Universidade Nova de Lisboa
Instituto de Higiene e Medicina Tropical

Malaria vectorial capacity and competence of *Anopheles atroparvus* Van Thiel, 1927 (Diptera, Culicidae): Implications for the potential re-emergence of malaria in Portugal

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LIST OF ABBREVIATIONS

a	- Man biting habit
ABI	- Absolute Breeding Index
C	- Vectorial capacity
CDC-CO_2	- CDC miniature light-traps baited with carbon dioxide
CMDT-LA	- Centro de Malária e Outras Doenças Tropicais-Laboratório Associado
d	- Index of larval association between two species
d.f.	- Degrees of freedom
DNA	- Deoxyribonucleic acid
Δ	- CDC-CO_2 light-trap efficiency
EIR	- Entomological Inoculation Rate
F	- Blood feeding frequency
FA-PVA	- Formic Acid-Polymerized Vinyl Alcohol solution
GBI	- General Breeding Index
HB	- Human baited landing (collections)
HBext	- Outdoor Human baited landing (collections)
HBI	- Human Blood Index
i	- Gonotrophic cycle
i_0	- First gonotrophic cycle
IHMT	- Instituto de Higiene e Medicina Tropical
IR	- Indoor resting (collections)
ITS2	- Internal Transcribed Spacer 2
K-S	- Kolmogorov-Smirnov normality test
K-S	- Kolmogorov-Smirnov statistics
Ku.	- Kurtosis
m	- Number of mosquitoes *per* human
ma	- Man biting rate
MADMTemp	- Monthly Average of Daily Mean Temperature
Md.	- Median
MoAbs	- Anti-IgG antibodies
MoAbs*	- Anti-IgG antibodies conjugated with a peroxidase enzyme
$\underline{n}$	- Parasite extrinsic incubation period
n	- Number of observations
N.	- Number
OR	- Outdoor resting (collections)
P	- P-value
p	- Daily survival rate

PCR - Polymerase Chain Reaction
PIRA - Primer Introduced Restriction Analysis
%RH - Percentage of Relative Humidity
R - Multiple correlation coefficient
R square/R^2 - coefficient of determination
RBI - Relative Breeding Index
rDNA - Ribosomal deoxyribonucleic acid
RFLP - Restriction Fragment Length Polymorphism
s - Standard deviation
Sk. - Skewness
SPSS - Statistical Package for the Social Sciences
S-W - Shapiro Wilk statistics
U - Mann-Whitney U statistics
UEM - Unidade de Entomologia Médica
UNL - Universidade Nova de Lisboa
UTC - Coordinated Universal Time
$\overline{X}$ - Arithmetic mean
X^2_y - Pearson's Chi-square statistics withYate's continuity correction

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ABSTRACT

In Western-European Countries the risk of malaria re-emergence under current environmental and social conditions is considered minimal. However, in the last decade the number of imported cases has increased and several autochthonous cases have been reported from malaria–free places. If the predicted global climate change or other environmental modification would cause a large increase in mosquito vectorial capacity, malaria re-emergence in Europe could become possible. To assess how environmental driven factors may be linked to the risk of re-introducing malaria in Portugal, one must start by characterising the current status of its former vectors. By studying the receptivity and infectivity of present-day mosquito populations, it will be possible to identify factors that may trigger disease emergence and spreading, as well as to provide entomological data to be used in the identification of environmental induced changes of epidemiological significance. Aiming at contributing to these goals, this study has focused on the following **objectives**: (i) to estimate *Anopheles atroparvus* Van Thiel, 1927 vectorial capacity towards malaria and analyse other bioecological parameters with relevance to the introduction of the disease; (ii) to determine *An. atroparvus* vector competence for tropical strains of *Plasmodium falciparum* Welch, 1897.

The region of Comporta presents a unique setting to assess the vector capacity and competence of *An. atroparvus* from Portugal. It was a former malaria hyperendemic region, where *P. falciparum* was the most prevalent malaria parasite. It is a semi-rural area with vast numbers of mosquito breeding sites and a highly mobile human population due mainly to tourism. It is also located fairly close to Lisbon which allows frequent visits to the study area.

Nine would be the maximum estimated number of new daily inoculations that could occur if an infective human host would be introduced in the area. This estimate was obtained for a sporogonic cycle of 11 days (compatible with *P. vivax* development under optimal conditions) and the highest man biting rate obtained in this study (38 bites *per* person *per* day). This value of C is similar to some obtained for other malaria vectors. However, due to the overestimation of most of the computed variables, one can foresee that the receptivity of the area to the re-emergence of the disease is very limited. With the exception of August 2001, the threshold of C=1 was only surpassed during winter/spring months, when parous rates were above 0.95 but abundances were lowest.

Out of 2,207 *An. atroparvus* that were sent to Nijmegen Medical Centre to be artificially infected with the tropical strains of *P. falciparum*, more than 790 specimens took one or two infected blood meals. *Anopheles atroparvus* females infection was successful in a single experiment. These specimens took two infective feeds with a seven days interval.

