes again predominant, demonstrating a decrease of the S-form larval population in the village. A similar temporal trend is observed for *An. arabiensis* in relation to *An. gambiae s.s.* (both molecular forms), but the phenomenon is not marked as with the other two taxa.

The different categories of larval habitats sampled are well exploited by the three taxa of *An. gambiae* complex. The histograms in Figure 10 (a) and (b) show the relative frequencies of *An. gambiae* M-form and *An. arabiensis*. No statistical differences were observed among the larval relative frequencies of the taxa on the various habitats. The relative frequencies of the *An. gambiae s.s.* molecular forms (reported as M-form frequencies) was calculated eliminating July from the analysis, because in this month the presence of the S-form was very scarce. The absence of data for the pool category is due to problems on the molecular identification of larval samples originating from these sites, as explained before.

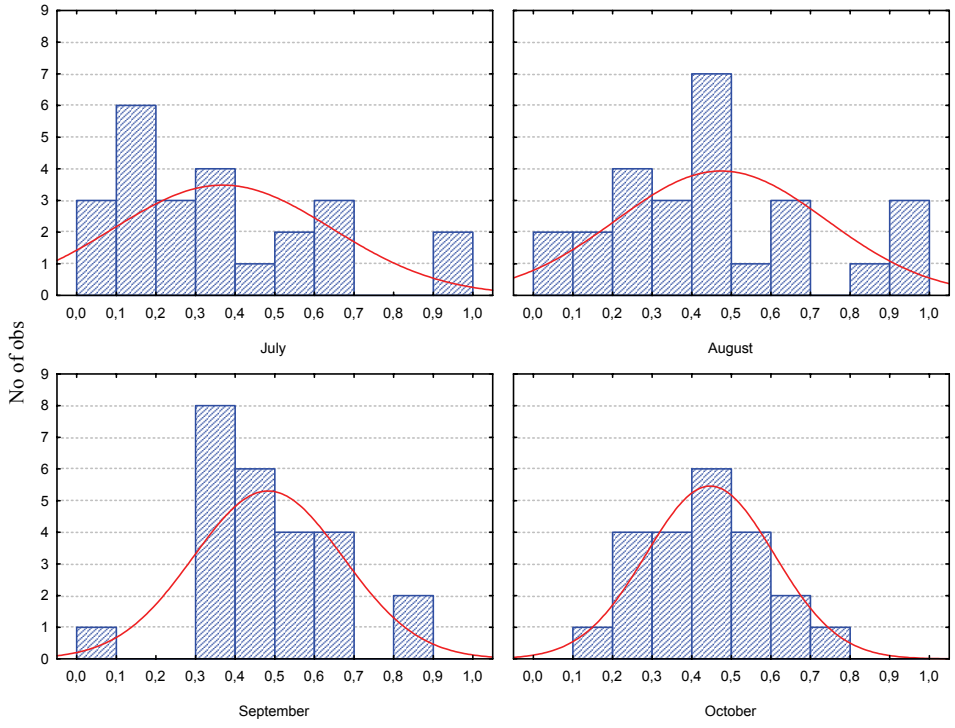
We considered the possibility that the relative frequency of a taxon in a specific larval habitat could be affected by the evolution of the rainy season. As an example, a larval habitat could be

more attractive for a particular taxon at early rainy season and less at late rainy season. To investigate this possibility, we made a factorial ANOVA considering the interaction between the month and the type of larval habitat, but the results of the statistics show no interactions between the two grouping factors.

Despite the absence of differences of taxa frequencies among the larval habitats, these show significant differences for the overall *An. gambiae s.l.* larval densities ($F_{(7, 89)}$=2.169; p=0.044). As shown in Figure 11, the major larval densities are in the stream pools (11.3 larvae/dip), while the less populated habitats are the pools (2.9 larvae/dip). Between these two extremes, there are the other larval habitats: stream (7.2 larvae/dip), puddle (5.6 larvae/dip), hoofprint (4.39 larvae/dip), lake margin (4.5 larvae/dip), borrow pit (3.8 larvae/dip), pit (3.3 larvae/dip).

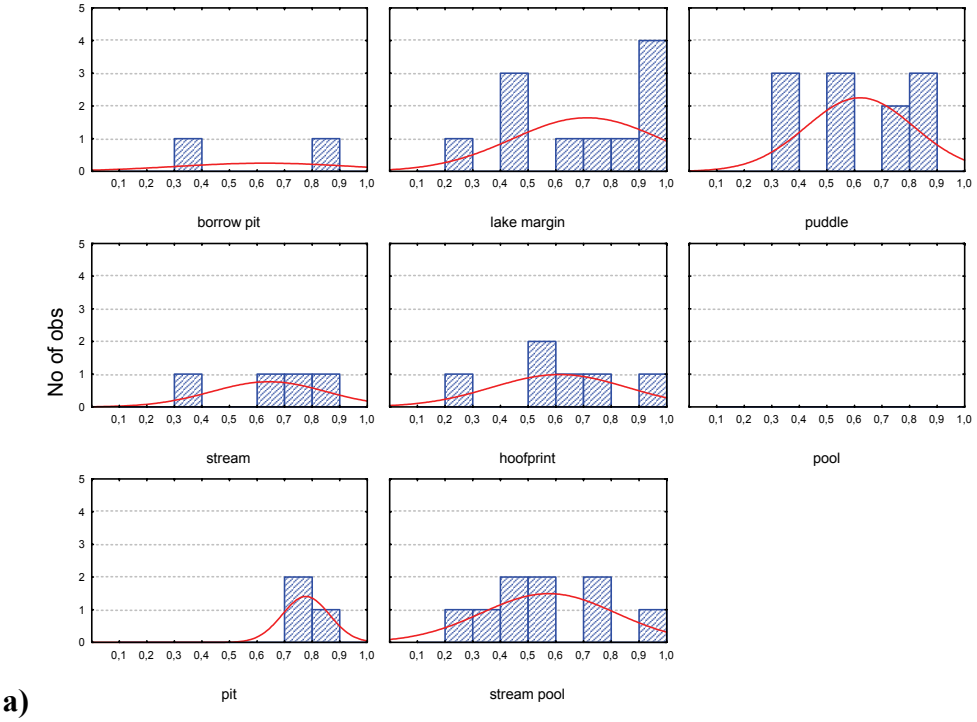

No of obs
July
August
September
October

a)

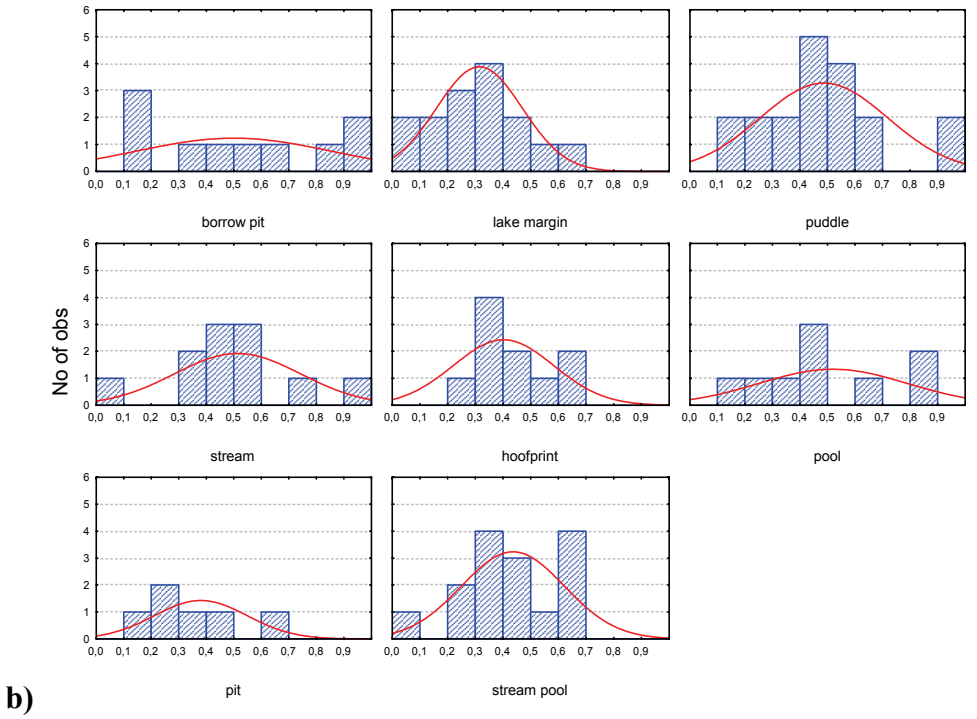

No of obs
July
August
September
October

b)

Figure 9: Larval population dynamics of Anopheles gambiae *M-form* (a) *and* An. arabiensis (b) *during the raining season 2003.The X-axis shows the relative frequencies per larval site, with a minimum number of 10 larvae identified, and the Y-axis shows the number of observations per frequency rank.*



a)



b)

*Figure 10: larval frequencies of Anopheles gambiae M-form (**a**) and Anopheles arabiensis (**b**) classified per category of larval habitat during the months August-October and July-October 2003, respectively. The X-axis shows the relative frequencies per larval site, with a minimum number of 10 larvae identified, and the Y-axis shows the number of observations per frequency rank.*

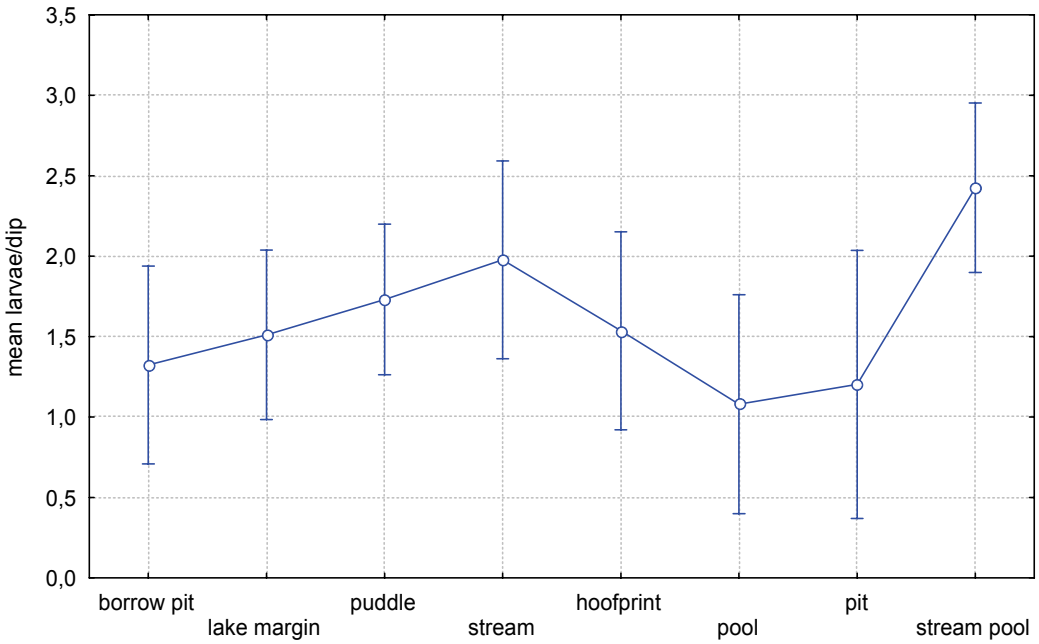


Figure 11: ANOVA of larval density (Y-axis, expressed in mean of larvae per dip) in relation of larval habitat categories. ($F_{(7, 89)}=2.169$; $p=0.044$). Vertical bars denote 95% confidence intervals. The values reported are relative to log-transformed data.

The table reported below shows the descriptive statistics for the physico-chemical variables measured on the breeding sites sampled.

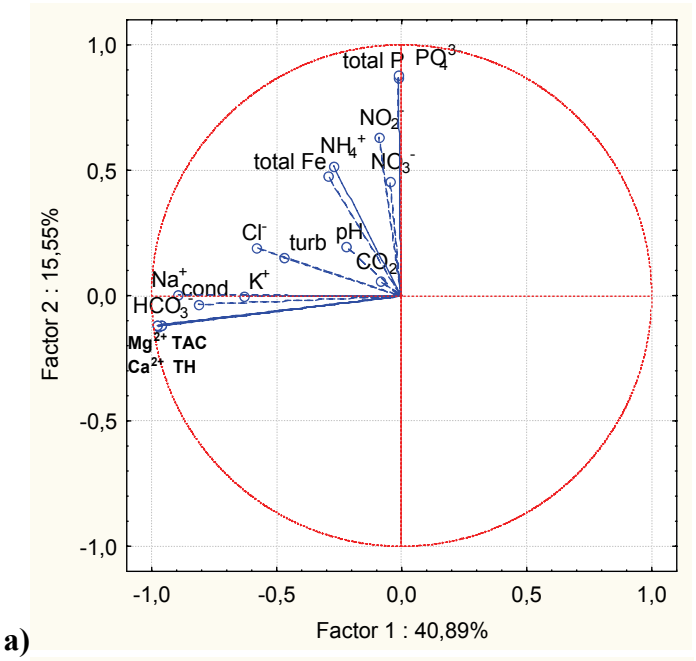
	Mean	Median	Mode	Frequency of Mode	Minimum	Maximum	Std.Dev.	Standard Error
conductivity (µS)	114,19	79,30	46,40	3	23,80	584,00	96,66	9,81
pH	7,96	7,80	7,80	11	7,00	9,60	0,62	0,06
turbidity (FTU)	1902,95	400,00	Multiple		1,14	53750,00	6627,22	672,89
CO_2 (mg/l)	6,64	5,79	4,63	19	1,16	35,89	4,80	0,49
TAC (°F)	5,54	3,60	3,20	6	1,10	45,00	6,08	0,62
TH (°F)	4,70	3,10	2,80	7	1,00	40,00	5,25	0,53
Ca^{2+} (mg/l)	9,74	6,40	6,40	8	1,90	80,00	10,66	1,08
Mg^{2+} (mg/l)	5,47	3,60	2,40	10	1,20	48,40	6,28	0,64
Na^{+} (mg/l)	5,50	4,17	2,50	11	0,66	37,00	5,63	0,57
K^{+} (mg/l)	1,23	0,66	0,33	33	0,33	4,58	1,07	0,11
total Fe (mg/l)	0,90	0,29	0,10	6	0,08	15,60	1,95	0,20
NH_4^{+} (mg/l)	0,82	0,42	0,22	7	0,06	15,30	1,69	0,17
HCO_3^{-} (mg/l)	67,56	43,90	39,00	6	13,40	549,00	74,18	7,53
Cl^{-} (mg/l)	2,28	1,88	1,50	5	0,67	9,73	1,45	0,15
NO_2^{-} (mg/l)	0,29	0,16	Multiple		0,01	4,22	0,59	0,06
NO_3^{-} (mg/l)	4,51	1,76	1,32	12	0,00	57,20	7,10	0,72
PO_4^{3-} (mg/l)	0,30	0,15	0,08	10	0,03	2,50	0,38	0,04
total P (mg/l)	0,10	0,05	0,03	18	0,01	0,81	0,12	0,01

Is evident the chemical heterogeneity of these water collections, with values often dispersed over hundreds of units. As reported in material and methods, all the statistical analyses described below are made with normalized (log-transformed) parameters with a month-stratified standardization, to eliminate the temporal effect of the rainy season on the variables measured.