Blood fed females were kept always at 26ºC with the exception of a 19 hours period that occurred two hours after the second blood meal and during which mosquitoes were placed at 21ºC. Out of the 37 mosquitoes that were dissected, five presented oocysts in their midguts. Prevalence of infection was 13.5% and the mean number of oocysts *per* infected female was 14, ranging between 2 to 75 oocysts *per* infected midgut.

It was confirmed that *An. atroparvus* is, at the most, a low competent vector regarding tropical strains of *P. falciparum*. Artificial infection experiments were not carried out beyond the oocysts phase, thus no conclusion can be drawn regarding sporozoite formation and invasion of salivary glands. Nevertheless, *An. atroparvus* complete refractoriness to tropical *P. falciparum* strains seems less certain than at the beginning of this study.

This study has produced an update on the bionomics of *An. atroparvus* in Portugal and, for the first time, a comprehensive assessment of its vectorial capacity and competence for the transmission of human malaria parasites. It was also attempted to determine if the biology and behaviour of this species has suffered any major switches since the time malaria was an endemic disease in Portugal. The results obtained in this study support the idea that the establishment of malaria in Portugal is a possible but unlikely event in the present ecological conditions.

RESUMO

O risco de re-emergência da malária em países da Europa Ocidental no actual contexto sócio-económico e ambiental é considerado mínimo. No entanto, durante a última década o número de casos de malária importados de regiões endémicas tem aumentado consideravelmente e vários países têm reportado o aparecimento esporádico de casos clínicos autóctones. Face às mudanças climáticas globais observadas teme-se que estas ou outras possíveis alterações ambientais induzam modificações biológicas e/ou etológicas nas espécies de mosquito vectoras do parasita aumentando a sua capacidade vectorial para a transmissão da doença. Para efectuar uma avaliação do possível impacte de alterações ambientais na re-emergência da malária é necessário começar por caracterizar a real situação das suas antigas espécies vectoras. Assim, ao avaliar a presente capacidade e competência vectorial destas espécies poder-se-á identificar os factores que poderão favorecer a re-introdução e disseminação da doença assim como obter dados entomológicos que permitam perceber quais serão as alterações ambientais de maior impacte epidemiológico. Foi com a finalidade de contribuir para o esclarecimento de algumas destas questões que se efectuou o estudo aqui apresentado. Os objectivos principais foram: (i) estimar a capacidade vectorial de *Anopheles atroparvus* Van Thiel, 1927 em relação à malária e analisar outros parâmetros bio-ecológicos com possível relevância para a re-introdução da doença; (ii) determinar a competência vectorial da mencionada espécie em relação à transmissão de estirpes tropicais de *Plasmodium falciparum* Welch, 1897.

A região da Comporta apresenta algumas características que a tornam ideal como área de estudo para a concretização dos objectivos propostos: (i) foi uma antiga região palúdica, onde a malária era hiperendémica e *P. falciparum* a espécie de parasita mais prevalente; (ii) é uma área semi-rural com vastos potenciais criadouros de anofelíneos e é uma zona de lazer que por tal apresenta fluxos sazonais de turistas; (iii) a sua localização é relativamente próxima de Lisboa o que facilita as deslocações regulares à área de estudo.

Em relação ao estudo da capacidade vectorial (C), nove seria o número máximo de inoculações potencialmente infectantes que a população local de *An. atroparvus* poderia ter originado se um portador de gametócitos tivesse permanecido na área por um único dia. Esta estimativa foi calculada para um ciclo esporogónico de 11 dias (compatível com desenvolvimento de *P. vivax* em condições ideais) e para a taxa de agressividade para o Homem máxima determinada ao longo do estudo (38 picadas/dia/Homem). Este valor de C é semelhante a estimativas obtidas para outras espécies vectoras de malária. No entanto, com excepção do mês de Agosto de 2001, o valor limite de C=1 só foi ultrapassado durante os meses de Inverno e Primavera. Isto deveu-se a elevadas taxas de paridade das fêmeas de *An. atroparvus*, acima de 0.95. No entanto, foi também durante estes meses que *An. atroparvus* registou os menores valores de abundância. Assim, e considerando que os demais parâmetros

que compõem a capacidade vectorial foram sobrestimados, poder-se-á concluir que a receptividade da região da Comporta à introdução da malária é relativamente baixa.