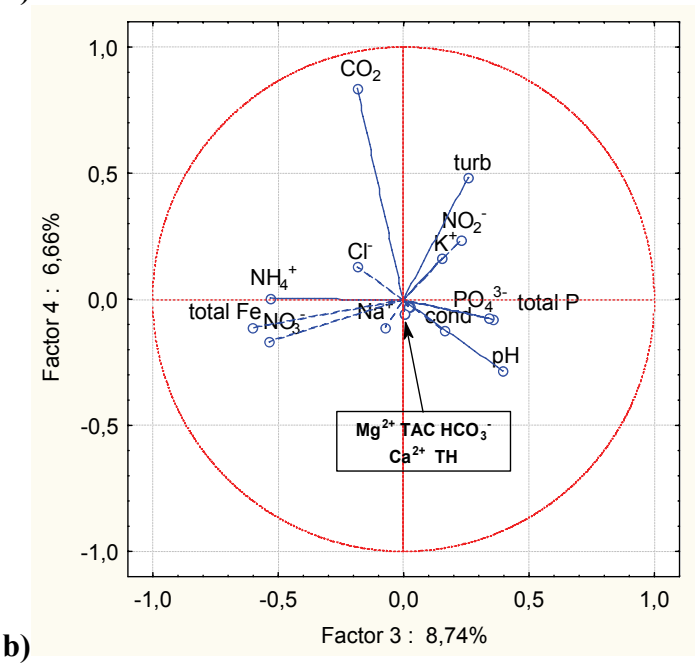
The Principal Component Analysis (PCA) of the physico-chemical variables measured on breeding sites sampled identifies the most important parameters describing the diversity of sites. The first four factors include 72% of the total variance, as reported in the table below.

Number Value	Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative % Variance
1	7,36	40,89	7,36	40,89
2	2,80	15,55	10,16	56,44
3	1,57	8,74	11,73	65,19
4	1,20	6,66	12,93	71,85
	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
conductivity	-0,300	-0,020	0,132	-0,114
pH	-0,082	0,116	0,314	-0,259
turbidity	-0,174	0,090	0,207	0,441
CO_2	-0,031	0,033	-0,147	0,764
TAC	-0,360	-0,072	0,002	-0,051
TH	-0,358	-0,073	0,016	-0,027
Ca^{2+}	-0,355	-0,071	0,019	-0,025
Mg^{2+}	-0,355	-0,072	0,014	-0,026
Na^{+}	-0,329	0,002	-0,058	-0,101
K^{+}	-0,232	-0,003	0,123	0,148
total Fe	-0,108	0,284	-0,481	-0,101
NH_4^{+}	-0,100	0,309	-0,424	0,005
HCO_3^{-}	-0,360	-0,072	0,002	-0,050
Cl^{-}	-0,215	0,113	-0,147	0,117
NO_2^{-}	-0,033	0,377	0,183	0,212
NO_3^{-}	-0,017	0,273	-0,427	-0,154
PO_4^{3-}	-0,004	0,520	0,282	-0,074
total P	-0,006	0,524	0,269	-0,067

The projection of the variables on the factor-planes elaborated with factor 1 vs. factor 2, and with factor 3 vs. factor 4 (Fig. 12) illustrates which are the most important variables.



a)



b)

Figure 12: Projection of the physico-chemical variables on the factor-plane elaborated with the factor 1 and 2 (a)*, and with the factor 3 and 4* (b) *obtained by the PCA.*

Conductivity, TAC, TH, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, HCO_3^{-}, Cl^{-} give the stronger contribute to the factor 1 (40,89% of total variance); total P, PO_4^{3-}, NO_2^{-}, NO_3^{-}, NH_4^{+} and the total Fe are mostly

linked to factor 2 (15,55%); the factor 3 (8,74%) is the result of contribution of the same variables of the factor 2, with also pH and turbidity; factor 4 (6,66%) resulted principally by the contribution of CO_2 and turbidity.

Then, the most important variables that contribute to the variance of our sample are inorganic salt correlated with the hardness of water. Considerable but less important are those correlated with biotic activity and pollution. A minor importance has CO_2, pH and turbidity.

No clusterization is evident among the larval habitats for the first four factors of the PCA, as shown by the projection of the sites sampled on the factor-planes (Fig. 13). Nonetheless, the lake margin and the pit are the most homogeneous habitats, with a concentration of points to the positive values of the factor 1. This means that such larval sites have low concentration of carbonate salts and consequently low conductivity and hardness. To the contrary, puddles, pools, and stream pools are breeding sites chemically more heterogeneous, varying on all the range values of the PCA factors. Then, for what concern the chemical aspects of the sites sampled, apart the lake margins and the pits, the *An. gambiae* breeding sites are too heterogeneous to be classified among different categories of larval habitats in a rigorous way.

Therefore, no clusterization is evident also for breeding sites with predominance of a specific taxon, as shown by figures 14 and 15.

These results could explain the lack of repartition of the taxa on larval categories. Is possible that the different distribution of the two molecular forms of *An. gambiae s.s.*, observed on season 1996 and 2000, was due to more marked differences on chemical profiles of breeding sites sampled.

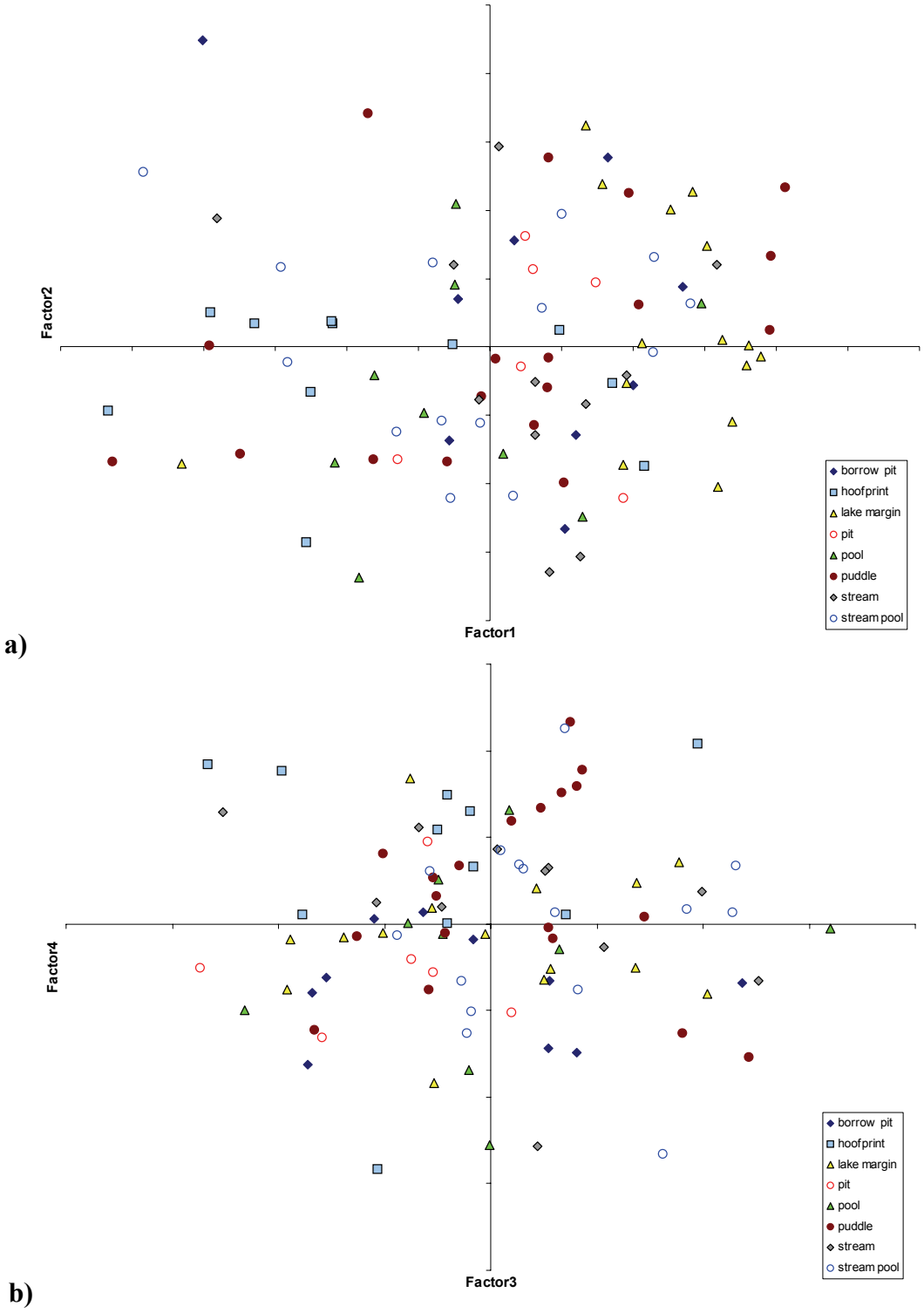
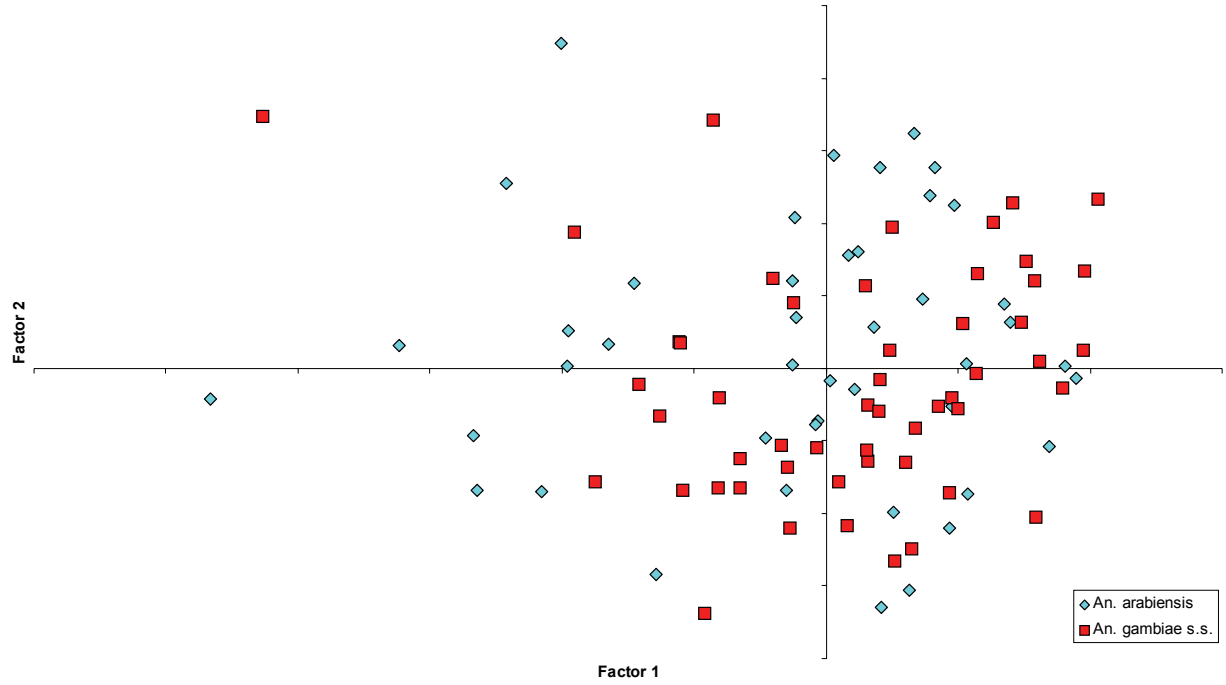
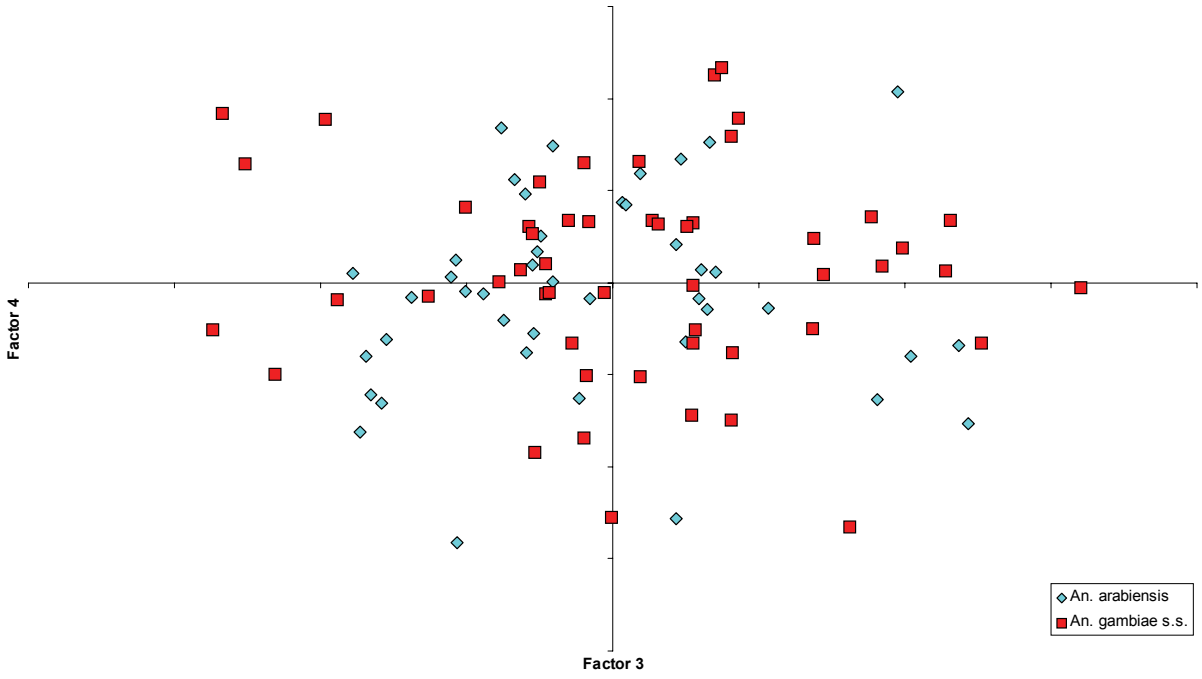


Figure 13: Scatterplot of larval habitats on the factor-planes elaborated with the factor 1 and 2 (a)*, and with the factor 3 and 4* (b) *obtained by the PCA.*



a)



b)

Figure 14: Scatterplot of breeding sites with majority of An. arabiensis *or* An. gambiae s.s. *on the factor-planes elaborated with the factor 1 and 2* (a)*, and with the factor 3 and 4* (b) *obtained by the PCA.*

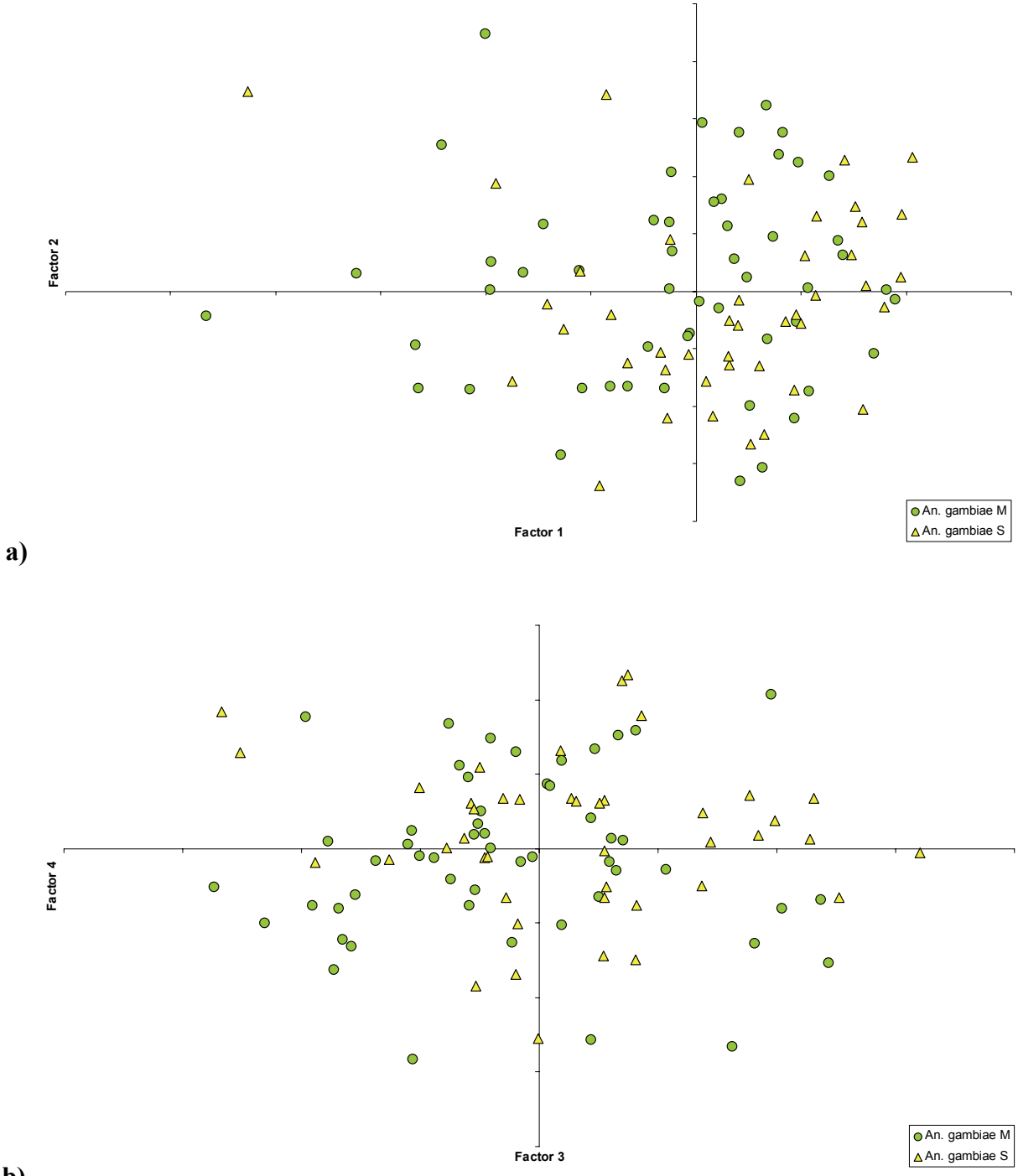


Figure 15: Scatterplot of breeding sites with majority of An. gambiae *M-form or* An. gambiae *S-form on the factor-planes elaborated with the factor 1 and 2* (a)*, and with the factor 3 and 4* (b) *obtained by the PCA.*

Also, the samples of 1996 and 2000 were made in a short period of time, at the end of rainy season, when the differences among the sites were enhanced by the scarcity of precipitations. However, the differences observed in 2000 were principally due to the lake margin, where the *An. gambiae* M-form was more abundant than in the other sites. In our sample, the lake margin is the most chemically homogeneous habitat, where was observed the major proportion of *An. gambiae* M-form (52%, see table at page 42). Considering this, our observations are partially in accord with these precedent data, even if not statistically significant.

The table reported below shows the results for the linear correlation analysis between the entomological parameters (*An. arabiensis* and *An. gambiae* M-form relative frequencies, total larval density) and the physico-chemical parameters.

	An. arabiensis relative frequency		*An. gambiae* M-form relative frequency		total larval density	
	r	*p*	r	*p*	r	*p*
conductivity	0,093	0,429	0,094	0,473	0,051	0,622
pH	0,022	0,852	-0,081	0,536	0,391	0,00007
turbidity	0,054	0,645	-0,099	0,447	0,197	0,066
CO_2	0,294	0,011	-0,116	0,372	0,056	0,587
TAC	0,111	0,342	0,041	0,756	0,147	0,150
TH	0,100	0,392	0,043	0,741	0,155	0,128
Ca^{2+}	0,078	0,506	0,015	0,908	0,132	0,198
Mg^{2+}	0,108	0,355	0,063	0,632	0,176	0,085
Na^{+}	0,073	0,536	-0,037	0,775	0,175	0,086
K^{+}	0,115	0,326	0,131	0,316	0,076	0,460
total Fe	-0,096	0,415	0,188	0,147	-0,051	0,620
NH_4^{+}	-0,055	0,641	0,160	0,219	-0,011	0,914
HCO_3^{-}	0,110	0,346	0,038	0,770	0,146	0,152
Cl^{-}	-0,053	0,650	0,081	0,533	0,134	0,190
NO_2^{-}	-0,069	0,559	-0,143	0,272	0,117	0,254
NO_3^{-}	-0,087	0,456	0,252	0,050	0,143	0,161
PO_4^{3-}	-0,134	0,252	-0,003	0,980	-0,029	0,774
total P	-0,140	0,230	0,001	0,991	-0,038	0,711

Very few chemical variables are significantly correlated with the relative frequencies of the taxa (evidenced in red in the table), all with low Pearson's correlation coefficients (*r*). The only significant results are CO_2 and NO_3^- directly correlated with *An. arabiensis* and *An. gambiae* M-form relative frequencies, respectively, and pH directly correlated with larval density. The low Pearson's *r* calculated for these three correlations are probably due to the background noise of the data sets, as shown by figures 16, 17, and 18.

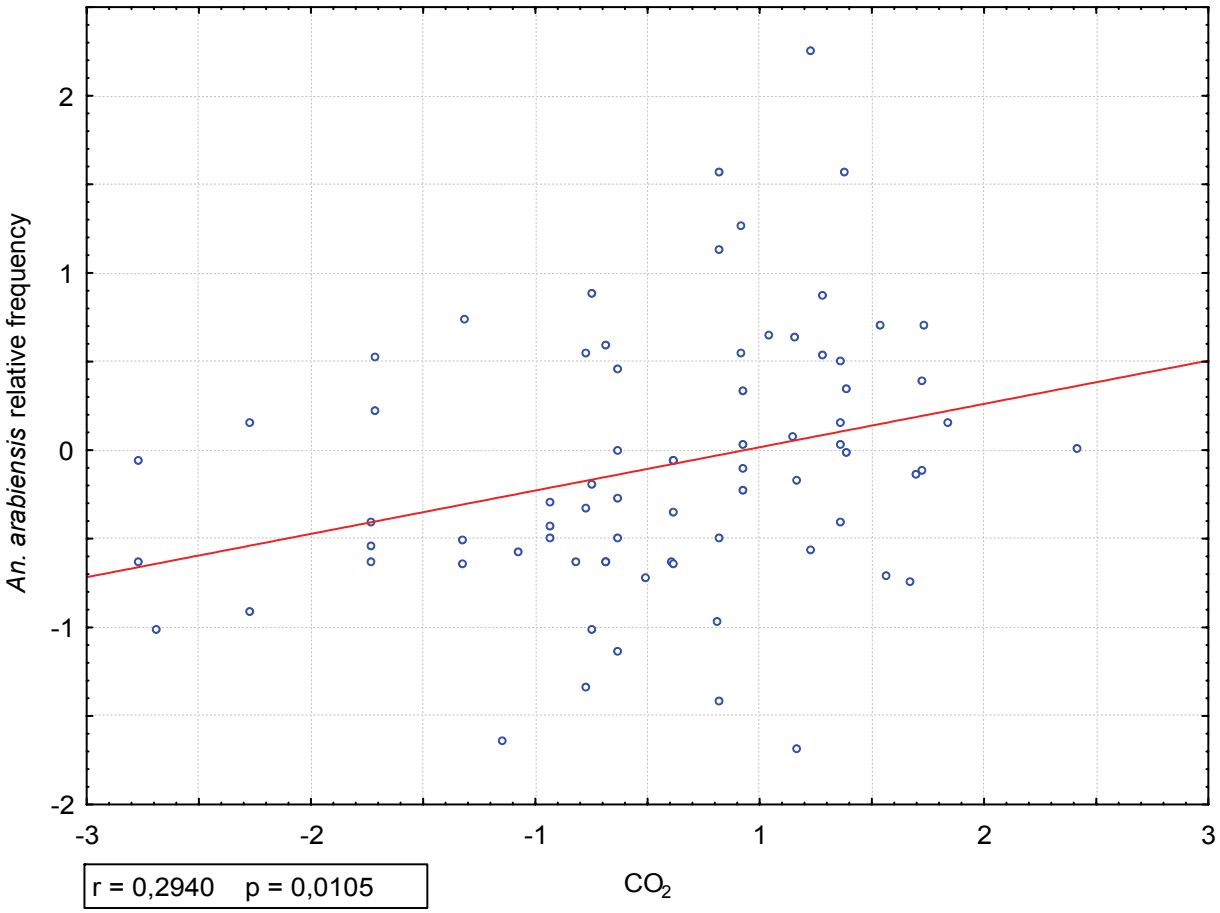


Figure 16: Scatterplot of An. arabiensis *relative frequency vs. concentration of CO_2 in solution.*