Dos 2207 exemplares de *An. atroparvus* que foram enviados para "Nijmegen Medical Centre" para integrarem os ensaios de infecção artificial com estirpes tropicais de *P. falciparum*, mais de 790 efectuaram pelo menos uma refeição sanguínea infectante. No entanto, em apenas um ensaio foi conseguida a infecção experimental de fêmeas de *An. atroparvus*. Neste ensaio os espécimes efectuaram duas alimentações infectantes com um intervalo de sete dias entre cada alimentação. As fêmeas alimentadas foram mantidas a 26ºC, com excepção de um período de 19 h que ocorreu duas horas após a segunda alimentação. Durante esse período as fêmeas recém alimentadas foram mantidas a 21ºC. Das 37 fêmeas dissecadas, cinco apresentavam oocistos no estômago. A prevalência de infecção foi de 13.5% e o número médio dos oocistos por fêmea infectada foi de 14, variando entre 2 e 75 oocistos por estômago infectado. Assim, confirmou-se que *An. atroparvus* é um vector pouco competente para o desenvolvimento de estirpes tropicais de *P. falciparum*. As experiências não foram prolongadas para além da fase de oocisto e, assim, nenhuma conclusão pode ser retirada a respeito da formação dos esporozoitos e da sua capacidade de invasão das glândulas salivares do mosquito. No entanto, face aos resultados obtidos poder-se-á dizer que a hipótese de *An. atroparvus* ser uma espécie completamente refractária à infecção por estirpes tropicais de *P. falciparum* poderá não corresponder à total realidade.

Este estudo permitiu um conhecimento actualizado da biologia e etologia da espécie *An. atroparvus* bem como, pela primeira vez, uma avaliação detalhada de sua capacidade e competência vectorial para a transmissão de parasitas de malária humana. Por comparação com os estudos efectuados durante o período endémico de malária, tentou-se ainda determinar se algum dos parâmetros bio-ecologicos analisados terá sofrido alterações significativas que de algum modo justifiquem uma modificação na receptividade e infectividade da população local de *An. atroparvus* em relação à malária.

Como conclusão poder-se-á dizer que os resultados obtidos neste estudo suportam a ideia que a re-emergência da malária em Portugal é um evento possível mas improvável nas circunstâncias ecológicas actuais.

Chapter I

GENERAL INTRODUCTION

I.1. MALARIA BASIC CONCEPTS AND DEFINITIONS

Human malaria is an infectious parasitic disease caused by four species of the genus *Plasmodium* and transmitted by mosquito females of the genus *Anopheles*. Three of the *Plasmodium* species: *Plasmodium falciparum* Welch, 1897, *Plasmodium ovale* Stephens, 1922 and *Plasmodium vivax* (Grassi & Feletti, 1890) are exclusive parasites of Man. *Plasmodium malariae* (Laveran, 1881) is common to humans and apes (Garnham, 1988).

Malaria is an acute febrile illness. Its severity depends both on the parasite species and strain as well as on patient age, immunity status, genetic constitution, health and nutritional state. The most common recognized malaria symptom is the febrile paroxysm. This starts with a sudden feeling of cold and mild shivering, the beginning of the cold phase. Symptoms rapidly evolve to violent teeth chattering and shaking of the whole body. After 15 to 60 min, patients pass to the second, hot, stage. It starts with the feeling of some waves of warmth. Patients quickly become unbearably hot and body temperature may reach 40-41ºC. During this phase which usually lasts for 2-6 h, spleen enlargement (splenomegaly) may already be palpable. In the third phase, the patients start sweating profusely. The fever and other symptoms diminish during the next 2-4 h after which the exhausted patients tend to fall asleep. The total duration of the typical paroxysm is 8-12 h. Headaches, body aching, nausea and vomiting are other common symptoms associated with malaria (Knell, 1991; Warrel, 2002).

In untreated patients infection becomes synchronized and the febrile paroxysms present a typical periodicity. Infections by *P. falciparum*, *P. vivax* and *P. ovalae* usually originate febrile paroxysms on alternate days while *P. malariae* causes febrile symptoms every 72 h. Based on the period of time between febrile episodes, the first group of species is responsible for what is called the tertian fevers and *P. malariae* for the quartan fever. Due to the high mortality of *falciparum* malaria, the disease caused by this parasite is also known as the malignant tertian fever. In Portugal, the disease was known by "sezões" or "tremedeira" terms derived from the disease symptoms (Faustino, 2006).

In malaria endemic regions, due to acquired immunity, human populations may present some degree of tolerance to infection showing trivial or no symptoms of the disease. However, in poorly immune individuals, malaria is a potential fatal disease. Most of the pernicious complications of the disease are associated with *P. falciparum* infections. Hyperpyrexia, severe anaemia, renal failure and cerebral malaria may develop at any stage of the disease. Of these, cerebral malaria is the most familiar presentation of life threatening malaria and a frequent cause of mortality in young children and non-immune adults (Harinasuta & Bunnag, 1988; Warrel, 2002).

Treatment of malaria depends on the severity of the disease and sometimes on the species and geographic origin of the parasite. Several antimalarial drugs of natural and synthetic origin have been developed through time. At the present the compounds more frequently used in the treatment and prophylaxis of malaria are the antifolate drugs (pyrimethamine, sulfadoxine), the derivates of Quinghaosu (artemisin) and compounds of the quinoline class (quinine, mefloquine, chloroquine, primaquine) (Cowman & Foote, 1990; Arav-Boger & Shapiro, 2005).

I.1.1. MALARIA PARASITES: SYSTEMATIC POSITION AND LIFE CYCLES

According to Ayala *et al*. (1998) the four species of parasites that cause human malaria are classified as:

Kingdom Protista
Phylum Apicomplexa
Class Hematozoa
Order Haemosporida
Family Plasmodiidae

Genus *Plasmodium* - with species in which there is asexual multiplication by division in other cells besides the erythrocytes of the vertebrate host. Several species of Culicidae act as the invertebrate host of these parasites. The genus *Plasmodium* is according to Gilles (1993) divided in three subgenera:

-Subgenus *Plasmodium*: with 20 species including *P. malariae* (Laveran, 1881), *P. ovale* Stephens, 1922 and *P. vivax* (Grassi & Felleti, 1890);
-Subgenus *Laverania*: in which *P. falciparum* Welch, 1897 is included;
-Subgenus *Vinckeia*: with several species responsible for rodent malaria.

The life cycle of all human Plasmodia are similar and consist of a sequence of two phases: one sexual phase with multiplication which takes place inside the mosquito and another asexual phase that takes place in the human host (Figure I.1.). The latter can be divided in two stages: one that involves the development and multiplication of the parasite in the liver parenchyma cells – exoeritrocytic or hepatic schizogony – and another that occurs in the red blood cells – erytrocytic schizogony. The term sporogonic or extrinsic cycle is frequently used as referring to the parasite life phase inside the mosquito while the denomination schizogonic cycle is frequently applied to the parasite life stages inside the vertebrate host.

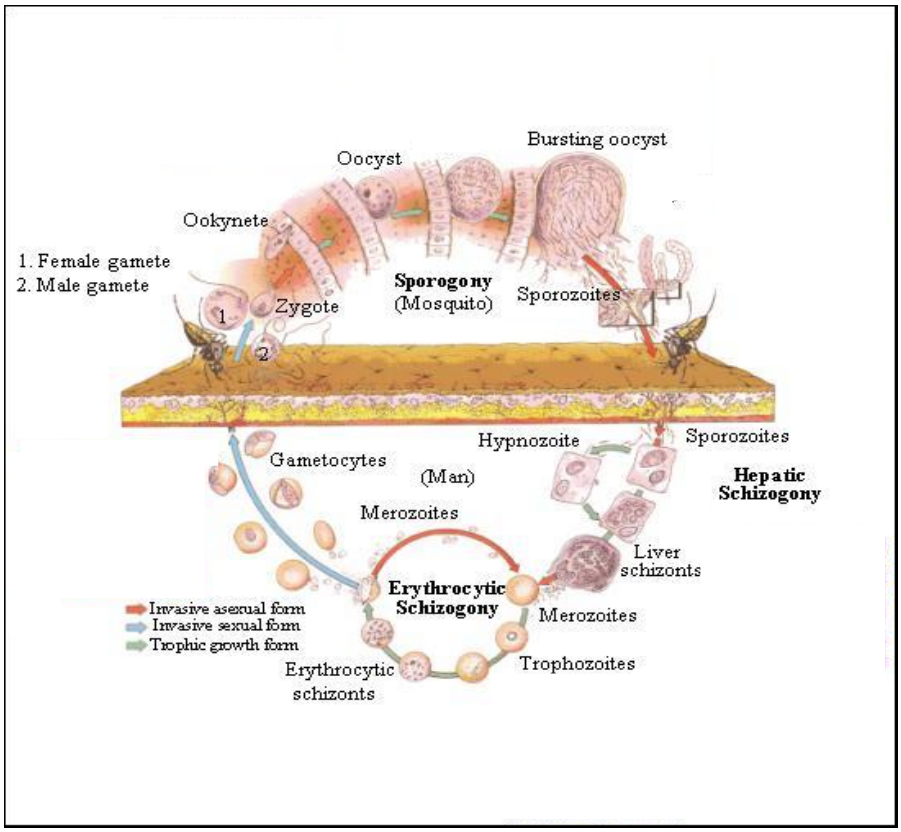


Figure I.1. The life-cycle of human malaria parasites.
Adapted from Knell, 1991.

Sporogony: The gametocytes present in an infected human host when ingested by a *Anopheles* female during a blood meal differentiate into free gametes, male and female. Fertilization, that takes place in the lumen of the mosquito midgut, originates the zygote. This is the only diploid phase of the parasite life-cycle where meiosis occurs. The zygote develops into the invasive ookinete which settles into the stomach wall and becomes an oocyst, a rounded globular pigmented body. The oocyst grows and divides by repeated mitosis to produce thousands of elongated mobile forms, the sporozoites. The mature oocyst burst and the free sporozoites migrate through the mosquito haemocoel. The sporozoites reach the mosquito salivary glands and penetrate them.
Hepatic schizogony: When the mosquito female feeds again on a human the sporozoites are injected into the blood. They invade the liver cells and differentiate into hepatic trophozoites. In *P. ovale* and *P. vivax*, some trophozoites differentiate into dormant forms, the hypnozoites. The hepatic trophozoites grow and divide to produce thousands of invasive merozoites that are released into the bloodstream.
Erythrocytic schizogony: Merozoites invade red blood cells and become erythrocytic trophozoites. These will grow, feeding on the haemoglobin of the erythrocyte, and then divide originating erythrocytic schizonts. Each schizont by cytoplasmatic segmentation originates 8-16 new merozoites. The red cell bursts, the merozoites are released into the bloodstream and the cycle starts again by the invasion of new erythrocytes. As the disease progress a few merozoites will differentiate into gametocytes. However, these will not develop further unless taken up by a mosquito female and initiating then the sporogonic phase.