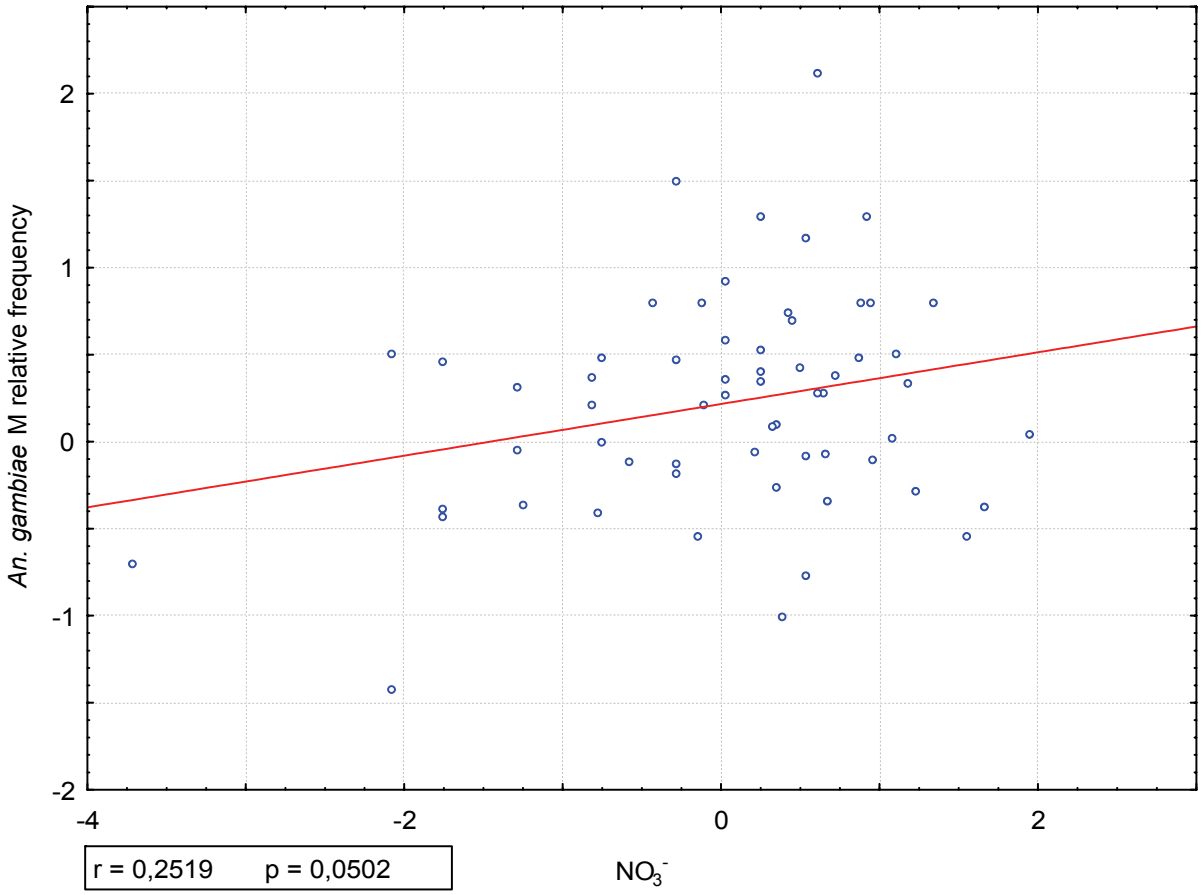


Figure 17: Scatterplot of An. gambiae M-form relative frequency vs. concentration of NO_3^- in solution.

The single correlation analysis describes the intensity of the association of two variables in a one-to-one way. As already observed in the introduction, a single cue is probably less informative respect to a complex pattern of stimuli. Then, this test cannot reveal the possible interactions of cues pattern on the oviposition site choice. To take in consideration the effect of all the variables contemporaneously, considering also the between-variable effects, a multiple regression analysis was made.

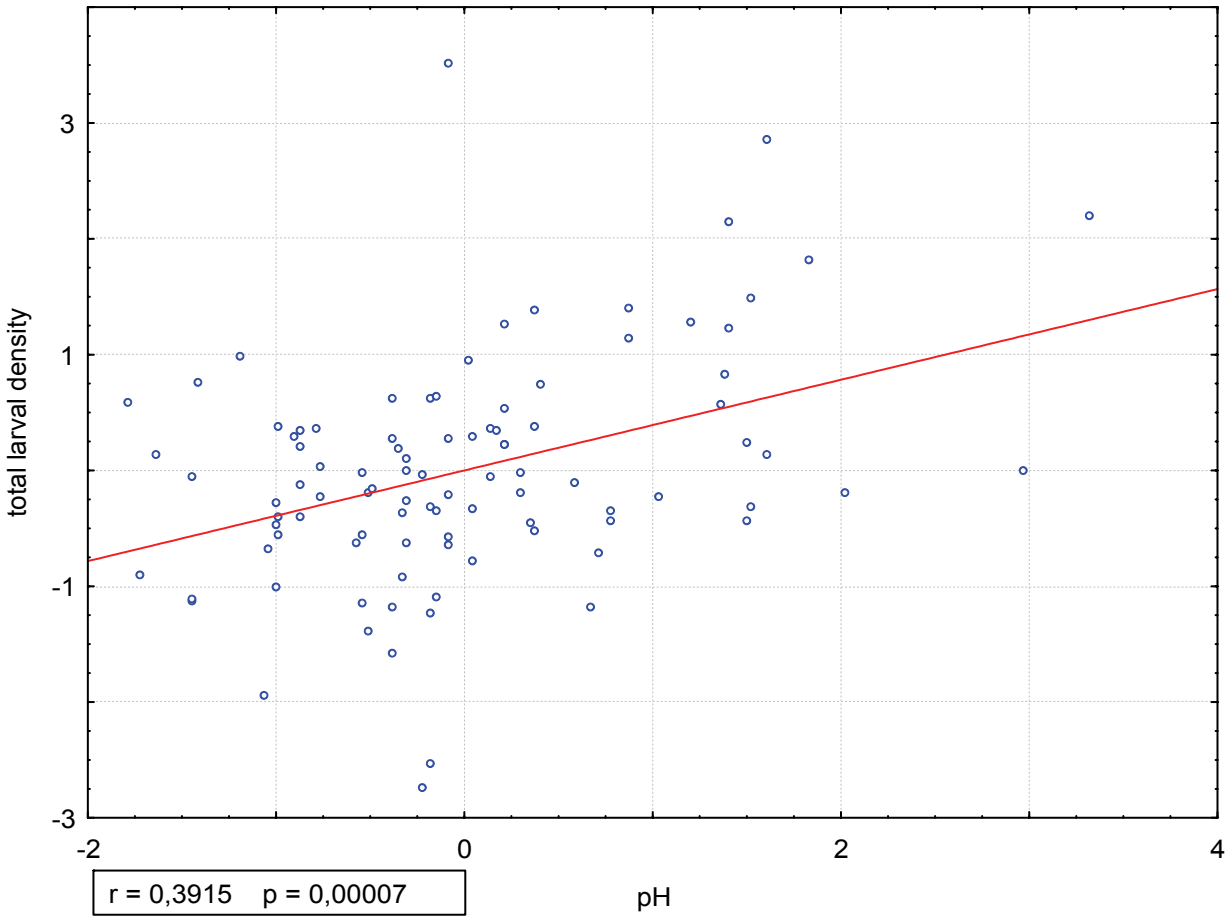


Figure 18: Scatterplot of total larval density vs. pH .

The results of this statistic (Tab. 1) are substantially in accord to the single linear regression results. Two regression models have a significant *p* (*An. arabiensis* relative frequency, total larval density), while no statistically significant model could be made for relative frequencies of *An. gambiae* molecular forms. *An. arabiensis* relative frequency is significantly correlated with the CO_2 in solution (p=0.006), with a positive Beta (0.32) that confirms a direct correlation. In this model are also included three other variables with a F-to-enter >1 (total Fe, Mg^{2+}, Ca^{2+}), but in this case with a not significant *p*-value. The total larval density is confirmed to be directly correlated with pH

(Beta=0,405; p<0,001). Other variables increases the multiple R of the regression (NO_3^-, total P, NO_2^-, Mg^{2+} conductivity, total Fe, TH), but their contribute is too little to have a significant p-value.

***An. arabiensis* relative frequency** R= 0,380 N=75 F(4,70)=2,952 p<0,026 Std.Error of estimate: 0,706									
	Multiple R	**Multiple R-square**	**R-square change**	**F-to-enter**	**p-level**	**Beta**	**Std.Err. of Beta**	**t(70)**	**p-level**
CO_2	0,294	0,086	0,086	6,906	0,010	0,320	0,112	2,858	0,006
total Fe	0,327	0,107	0,021	1,658	0,202	-0,197	0,118	-1,660	0,101
Mg^{2+}	0,357	0,127	0,020	1,660	0,202	0,685	0,468	1,463	0,148
Ca^{2+}	0,380	0,144	0,017	1,386	0,243	-0,549	0,466	-1,177	0,243
Total larval density R= 0,507 N=97 F(7,89)=4,271 p<0,0004 Std.Error of estimate: 0,884									
	Multiple R	**Multiple R-square**	**R-square change**	**F-to-enter**	**p-level**	**Beta**	**Std.Err.**	**t(89)**	**p-level**
pH	0,392	0,153	0,153	17,043	0,00008	0,405	0,097	4,164	0,00007
NO_3^-	0,411	0,169	0,016	1,755	0,189	0,161	0,102	1,574	0,119
total P	0,429	0,184	0,015	1,644	0,203	-0,124	0,112	-1,105	0,272
NO_2^-	0,446	0,199	0,015	1,726	0,192	0,120	0,106	1,131	0,261
Mg^{2+}	0,456	0,208	0,009	1,024	0,314	1,011	0,683	1,481	0,142
conductivity	0,485	0,235	0,027	3,174	0,078	-0,254	0,150	-1,689	0,095
total Fe	0,498	0,248	0,013	1,531	0,219	-0,121	0,107	-1,138	0,258
TH	0,507	0,257	0,009	1,023	0,315	-0,702	0,694	-1,011	0,315

Table 1: Significant multiple regression models for the entomological variables in relation to the physico-chemical variables.

We performed also multiple regression models correlating the first four factors of the PCA (pag. 49) with the entomological variables. The PCA factors are the results of the relative contribution of all chemical variables. Then, the correlation of entomological variables with one or more PCA factors is indirectly a correlation with the chemical variables that give the major contribute to the calculation of the factors. We didn't find any significant multiple regression model

among the entomological variables and the PCA factor, as expected considering the lack of clusterization observed on PCA factors scatterplots of figures 14 and 15.

The table reported below shows descriptive statistics of the isolated bacteria by cultivation methods (N=35, expressed in CFU/ml at 168 hours of cultivation) of the breeding sites sampled.

	Mean	Median	Mode	Frequency of Mode	Minimum	Maximum	Std.Dev.	Standard Error
Gram+ cocci (CFU/ml)	1235,89	32	6	3	1	20091	4032,87	681,68
Endospore form. Gram+ (CFU/ml)	1591,11	41	6	3	0	20012	4928,79	833,12
Regular Gram+ rods (CFU/ml)	575,34	0	0	24	0	10005	2354,72	398,02
Irregular Gram+ rods (CFU/ml)	307,83	0	0	24	0	10000	1688,69	285,44
Gram- aerobic/microaero. (CFU/ml)	292,57	0	0	24	0	10000	1689,22	285,53
Enterobacteriaceae (CFU/ml)	20,80	0	0	21	0	233	54,35	9,19
unidentified Gram- (CFU/ml)	912,66	17	0	7	0	10223	2853,99	482,41
unidentified Gram var. (CFU/ml)	296,80	0	0	26	0	10300	1740,59	294,21

The identification of cultured bacteria encountered some problems due to the non-strict selection of the different media. In fact, excluded Baird-Parker and Streptococcal KC, in all media were isolated a variety of different strains, and not only the species for which the single medium could be selective. Because of these limitations, the identification of strains was confined to a sub-sample (N=35) of the breeding sites collected, using a broad classification of systematic groups, referring to the identification keys of Bergey's manual of determinative bacteriology (Holt *et al.*, 2000). A total of 397 bacterial strains were isolated from 35 water samples (11.3 strains/sample), classified in 8 bacterial groups, as showed in the table. The last two groups (unidentified Gram negatives and unidentified Gram variables) are not present in the Bergey's Manual and were

introduced to classify, respectively, Gram negative and Gram variable bacteria that were not identifiable.

It is evident that these results represent a baseline set of information that needs deeper investigations to assess the role of bacteria on the *Anopheles gambiae* species oviposition site choice. Then, a 16S ribosomal DNA analysis of the strain isolated will be made before the utilization of these strains in laboratory experiment of oviposition behaviour.

As observed for the physico-chemical results, the microbiological analysis shows a wide dispersion of data. All the groups range from 0 to 10^4 CFU/ml, with a negative binomial distribution of data. To describe the diversity and homogeneity of bacterial groups among the water samples analyzed, we used the Shannon (H) and Pielou indexes (*e*), respectively. These indexes are normally used to describe the communities of species in an ecosystem (Odum, 1971). Obviously, in this case we don't have a community of true species, but equally such indexes could give us an idea of our sample diversity.

	Mean	Median	Minimum	Maximum	Std.Dev.	Std.Err.
H	0,862	0,889	0,006	1,746	0,462	0,078
e	0,602	0,684	0,004	0,919	0,292	0,049

The descriptive statistics of H and *e* above reported shows the great heterogeneity of the breeding sites for both indexes.