While the duration of the hepatic schizogony varies mainly according to the parasite species, the period necessary for the extrinsic cycle to take place is dependent on both the parasite species and the temperature to which the mosquito is subjected. Some findings support the idea that the mosquito species may also have an effect on the duration of the sporogony but in a much smaller scale (Molineaux, 1988). The duration of the sporogonic

cycle, also denoted as extrinsic incubation period ($\underline{n}$), can be estimated based on Moshkovsky method (*fidé* Detinova, 1963) according to the formula:

$$\underline{n} = T/(t_a - t_m)$$

where T=105, 111 and 144 for *P. vivax, P. falciparum* and *P. malariae* respectively; t_a refers to the actual average temperature in degrees centigrade; t_m=14.5 for *P. vixax* and t_m=16 for *P. falciparum* and *malariae*.

I.1.2. MALARIA VECTORS

In 1898, Sir Ronald Ross described for the first time the sporogonic cycle of *Plasmodium* parasites (Bruce-Chwatt, 1988). Finally, one of the most puzzling medical mysteries of his time was solved and the fundamental role of mosquitoes as malaria vectors was clearly exposed. Although Ross observations were made on bird malaria (*Plasmodium relictum* (Grassi & Felleti, 1891)) transmitted by *Culex quinquefastiatus* Say, 1833, the complete cycles of *P. falciparum* and *P. vivax* were soon described in *Anopheles* species by Grassi and collaborators (Grassi *et al.*, 1989; Grassi, 1900 *fidé* Bruce-Chwatt, 1988). Nowadays, it is known that several genera of mosquitoes can transmit malaria. However, only females of the genus *Anopheles* can act as vectors of the human *Plasmodium* parasites.

I.1.2.1. Systematic position of the genus *Anopheles*

According to the classification of Richards & Davies (1977), the systematic position of the genus *Anopheles* is:

Kingdom Animalia
Phylum Arthropoda
Class Insecta
Order Diptera
Family Culicidae
Subfamily Anophelinae

Genus *Anopheles:* with 97% of all Anopheline species (Krzywinski *et al.*, 2001), this genus is the largest and the most diversified of the three genera recognized in the Anophelinae subfamily: *Bironella*, *Chagasia* and *Anopheles*. (Senevet, 1958; Krzywinski & Besansky, 2003). The genus includes 444 formally named and 40 provisionally designated extant species (Harbach, 2004). These are divided into four small Neotropical (distributed

throughout Central and South America) subgenera: *Kerteszia*, *Lophopodomyia*, *Nyssorhynchus* and *Stethomyia*; and two larger subgenera: *Cellia* in the Old World (Europe, Africa and Asia) and *Anopheles* with worldwide distribution. Of all the Anopheline species, *ca.* 70 may play some role as malaria vectors, but only 40 of these are of major importance (Service & Townson, 2002). While the main malaria vectors in Africa (*An. gambiae s.l.* and *An. funestus s.l.*) belong to the subgenus *Cellia*, in Europe malaria transmission is mainly carried out by species of the subgenus *Anopheles*.

-Subgenus *Cellia*: Adults of this subgenus usually present wings with distinct pale markings, including a series of spots along the costa vein (Evans, 1938 *fidé* Gillies & De Meillon, 1968).

-Subgenus *Anopheles*: Adults usually have dark wings with less than four main dark areas. Pale scales of veins when present intermingled with dark ones not forming distinct pale areas (Evans, 1938 *fidé* Gillies & De Meillon, 1968). This subgenus includes the *Anopheles maculipennis* complex, in which almost all malaria vector species of Europe are included.

I.1.2.2. *Anopheles* life cycle and morphology

The following descriptions of *Anopheles* life cycle and external morphology, unless stated otherwise, were based on the works of Knell (1991), Service & Townson (2002) and White (2003). These descriptions cannot be seen as exclusive and diagnostic of the genus *Anopheles* since in most cases refer to criteria that differentiate the Anophelines (subfamily Anopheline) from the other Culicids and therefore are shared by all the three Anopheline genera. However, due to the limited representation of *Bironella* and *Chagasia* regarding their number of species and geographic location (*Bironella* - Australian region; *Chagasia* - Neotropical region), the descriptions hereby presented can be regarded as exclusive of the genus *Anopheles* for the Holartic region (Europe, Asia and North America), the zoogeographic region where all the members of the *An. maculipennis* species complex are distributed.