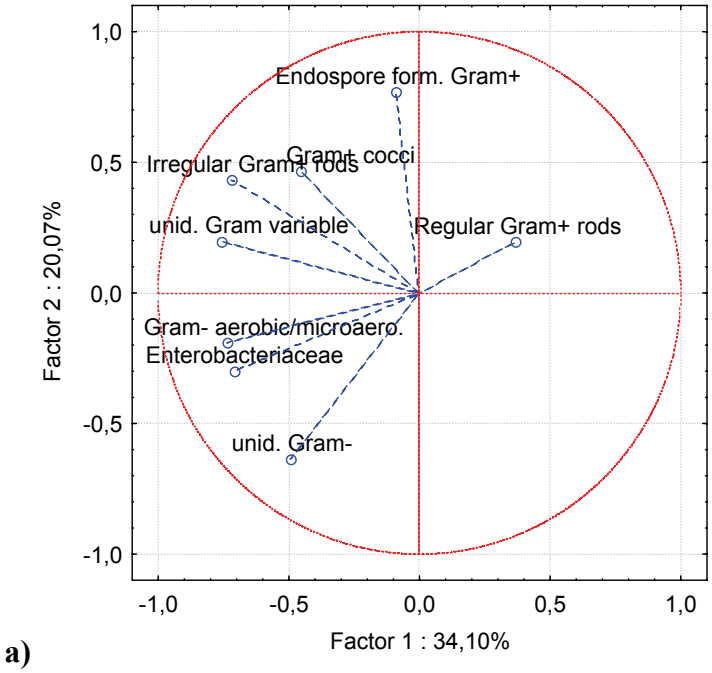
The single correlation analysis of H and *e* versus the entomological variables demonstrate a total lack of correlations. This possibly means that there are no relations between the *Anopheles*

gambiae taxa and the diversity of bacterial community in the breeding sites, at least with this broad classification of bacterial groups.

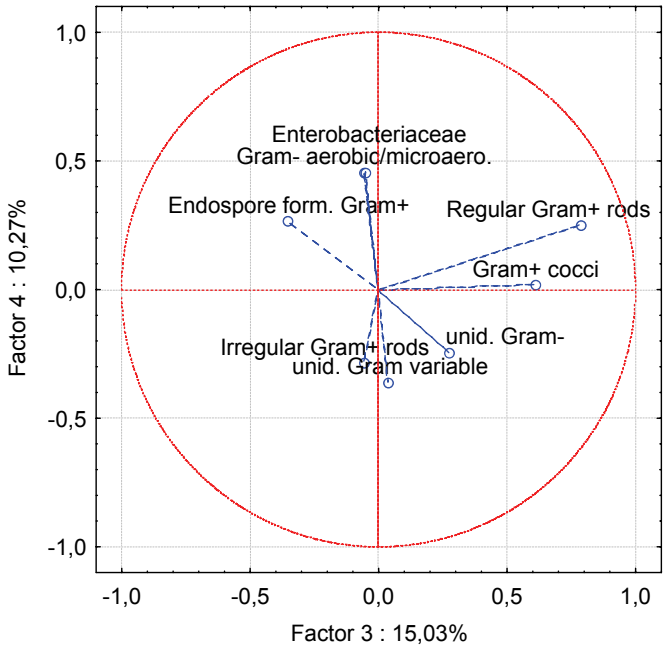
The results of PCA analysis show the lack of predominance of a specific bacterial group to the contribution of variance of the sample (see table below and figure 19).

Number Value	Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative % Variance
1	2,73	34,10	2,73	34,10
2	1,61	20,07	4,33	54,17
3	1,20	15,03	5,54	69,20
4	0,82	10,27	6,36	79,47
	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
Gram+ cocci	-0,275	0,369	0,557	0,020
Endospore form. Gram+	-0,053	0,608	-0,325	0,296
Regular Gram+ rods	0,223	0,154	0,716	0,277
Irregular Gram+ rods	-0,436	0,340	-0,051	-0,316
Gram- aerobic/microaero.	-0,446	-0,151	-0,053	0,503
Enterobacteriaceae	-0,428	-0,237	-0,047	0,504
unidentified Gram-	-0,300	-0,502	0,249	-0,269
unidentified Gram variable	-0,458	0,156	0,033	-0,396

Also, the larval habitats not clusterize with any of the four PCA factors (figure 20). The same situation is observed also for breeding sites with predominance of a specific taxon (figures 21, 22).



a)



b)

Figure 19: Projection of the bacterial groups on the factor-plane elaborated with the factor 1 and 2 (a)*, and with the factor 3 and 4* (b) *obtained by the PCA.*

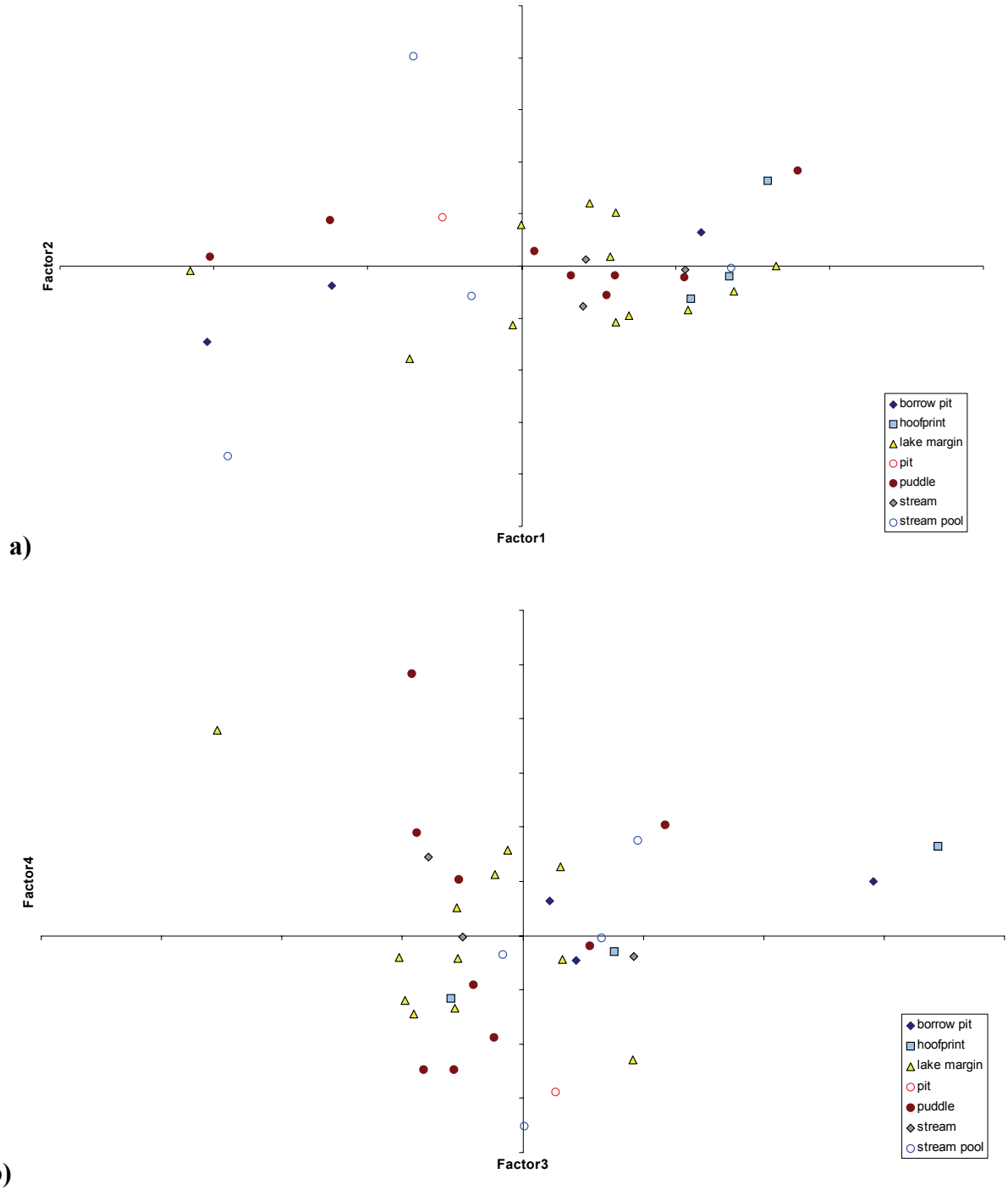
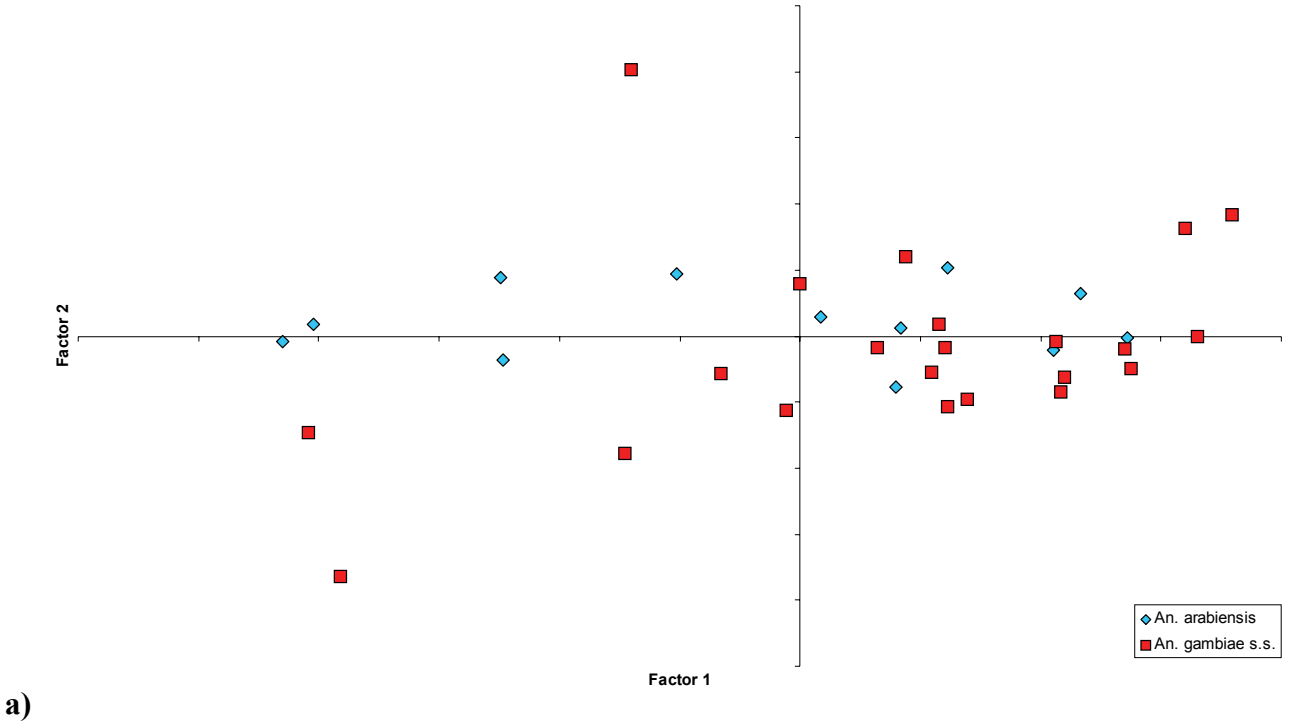
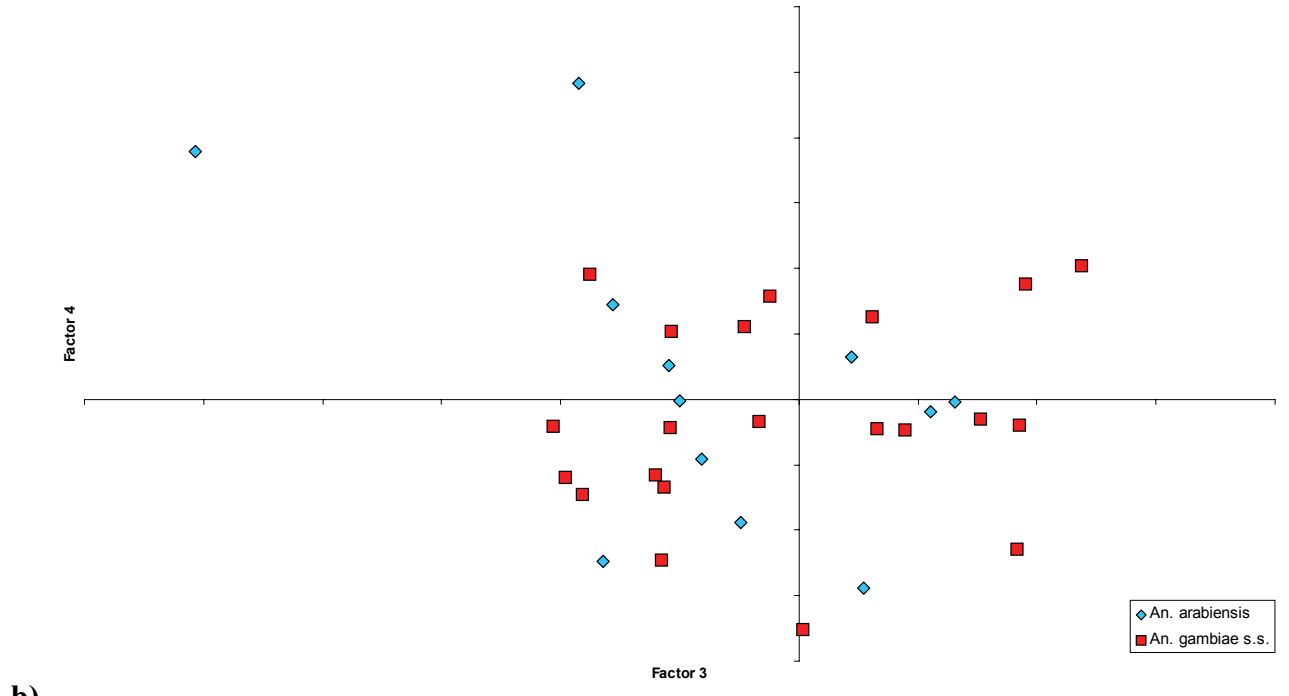