As it happens in all the other mosquito genera, *Anopheles* mosquitoes pass through four distinct stages of growth and metamorphosis: egg, larva, pupa and adult or imago (Figure I.2.). The first three immature stages are aquatic and the adult stage is terrestrial.

Anopheles eggs are ovoid or boat-shaped *ca.* 0.5 mm long. In almost all species, eggs present two lateral air-filled floats which allow them to float on the water surface after being individually laid by the adult female. The delicate outer layer of the egg (exocorion) may present ornamentation patterns on its dorsal surface which can be useful for the identification of some species. The egg takes one to two days to hatch and originate a larva.

The body of the larvae is composed by a distinct head, a broad and flat thorax and an abdomen with 10 segments. The head bears a pair of eyes and conspicuous mouth brushes with numerous flexible hairs. The thorax has several groups of setae having species diagnostic importance. The abdomen presents only nine visible segments. The 8th and 9th abdominal segments are fused and bear a pair of rounded spiracular openings of the respiratory system. This is the most obvious diagnostic characteristic of *Anopheles* larvae since other Culicids have their spiracular openings at the tip of a prominent tube, the respiratory siphon. Other distinct features of *Anopheles* larvae are the presence of abdominal palmate hairs or small accessory tergal plates. The larva passes by three successive ecdyses going through four instars (from L1 to L4) before becoming a pupa.

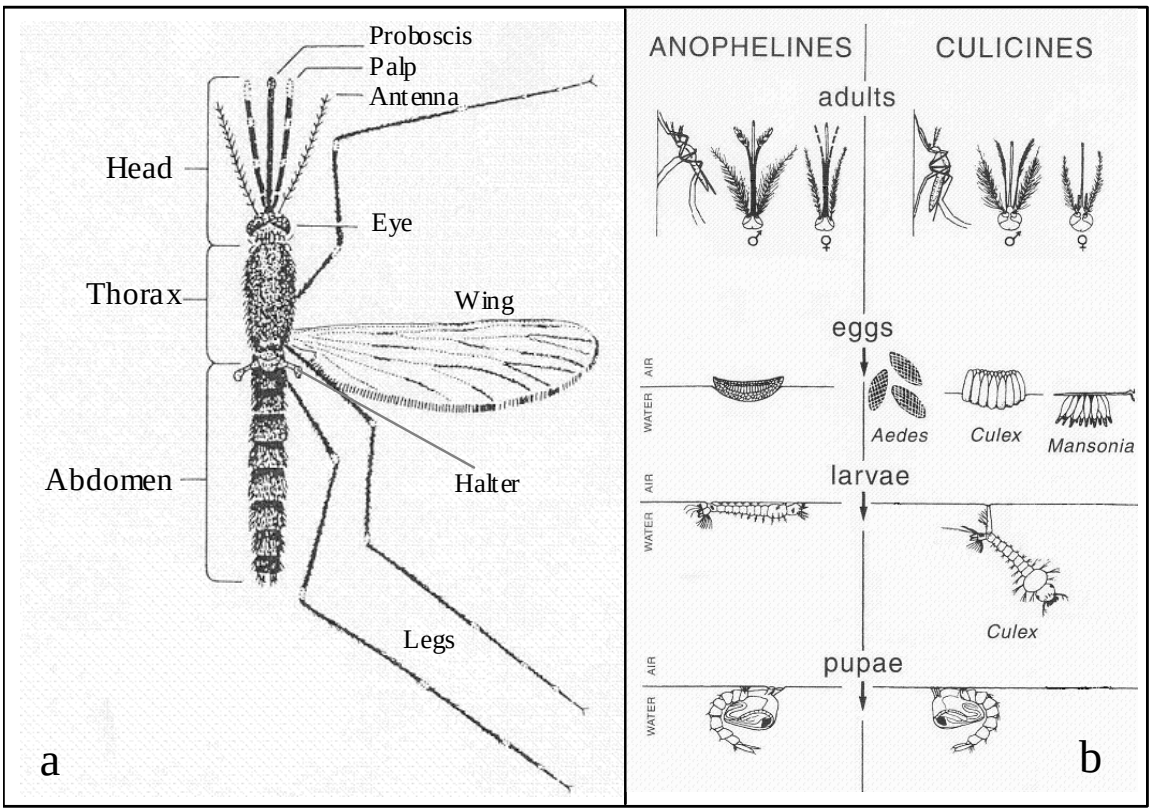


Figure I.2. General mosquito anatomy (a); morphological differences between Anophelines and Culicines (b). Adapted from WHO, 1994 and Service, 1993.

The pupae have a comma-shaped body composed by a cephalotorax, with a pair of short apical flared respiratory trumpets, and an abdomen with eight visible segments and a pair of terminal, oval-shaped, flatted paddles. The distinction of pupae of different genera of Culicids is not obvious. However, in Anopheline pupae, short peg-like hairs are usually present at the posterior corners of most abdominal segments. Pupae do not feed and after one or two days metamorphose to adult.