Figure 20: Scatterplot of larval habitats on the factor-planes elaborated with the factor 1 and 2 (a)*, and with the factor 3 and 4* (b) *obtained by the PCA.*

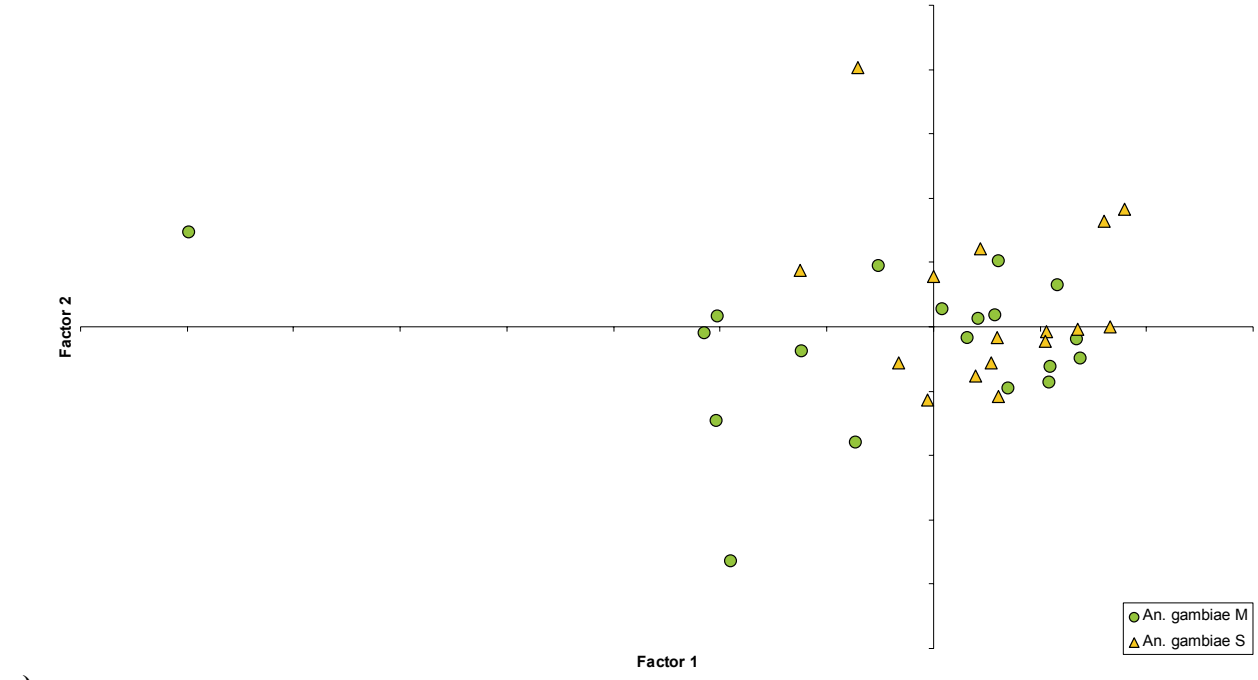


a)

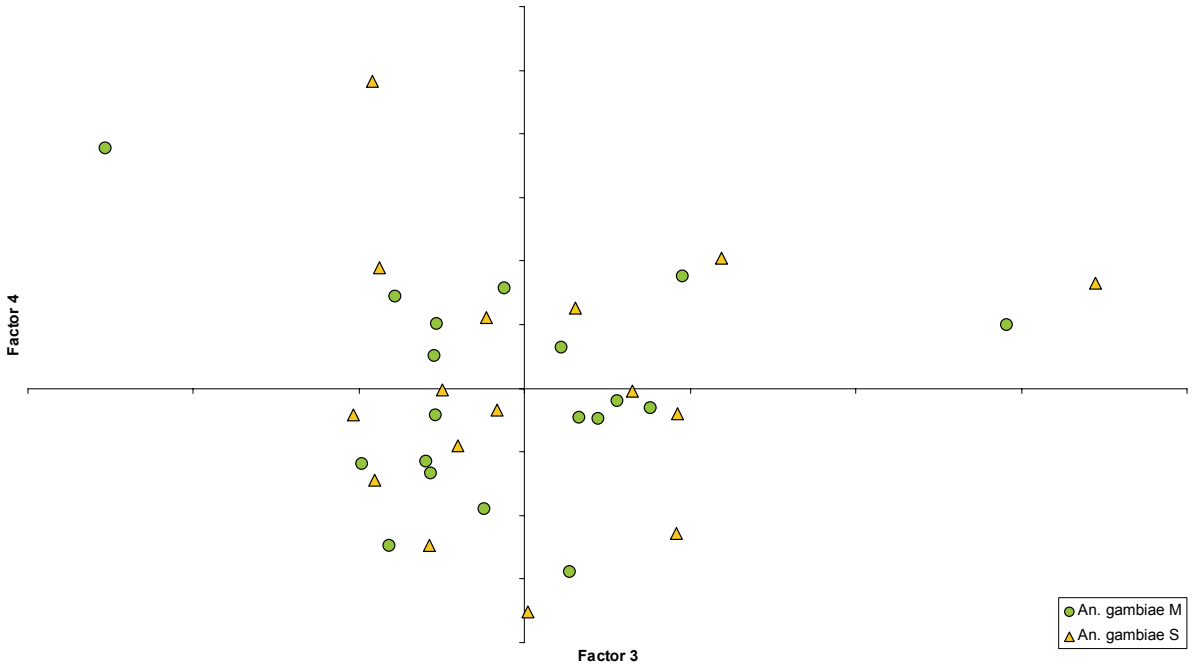


b)

Figure 21: Scatterplot of breeding sites with majority of An. arabiensis *or* An. gambiae s.s. *on the factor-planes elaborated with the factor 1 and 2* (a)*, and with the factor 3 and 4* (b) *obtained by the PCA.*



a)



b)

Figure 22: Scatterplot of breeding sites with majority of An. gambiae *M-form or* An. gambiae *S-form on the factor-planes elaborated with the factor 1 and 2* (a)*, and with the factor 3 and 4* (b) *obtained by the PCA.*

The results of the single correlation analysis of the bacterial groups versus the entomological variables, reported in the table below, shows a situation similar to this observed with physico-chemical variables.

	An. arabiensis relative frequency		*An. gambiae* M-form relative frequency		total larval density	
	r	*p*	r	*p*	r	*p*
Gram+ cocci	0,232	0,180	-0,297	0,083	0,266	0,122
Endospore form. Gram+	0,264	0,126	0,038	0,831	0,002	0,990
Regular Gram+ rods	0,043	0,805	-0,205	0,239	0,371	0,028
Irregular Gram+ rods	0,396	0,019	0,167	0,339	0,137	0,433
Gram- aerobic/microaerophilic	0,248	0,152	0,314	0,066	-0,065	0,712
Enterobacteriaceae	0,152	0,385	0,362	0,033	-0,184	0,291
unidentified Gram-	0,153	0,381	0,393	0,019	0,095	0,586
unidentified Gram variable	0,226	0,192	0,15	0,391	-0,057	0,746

The total larval density directly correlates with regular Gram positive rods, and *An. arabiensis* relative frequency shows a significantly direct correlation with irregular Gram positive rods. Direct correlations are observed with *An. gambiae* M-form relative frequency vs. unidentified Gram-negative bacteria. Table 2 report the statistically significant results obtained with the multiple regression analysis. The multiple regression model for *An. arabiensis* relative frequency confirms the results obtained with single regressions. In fact, the only variable included is the irregular Gram positive rods group. The *An. gambiae* molecular forms relative frequencies have a good correlation with Gram positive cocci (direct for S-form, inverse for M-form). Also included in the model are:

unidentified Gram negatives, endospore forming Gram positives, Enterobacteriaceae and irregular Gram positive rods, all with non significant Betas. For what concern the entomological densities, the regression model for total larval density included regular and irregular Gram positive rods, with direct correlations, but only the first group have a significant *p*-value. Also, the *An. arabiensis* density result directly associated to the same variables, both significant in this case. Finally, the *An. gambiae* S-form density correlates directly with Gram positive cocci with a significant p-value, and have also an inverse correlation with Gram negative aerobic/microaerophilic bacteria, Enterobacteriaceae and irregular Gram positive rods, but in all cases without a significant *p*-value.

***An. arabiensis* relative frequency** R= 0,396 N=35 F(1,33)=6,140 p<0,018 Std.Error of estimate: 0,492									
	Multiple R	**Multiple R-square**	**R-square change**	**F-to-enter**	**p-level**	**Beta**	**Std.Err. of Beta**	**t(33)**	**p-level**
Irregular Gram+ rods	0,396	0,157	0,157	6,140	0,019	0,396	0,160	2,478	0,019
***An. gambiae* M-form relative frequency** R= 0,629 N=35 F(5,29)=3,793 p<0,009 Std.Error of estimate: 0,494									
	Multiple R	**Multiple R-square**	**R-square change**	**F-to-enter**	**p-level**	**Beta**	**Std.Err. of Beta**	**t(29)**	**p-level**
unidentified Gram-	0,393	0,155	0,155	6,042	0,019	0,361	0,192	1,876	0,071
Gram+ cocci	0,495	0,245	0,090	3,829	0,059	-0,439	0,163	-2,688	0,012
Endospore form. Gram+	0,571	0,326	0,081	3,702	0,064	0,204	0,181	1,124	0,270
Enterobacteriaceae	0,612	0,374	0,049	2,340	0,137	0,236	0,166	1,416	0,167
Irregular Gram+ rods	0,629	0,395	0,021	1,006	0,324	0,181	0,181	1,003	0,324
Total larval density R= 0,444 N=35 F(2,32)=3,933 p<0,030 Std.Error of estimate: 0,775									
	Multiple R	**Multiple R-square**	**R-square change**	**F-to-enter**	**p-level**	**Beta**	**Std.Err. of Beta**	**t(32)**	**p-level**
Regular Gram+ rods	0,371	0,138	0,138	5,277	0,028	0,438	0,164	2,668	0,012
Irregular Gram+ rods	0,444	0,197	0,059	2,370	0,133	0,253	0,164	1,540	0,133

Table 1: Significant multiple regression models for the entomological variables in relation to the bacterial groups.

A biological interpretation of these data is not easy to find, because of their heterogeneity. However, some speculations can be made.

High larval densities are associated to breeding sites with alkaline water (Table 3). This seems to be true particularly for *An. arabiensis* and *An. gambiae* M-form. High salt concentrations are generally associated with the larval densities of all taxa. In particular, *An. gambiae* M-form is associated with the hardness of water, which obviously is cause of high pH level. However, the fact

that these ions are associated only with *An. gambiae* M-form led to hypothesize that they could not be the only cause of high pH values. This is confirmed by PCA analysis made to characterize the breeding sites, where pH is not strictly associated with carbonate salts (Figure 12). This means that the association between pH and *An. arabiensis* is probably independent from these salts. The only bacterial group associated with larval densities are the regular Gram positive rods. *An. arabiensis* is the only taxon associated to this bacterial group (Table 3).

An. arabiensis		*An. gambiae* M-form		*An. gambiae* S-form		Total larval density	
+	-	+	-	+	-	+	-
Irregular Gram+ rods	Regular Gram+ rods	unident. Gram-	Gram+ cocci	Gram+ cocci	unident. Gram-	Regular Gram+ rods	
CO_2		Enterobact.	CO_2	Na^+	Enterobact.	pH	
pH		NO_3^-			NO_3^-	Mg^{2+}	
Na^+		TAC					
		TH					
		Ca^{2+}					
		Mg^{2+}					
		Na^+					
		HCO_3^-					
		pH					

Table 3: Summary of correlations of the Anopheles gambiae complex taxa and larval density versus the physico-chemical and microbiological variables. Columns (+) *and* (-) *regroup the positive and negative correlations, respectively. In red are highlighted the variables associated with the specific presence of a taxon, while in black are reported the variables implicated in larval densities only.*

High values of CO2 in solution are positively associated with the presence of *An. arabiensis* and with low densities of *An. gambiae* M-form. Another marker of presence of *An. arabiensis* is the presence of irregular Gram-positive rods, which seems to be a selective marker of this species, being *An. arabiensis* the only correlated with them.

Nitrates, Enterobacteriaceae, unidentified Gram-negative bacteria and Gram-positive cocci seem to be specific marker of the presence of the two *An. gambiae s.s.* molecular forms. In fact, the first three are directly correlated with the presence of the M-form and inversely with the presence of S-form, while the opposite is for Gram-positive cocci.

These results are evidently statistical associations that need successive investigations to understand the cause-effect relation of the variables implicated. Moreover, the microbiological data are supported by a low number of identifications that force to be prudent in the interpretation of the results. Also, the bacterial groups considered probably contain too many species to make any ecological consideration. Despite these premises, our results are of great interest because are the first information on the potential role of ecological markers on the niche partitioning of *Anopheles gambiae* complex malaria vectors in West Africa, and are an important step on the understating of ecological mechanism on incipient speciation of *An. gambiae* taxa, that represent a really important model of evolution in insects.

Our data are also of interest in relation of the development of new control tools of malaria in Africa. In fact, further identification of specific bacteria associated with the presence of a defined taxon would give the possibility to transform this bacterial species with a construct containing toxin genes such as this of the *Bacillus thuringiensis*, which is specific for the Nematocera larvae. This would be a self-maintaining control tool that may have a great efficacy in an environment where the larval control strategies are very difficult to apply and manage.

REFERENCES

Ahmadi, A., and G. A. H. McClelland. 1983. Oviposition attractants of the western treehole mosquito, *Aedes sierrensis*. Mosq News 43: 343-345.

Alilio, M. S., I. C. Bygbjerg, and J. G. Breman. 2004. Are multilateral malaria research and control programs the most successful? Lessons from the past 100 years in Africa. Am J Trop Med Hyg 71: 268-78.

Bates, M. 1940. Oviposition experiments with anopheline mosquitoes. Am J Trop Med 20: 569-583.

Beattie, M. V. G. 1932. The physico-chemical factors of water in relation to mosquito breeding in Trinidad. Bull Entomol Res 23: 477-496.

Bellamy, R. E., W. C. Reeves, and R. P. Scrivani. 1968. Experimental cyclic transmission of St. Louis encephalitis virus in chickens and Culex mosquitoes through a year. Am J Epidemiol 87: 484-95.

Bentley, M. D., and J. F. Day. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. Annu Rev Entomol 34: 401-21.

Bentley, M. D., I. N. McDaniel, H. P. Lee, et al. 1976. Studies of *Aedes triseriatus* oviposition attractants produced by larvae of *Aedes triseriatus* and *Aedes atropalpus* (Diptera: Culicidae). J Med Entomol 13: 112-115.

Bentley, M. D., I. N. McDaniel, M. Yatagai, et al. 1979. *p*-cresol: an oviposition attractant of *Aedes triseriatus*. Environ Entomol 10: 186-189.

Bentley, M. D., I. N. McDaniel, M. Yatagai, et al. 1981. Oviposition attractants and stimulants of *Aedes triseriatus* (Say) (Diptera: Culicidae). Environ Entomol 10: 186-189.

Benzon, G. L., and C. S. Apperson. 1988. Reexamination of chemically mediated oviposition behavior in Aedes aegypti (L.) (Diptera: Culicidae). J Med Entomol 25: 158-64.

Bidlingmayer, W. L. 1975. Mosquito flight paths in relation to the environment. Effects of vertical and horizontal visual barriers. Ann Entomol Soc Am 68: 51-57.

Blackwell, A., and S. N. Johnson. 2000. Electrophysiological investigation of larval water and potential oviposition chemo-attractants for Anopheles gambiae s.s. Ann Trop Med Parasitol 94: 389-98.

Blackwell, A., A. J. Mordue, B. S. Hansson, et al. 1993. A behavioural and electrophysiological study of oviposition cues for *Culex quinquefasciatus*. Physiol Entomol 18: 343-348.

Bryan, J. H., M. A. Di Deco, V. Petrarca, et al. 1982. Inversion polymorphism and incipient speciation in *Anopheles gambiae s.s.* in Gambia, West Africa. Genetica 59: 167-176.

Buxton, P. A., and G. H. E. Hopkins. 1927. Researches in Polynesia and Melanesia: an account of investigations in Samoa, Tonga, the Ellice Group, and the New Hebrides, in 1924, 1925. Parts I-IV. Mem Ser London Sch Hyg Trop Med 1: xi+260.

Chadee, D. D., A. Lakhan, W. R. Ramdath, et al. 1993. Oviposition response of Aedes aegypti mosquitoes to different concentrations of hay infusion in Trinidad, West Indies. J Am Mosq Control Assoc 9: 346-8.

Charlwood, J. D., and D. Edoh. 1996. Polymerase chain reaction used to describe larval habitat use by Anopheles gambiae complex (Diptera: Culicidae) in the environs of Ifakara, Tanzania. J Med Entomol 33: 202-4.

Clements, A. N. 1999. The Biology of Mosquitoes. CABI Publishing, New York.

Collins, F. H., S. M. Paskewitz, and V. Finnerty. 1990. Ribosomal RNA genes of the *Anopheles gambiae* species complex. Springer-Verlag, New York.

Collins, L. E., and A. Blackwell. 2002. Olfactory cues for oviposition behavior in Toxorhynchites moctezuma and Toxorhynchites amboinensis (Diptera: Culicidae). J Med Entomol 39: 121-6.

Collins, R. T., and A. Blackwell. 1998. Electroantennogram studies of potential oviposition attractans fot *Toxorhynchites moctezuma* and *T. amboinensis* mosquitoes. Physiol Entomol 23: 214-219.

Coluzzi, M. 1984. Heterogeneities of the malaria vectorial system in tropical Africa and their significance in malaria epidemiology and control. Bull World Health Organ 62 (suppl.): 107-113.

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

Coluzzi, M., A. Sabatini, A. della Torre, et al. 2002. A polytene chromosome analysis of the Anopheles gambiae species complex. Science 298: 1415-8.

Coluzzi, M., A. Sabatini, V. Petrarca, et al. 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Trans R Soc Trop Med Hyg 73: 483-497.

Costantini, C., S. Li, A. della Torre, et al. 1996a. Density, survival and dispersal of *Anopheles gambiae* complex mosquitoes in a West African Sudan savanna village. Med Vet Entomol 10: 203-219.

Costantini, C., N. F. Sagnon, A. della Torre, et al. 1999. Mosquito behavioural aspects of vector-human interactions in the *Anopheles gambiae* complex. Parassitologia 41: 209-217.

Dadd, R. H., and J. E. Kleinjan. 1974. Autophagostimulant from *Culex pipiens* larvae: distinction from other mosquito larval factors. Environ Entomol 3: 21-28.

Davidson, G. 1964. The Five Mating-Types in the Anopheles Gambiae Complex. Riv Malariol 43: 167-83.

Davidson, G. 1964. Anopheles Gambiae, a Complex of Species. Bull World Health Organ 31: 625-34.

Day, J. F., and J. D. Edman. 1988. Host location, blood-feeding, and oviposition behavior of Culex nigripalpus (Diptera: culicidae): their influence on St. Louis encephalitis virus transmission in southern Florida. Misc Publ Entomol Soc Am 68: 1-8.

della Torre, A., C. Costantini, N. J. Besansky, et al. 2002. Speciation within *Anopheles gambiae*: the glass is half full. Science 298: 115-117.

della Torre, A., C. Fanello, M. Akobeto, et al. 2001. Molecular evidence of incipient speciation within *Anopheles gambiae* s.s. in West Africa. Insect Mol Biol 10: 9-18.

Dethier, V. G., L. Barton Brown, and C. N. Smith. 1960. The designation of chemicals in terms of the responsesthey elicit from insects. J Econ Entomol 53: 134-136.

Du, Y. J., and J. G. Millar. 1999. Electroantennogram and oviposition bioassay responses of Culex quinquefasciatus and Culex tarsalis (Diptera: Culicidae) to chemicals in odors from Bermuda grass infusions. J Med Entomol 36: 158-66.

Fanello, C., F. Santolamazza, and A. della Torre. 2002. Simultaneous identification of species and molecular forms of the Anopheles gambiae complex by PCR-RFLP. Med Vet Entomol 16: 461-4.

Favia, G., A. della Torre, M. Bagayoko, et al. 1997. Molecular identification of sympatric chromosomal forms of *An. gambiae* and further evidence of their reproductive isolation. Insect Mol Biol 6: 377-383.

Favia, G., G. Dimopoulos, A. della Torre, et al. 1994. Polymorphisms detected by random PCR distinguish between different chromosomal forms of Anopheles gambiae. Proc Natl Acad Sci U S A 91: 10315-9.

Favia, G., A. Lanfrancotti, L. Spanos, et al. 2001. Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of Anopheles gambiae s.s. Insect Mol Biol 10: 19-23.

Fontenille, D., L. Lochouarn, N. Diagne, et al. 1997. High annual and seasonal variation in malaria transmission by anophelines and vector species composition in Dielmo, a holoendemic area in Senegal. Am J Trop Med Hyg 56: 247-253.

Gentile, G., M. Slotman, V. Ketmaier, et al. 2001. Attempts to molecularly distinguish cryptic taxa in *Anopheles gambiae* s.s. Insect Mol Biol 10: 25-32.

Gillies, M. T., and M. Coetzee. 1987. A supplement to the Anophelinae of Africa south of the Sahara (Afrotropical Region). Publ S Afr Inst Med Res 55: 1-143.

Gillies, M. T., and B. De Meillon. 1968. The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). Publ S Afr Inst Med Res 54: 1-343.

Gimnig, J. E., M. Ombok, L. Kamau, et al. 2001. Characteristics of larval anopheline (Diptera: Culicidae) habitats in Western Kenya. J Med Entomol 38: 282-8.

Gimnig, J. E., M. Ombok, S. Otieno, et al. 2002. Density-dependent development of Anopheles gambiae (Diptera: Culicidae) larvae in artificial habitats. J Med Entomol 39: 162-72.

Gjullin, C. M., J. O. Johnsen, and F. W. Plapp. 1965. The effects of odors released by various waters on the oviposition sites selected by two species of *Culex*. Mosq News 25: 268-271.

Hasselschwert, D., and C. L. Rockett. 1988. Bacteria as ovipositional attractants for *Aedes aegypti* (Diptera: Culicidae). Great Lakes Entomol 21: 163-168.

Hay, S. I., C. A. Guerra, A. J. Tatem, et al. 2004. The global distribution and population at risk of malaria: past, present and future. The Lancet infectious disease 4: 327-336.

Hazard, E. I., M. S. Mayer, and K. E. Savage. 1967. Attraction and oviposition stimulation of gravid female mosquitoes by bacteria isolated from hay infusions. Mosq News 27: 133-136.

Herms, W. B., and S. B. Freeborn. 1921. The egg laying habits of Californian anophelines. J Parasitology 7: 69-79.

Holding, P. A., and R. W. Snow. 2001. Impact of Plasmodium falciparum malaria on performance and learning: review of the evidence. Am J Trop Med Hyg 64: 68-75.

Holstein, M. H. 1954. Biology of *Anopheles gambiae*, research in French West Africa. World Health Organization, Geneva.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, et al. 2000. Bergey's Manual of determinative bacteriology. Lippincott Williams & Wilkins, Philadelphia.

Hudson, A. 1956. The behaviour of the feemale mosquito in selecting water for oviposition. J Exp Biol 33: 478-492.

Hudson, A., and J. McLintock. 1967. A chemical factor that stimulates oviposition by *Culex tarsalis* Coquillett (Diptera, Culicidae). Anim Behav 15: 336-341.

Hwang, Y. S., W. L. Kramer, and M. S. Mulla. 1980. Oviposition attractants and repellents of mosquitoes. Isolation and identification of oviposition repellents for *Culex* mosquitoes. J Chem Ecol 6: 71-80.

Hwang, Y. S., G. W. Schultz, H. Axelrod, et al. 1982. Ovipositional repellency of fatty acids and their derivatives against *Culex* and *Aedes* mosquitoes. Environ Entomol 11: 223-226.

Hwang, Y. S., G. W. Schultz, and M. S. Mulla. 1984. Structure-activity relationship of unsatured fatty acids as mosquito ovipositional repellents. J Chem Ecol 10: 145-151.

Ikeshoji, T. 1966a. Attractant and stimulant factors for oviposition of *Culex pipiens fatigans* in natural breeding-sites. Bull World Health Organ 35: 905-912.

Ikeshoji, T. 1966b. Studies on mosquito attractants and stimulants. Part I. Chemical factors determining the choice of oviposition site by *Culex pipiens fatigans* and *pallens*. Jap J Exp Med 36: 49-59.

Ikeshoji, T. 1966c. Studies on mosquito attractans and stimulants. Part II. A laboratory tecnique for obtaining mosquito eggs by forced oviposition. Jap J Exp Med 36: 61-65.

Ikeshoji, T. 1966d. Studies on mosquito attractants and stimulants. Part III. The presence in mosquito breeding waters of a factor which stimulates oviposition. Jap J Exp Med 36: 67-72.

Ikeshoji, T. 1975. Chemical analysis of wood creosote for species-specific attraction of mosquito oviposition. Appl Entomol Zool 10: 302-308.

Ikeshoji, T., and M. S. Mulla. 1970. Oviposition attractans for four species of mosquitoes in natural breeding waters. Ann Entomol Soc Am 63: 1322-1327.

Ikeshoji, T., K. Saito, and A. Yano. 1975. Bacterial production of the ovipositional attractants for mosquitoes on fatty acid substrates. Appl Entomol Zool 10: 239-242.

Ikeshoji, T., T. Umino, and S. Hirakoso. 1967. Studies on mosquito attractants and stimulants. Part IV. An agent producing stimulative effects for oviposition of *Culex pipiens fatigans* in field water and the stimulative effects of various chemicals. Jap J Exp Med 37: 61-69.

Isoe, J., and J. G. Millar. 1995. Characterization of factors mediating oviposition site choice by *Culex tarsalis*. J Am Mosq Control Assoc 11: 21-8.

Kaufman, M. G., E. D. Walker, T. W. Smith, et al. 1999. Effects of larval mosquitoes (Aedes triseriatus) and stemflow on microbial community dynamics in container habitats. Appl Environ Microbiol 65: 2661-73.

Kennedy, J. S. 1942. On water-finding and oviposition by captive mosquitoes. Bull Entomol Res 32: 279-301.

Kepner, R. L., Jr., and J. R. Pratt. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol Rev 58: 603-15.

Klowden, M. J. 1990. The endogenous regulation of mosquito reproductive behavior. Experientia 46: 660-670.

Kramer, W. L., Y. S. Hwang, and M. S. Mulla. 1980. Oviposition repellents of mosquitoes: negative responses elicited by lower aliphatic carboxylic acids. J Chem Ecol 6: 415-424.