In the adult, a rounded head attached to the thorax by a slender neck bears two prominent eyes, a pair of antennae, two maxillary palps and one piercing-sucking proboscis. The antenna, composed of 15 segments, carries numerous sense organs and presents sexual dimorphism, being plumose in males and feathered in females. The two palps, located one at each side of the proboscis, are also sensory structures. In contrast with Culicines, in which the female palps are short, in *Anopheles* the palps of both sexes are about as long as the proboscis and in males are club-shaped. The proboscis is formed by the labium, the labrum, paired mandibles and maxillae and the hollow hypopharynx. The thorax presents three pairs of legs, one pair of membranous wings and hind pair of wings transformed into drumstick-shaped, clubbed structures. The scale ornamentation of legs and wings has often species-specific patterns. Behind the main and larger part of the thorax (scutum) there is a small structure (scutellum) which in *Anopheles* is posteriorly evenly rounded and lacking lateral lobes. The abdomen has eight visible segments with an apical pair of small cerci, in females, and a pair of prominent claspers, in males.

I.1.2.3. Internal anatomy

Female *Anopheles* become infected and infectious with several pathogens due to their need of blood to develop eggs and produce an offspring. Thus, the internal structure and physiological mechanisms of digestive and reproductive systems in *Anopheles* are of upmost importance in the study of malaria transmission. The following description were based on the works of Knell (1991) and Service & Townson (2002)

Digestive system: This is divided in three main parts: (i) the foregut, formed by the pharynx and the oesophagus; (ii) the midgut, in which an expanded section is often called stomach, and; (iii) the hindgut. In the esophagus a ventral sac-like structure, the ventral diverticulum or crop, opens to the alimentary channel. Five Maphighian tubes, responsible for the initial phase of diuresis and nitrogen excretion are attached at the end of the midgut. These discharge the absorbed waste materials from the blood into the hindgut. The resultant fluid is modified in the rectum before being eliminated through the anus as urine (Figure I.3.).

Blood and sugary liquids, the two types of nourishment taken by females, are sucked through the labium to the esophagus. Sugary fluids go to the crop where they are stored until required, only then passing to the midgut for digestion. Blood goes directly to the stomach. When the female mosquito is feeding the hypopharynx injects saliva with anticoagulant and anaesthetic substances into the vertebrate host. The saliva is produced in a pair of three-lobed

salivary glands located in the anterior thorax. It is conducted from the glands by two ducts that form a salivary duct which in turn connects to the hypopharynx.

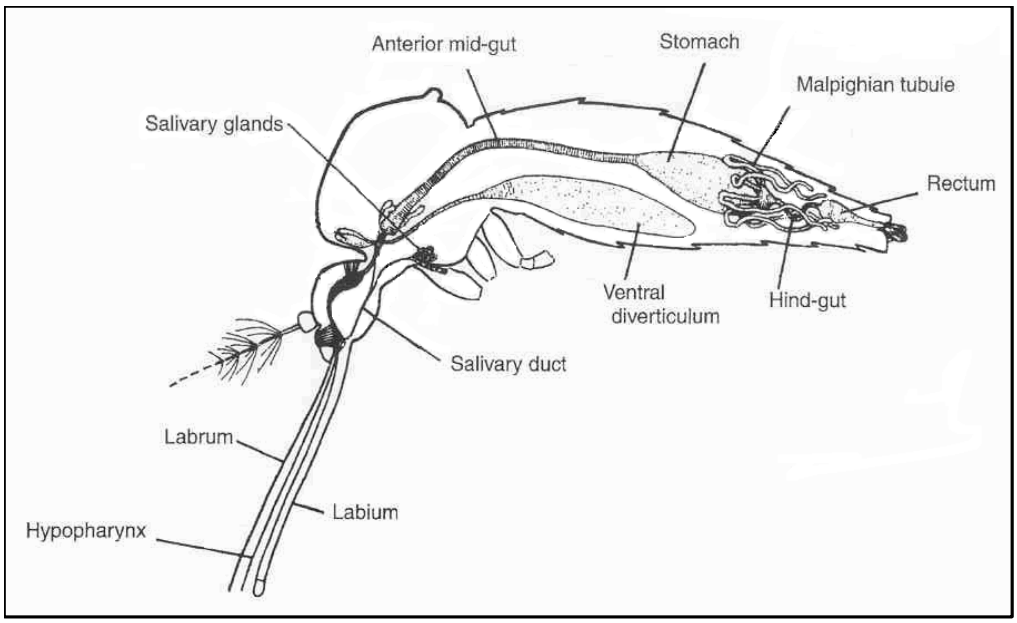


Figure I.3. Internal anatomy of a female mosquito digestive system.
Adapted from Service & Townson, 2002.

Reproductive system: In females a pair of ovaries, each composed by 50-200 ovarioles, is located in the posterior part of the abdomen. Each ovariole produces a single egg using the nutrients provided by the digestion of the blood meal. The ovariole consists of a hollow stalk, developing follicles and a terminal germarium. Oviducts of each ovary fuse into a common oviduct, into the distal part of which the sperm duct opens. This sperm duct comes from a sclerotized spherical structure, the spermatheca. The spermatheca stores, for the duration of the female's lifetime, the sperm introduced in it during mating. With few exceptions a female copulates only once.