Laurence , B. R., and J. A. Pickett. 1982. *erythro*-6-Acetoxy-hexadecanolide, the major component of a mosquito oviposition attractant pheromone. Chem Soc, Chem Commun: 59-62.

Laurence , B. R., and J. A. Pickett. 1985. An oviposition attractant pheromone in *Culex quinquefasciatus* Say (Diptera: Culicidae). Bull Entomol Res 75: 283-290.

Lincoln, R. J., G. A. Boxshall, and P. F. Clark. 1982. Dictionary of Ecology, Evolution and Systematics. Cambridge University Press, Cambridge, London and New York.

Linley, J. R. 1989. Laboratory tests of the effects of *p*-cresol and 4-methylcyclohexanol on oviposition by three species of *Toxorhynchites* mosquitoes. Med Vet Entomol 3: 347-352.

Macan, T. T. 1961. Factors that limit the range of freshwater animals. Biol Rev Camb Philos Soc 36: 151-98.

Mattingly, P. F. 1971. Ecological aspects of mosquito evolution. Parassitologia 13: 31-65.

Maw, M. G. 1970. Capric acid as a larvicide and an oviposition stimulant for mosquitoes. Nature 227: 1154-1155.

McCall, P. J., and G. Eaton. 2001. Olfactory memory in the mosquito Culex quinquefasciatus. Med Vet Entomol 15: 197-203.

McCrae, A. W. 1983. Oviposition by African malaria vector mosquitoes. I. Temporal activity patterns of caged, wild-caught, freshwater Anopheles gambiae Giles sensu lato. Ann Trop Med Parasitol 77: 615-25.

McCrae, A. W. 1984. Oviposition by African malaria vector mosquitoes. II. Effects of site tone, water type and conspecific immatures on target selection by freshwater Anopheles gambiae Giles, sensu lato. Ann Trop Med Parasitol 78: 307-18.

McDaniel, I. N., M. D. Bentley, H. P. Lee, et al. 1979. Studies of *Aedes triseriatus* (Diptera: Culicidae) oviposition attractants. Evidence for attractant production by kaolin-treated larvae. Can Entomol 111: 143-147.

Merzagora, L. 1993. Variazioni ecologiche ed epidemiologia della malaria in una zona di savana sudanese presso Ouagadougou, Burkina Faso., Dottorato di ricerca in sanità pubblica, Roma.

Mian, L. S., and M. S. Mulla. 1986. Survival and ovipositional response of *Culex quinquefasciatus* Say (Diptera: culicidae) to sewage effluent. Bull Soc Vector Ecol 11: 1944-1946.

Millar, J. G., J. D. Chaney, J. W. Beehler, et al. 1994. Interaction of the *Culex quinquefasciatus* egg raft pheromone with a natural chemical associated with oviposition sites. J Am Mosq Control Assoc 10: 374-9.

Millar, J. G., J. D. Chaney, and M. S. Mulla. 1992. Identification of oviposition attractants for *Culex quinquefasciatus* from fermented bermuda grass infusions. J Am Mosq Contr Ass 10: 374-379.

Minakawa, N., C. M. Mutero, J. I. Githure, et al. 1999. Spatial distribution and habitat characterization of anopheline mosquito larvae in Western Kenya. Am J Trop Med Hyg 61: 1010-6.

Minakawa, N., G. Sonye, M. Mogi, et al. 2004. Habitat characteristics of Anopheles gambiae s.s. larvae in a Kenyan highland. Med Vet Entomol 18: 301-5.

Mordue, A. J., A. Blackwell, B. S. Hansson, et al. 1992. Behavioural and electrophysiological evaluation of oviposition attractans for *Culex quinquefasciatus* Say (Diptera: Culicidae). Experientia 48: 1109-1111.

Muirhead-Thomson, R. C. 1951. Mosquito behaviour in relation to malaria trasmission and control in the tropics. Edward Arnold, London.

Murphey, F. J., and P. P. Burbutis. 1967. Straw infusions attractiveness to gravid female *Culex salinarius.* J Econ Entomol 60: 156-161.

Odum, E. P. 1971. Fundamentals of ecology. Saunders, Philadelphia.

Omer, S. M., and J. L. Cloudsley-Thomson. 1968. Dry season biology of Anopheles gambiae Giles in the Sudan. Nature 217: 879-880.

Omer, S. M., and J. L. Cloudsley-Thomson. 1970. Survival of *Anopheles gambiae* Giles through a 9-month dry season in Sudan. Bull World Health Organ 42: 319-330.

Osgood, C. E. 1971. An oviposition pheromone associated with the egg rafts of *Culex tarsalis*. J Econ Entomol 64: 1038-1041.

Otieno, W. A., T. O. Onyango, M. M. Pile, et al. 1988. A field trial of the synthetic oviposition pheromone with *Culex quinquefasciatus* Say (Diptera: Culiciade) in Kenya. Bull Entomol Res 78: 463-478.

Paskewitz, S. M., and F. H. Collins. 1990. Use of the polymerase chain reaction to identify mosquito species of the Anopheles gambiae complex. Med Vet Entomol 4: 367-73.

Pavlovich, S. G., and C. L. Rockett. 2000. Color,bacteria, and mosquito eggs as ovipositional attractants for *Aedes aegypti* and *Aedes albopictus* (Diptera:Culicidae). Great Lakes Entomol 33: 141-153.

Perry, A. S., and R. W. Fay. 1967. Correlation of chemical constitution and physical properties of fatty acid esters with oviposition response of *Aedes aegypti*. Mosq News 27: 175-183.

Petersen, J. J. 1969. Oviposition response of *Aedes sollicitans*, *Aedes taeniorhyinchus*, and *Psorophora confinnis* to seven inorganic salts. Mosq News 27: 472-483.

Petersen, J. J., and O. R. Willis. 1970. Oviposition responses of *Culex pipiens quinquefasciatus* and *Culex salinarius* in the laboratory. Mosq News 30: 438-444.

Petrarca, V., G. Petrangeli, P. Rossi, et al. 1986. Etude chromosomique d'*Anopheles gambiae* et *Anopheles arabiensis* à Ouagadougou (Burkina Faso) et dans quelques villages voisins. Parassitologia 28: 41-61.

Pile, M. M., M. S. J. Simmonds, and W. M. Blaney. 1991. Odour-mediated upwind flight of Culex quinquefasciatus mosquitoes elicited by a synthetic attractant. Physiol Entomol 16: 77-85.

Pile, M. M., M. S. J. Simmonds, and W. M. Blaney. 1993. Odour-mediated upwind flight of *Culex quinquefasciatus* mosquitoes elicited by a synthetic attractant: a reappraisal. Physiol Entomol 18.

Reisen, W. K., and T. F. Siddiqui. 1978. The influence of conspecific immatures on the oviposition preferences of the mosquitoes *Anopheles stephensi* and *Culex tritaeniorhynchus*. Pakistan J Zool 10: 31-41.

Reiter, P., M. A. Amador, and N. Colon. 1991. Enhancement of the CDC ovitrap with hay infusions for daily monitoring of Aedes aegypti populations. J Am Mosq Control Assoc 7: 52-5.

Rockett, C. L. 1987. Bacteria as ovipositional attractants for Culex pipiens (Diptera: Culicidae). Great Lakes Entomol 20: 151-155.

Rudolfs, W., and J. B. Lackey. 1929. The composition of water and mosquito breeding. Am J Hygiene 9: 160-180.

Sachs, J., and P. Malaney. 2002. The economic and social burden of malaria. Nature 415: 680-5.

Sagnon, N. F. 1999. Identification moleculaire dans l'etude de la distribution spatiale et de la structure de la population pre-imaginale des taxa du complexe *Anopheles gambiae*, vecteurs du paludisme au Burkina Faso., Dottorato di ricerca in sanità pubblica, Roma.

Scott, J. A., W. G. Brogdon, and F. H. Collins. 1993. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. Am J Trop Med Hyg 49: 520-9.

Service, M. W. 1993. Mosquito ecology: field sampling methods. Elsevier Applied Science, London and New York.

Shroyer, D. A., and D. P. Sanders. 1977. The influence of carbohydrate-feeding and insemination on oviposition of an Indiana strain of Aedes vexans (Diptera: culicidae). J Med Entomol 14: 121-7.

Smith, T. W., E. D. Walker, and M. G. Kaufman. 1998. Bacterial density and survey of cultivable heterotrophs in the surface water of a freshwater marsh habitat of Anopheles quadrimaculatus larvae (Diptera: Culicidae). J Am Mosq Control Assoc 14: 72-7.

Sokal, R. R., and F. J. Rohlf. 1995. Biometry: the principles and practice of statistics in biological research. W. H. Freeman and Company, New York.

Soman, R. S., and R. Reuben. 1970. Studies on the preference shown by ovipositing females *Aedes aegypti* for water containing immature stages of the same species. J Med Entomol 7: 485-489.

StatSoft, I. 2001. STATISTICA (data analysis software system). computer program, version 6. By StatSoft, I., Tulsa.

Stoddart, D. M. 1990. The scented ape: the biology and culture of human odour. Cambridge University Press, Cambridge.

Takken, W. 1991. The role of olfaction in host-seeking of mosquitoes: a review. Insect Sci Applic 12: 287-295.

Takken, W., and B. G. Knols. 1999. Odor-mediated behavior of Afrotropical malaria mosquitoes. Annu Rev Entomol 44: 131-57.

Takken, W., J. J. A. van Loon, and W. Adam. 2001. Inhibition of host-seeking response and olfactory responsiveness in *Anopheles gambiae* following blood feeding. J Insect Physiology 47: 303-310.

Taylor, C. E., Y. T. Toure, M. Coluzzi, et al. 1993. Effective population size and persistence of *Anopheles arabiensis* during the dry season in West Africa. Med Vet Entomol 7: 351-357.

Teklehaimanot, A., and A. Bosman. 1999. Opportunities, problems and perspectives for malaria control in sub-Saharan Africa. Parassitologia 41: 335-8.

Touré, T., V. Petrarca, and M. Coluzzi. 1983a. Il complesso *Anopheles gambiae* in Mali. Parassitologia 25: 367-370.

Toure, Y. T. 1985. Génétique écologique et capacité vectorielle des membres du complexe *Anopheles gambiae* au Mali., Doctoral d'Etat en Sciences Naturelles, Aix-Marseille.

Toure, Y. T., V. Petrarca, and M. Coluzzi. 1983b. Nuove entità del complesso *Anopheles gambiae* in Mali. Parassitologia 25: 1-3.

Touré, Y. T., V. Petrarca, S. F. Traore, et al. 1994. Ecological genetic studies in the chromosomal form Mopti of *Anopheles gambiae* s.str. in Mali, West Africa. Genetica 94: 213-223.

Touré, Y. T., V. Petrarca, S. F. Traore, et al. 1998. The distribution and inversion polymorphism of chromosomically recognized taxa of the *Anopheles gambiae* complex in Mali, West Africa. Parassitologia 40: 477-511.

Trexler, J. D., C. S. Apperson, L. Zurek, et al. 2003. Role of bacteria in mediating the oviposition responses of Aedes albopictus (Diptera: Culicidae). J Med Entomol 40: 841-8.

Trimble, R. M., and W. G. Wellington. 1980. Oviposition stimulant associated with fourth-instar larvae of *Aedes togoi* (Diptera: Culicidae). J Med Entomol 17: 509-514.

Vythilingam, I., S. Novi, and P. Seleena. 1999. Oviposition response of *Aedes aegypti* and *Aedes albopictus* in different types of water. Trop Biomed 16: 23-28.

W.H.O. 1975. Manual on practical entomology in malaria. Part II, methods and techniques. World Health Organization, Geneva.

W.H.O. 2003. The World health report 2003: shaping the future. World Health Organization, Geneva.

Walker, E. D., D. L. Lawson, R. W. Merritt, et al. 1991. Nutrient dynamics, bacterial populations, and mosquito productivity in tree hole ecosystems and microcosms. Ecology 72: 1529-1546.

Walker, E. D., and R. W. Merritt. 1993. Bacterial enrichment in the surface microlayer of an Anopheles quadrimaculatus (diptera: culicidae) larval habitat. J Med Entomol 30: 1050-2.

Wallace, J. R., and R. W. Merritt. 1999. Influence of microclimate, food, and predation on Anopheles quadrimaculatus (Diptera: Culicidae) growth and development rates, survivorship, and adult size in a Michigan pond. Environ Entomol 28: 233-239.

Wallis, R. C. 1954a. A study of oviposition activity of mosquitoes. Am J Hyg 60: 135-168.

Wallis, R. C. 1954b. The effedt of population density and of NaCl concentrations in test series in laboratory experiments with ovipositing *Aedes aegypti*. Mosq News 14: 200-204.

White, G. B. 1974. *Anopheles gambiae* complex and disease transmission in Africa. Trans R Soc Trop Med Hyg 68: 278-301.

White, G. B., S. A. Magayuka, and P. F. L. Boreham. 1972. Comparative studies on sibling species of the *Anopheles gambiae* Giles complex (Dipt., Culicidae): bionomics and vectorial activity of species A and species B at Segera, Tanzania. Bull Entomol Res 62: 295-317.

White, G. B., and P. Rosen. 1973. Comparative studies on sibling species of the *Anopheles gambiae* Giles complex (Diptera: Culicidae). II. Ecology of species A and B in savanna around Kaduna, Nigeria, during transition from wet to dry season. Bull Entomol Res 62: 613-625.

Wilton, D. P. 1968. Oviposition site selection by the tree-hole mosquito, *Aedes triseriatus* (Say). J Med Entomol 5: 189-194.