I.1.3. ASPECTS OF MOSQUITO PHYSIOLOGY AND BEHAVIOUR

Mosquitoes present a number of behavioural traits and physiological features that have medical importance. Most of these characteristics have been deeply studied especially in Anophelines and a whole lexicon has been developed around these subjects.

Some species of Anophelines can only copulate in open or very large areas. In these species, called *eurigamous*, males tend to form swarms. However, not all the species that swarm are eurigamous. One of these examples is the *stenogamous An. atroparvus* which by definition can copulate in a cage with $< 0.1\ m^3$ (Clements, 1999).

Blood feeding and egg production are intimately linked since the eggs develop and fill the abdomen as blood becomes digested. The term *gonotrophic cycle* is used to designate a specific period of the female's life. It is divided in three phases: (i) search for an appropriate host and blood meal intake; (ii) blood digestion and growth of egg follicles and; (iii) search for a suitable larval breeding site and oviposition.

Throughout the gonotrophic cycle the female's abdominal appearance changes. According to their abdominal condition females can be classified into seven classes, denoted as *Sella's stages* (Figure I.4.). In parallel the development of the ovarian follicles can also be classified into five different categories (Figure I.5.) known as Christophers *stages* (1911 *fidé* Detinova, 1963).

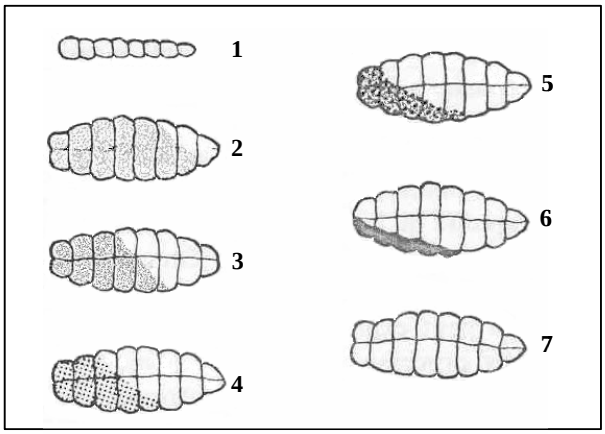


Figure I.4. Sella´s stages of gonotrophic development.
Stage 1: unfed; stage 2: recently blood fed; stages 3-5 half gravid stages; stages 6-7: gravid. Adapted from Detinova, 1963.

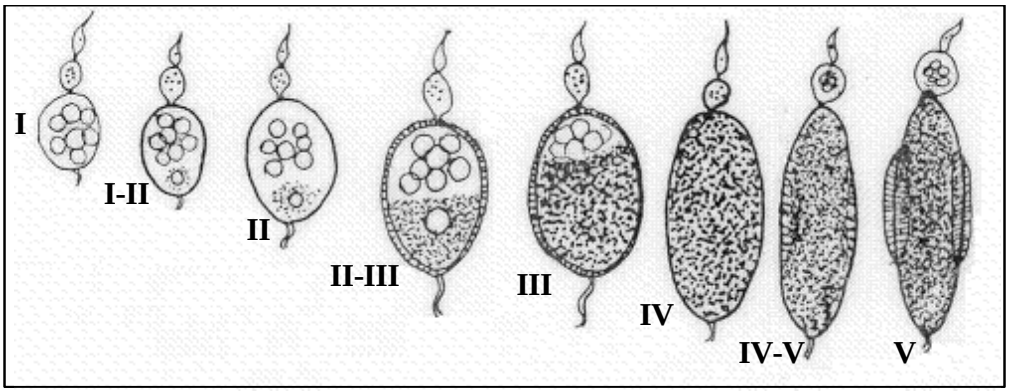


Figure I.5. Morphological appearance of an ovarian follicle during the five Christophers stages.
Based on the work of Detinova, 1963.

A species is considered *homodynamic* when it is able to continuously produce eggs if provided with adequate blood source and favourable ambient temperatures. In contrast, a *heterodymanic* species presents spontaneous ovarian diapauses (Roubaud, 1934).

According to their resting habits, in species called *endophilic*, mosquitoes usually rest indoors before or after the blood meal, while in *exophilic* species, engorged specimens are found resting outside (*e.g.* in the vegetation) (Clements, 1999). Similarly, *endophagy* refers to the mosquito's habit of blood feeding inside a man-made structure opposite to *exophagy* which refers to the preference of taking blood meals outdoors (Clements, 1999). As to host preferences, species can be denoted as *zoophilic* (*alt.* zoophagous) if mosquitoes blood feed preferentially in vertebrates beside humans or *anthropophilic* (*alt.* anthropophagous) if they use humans as the main blood source (Clements, 1999).

I.1.4. EPIDEMIOLOGICAL ASPECTS OF MALARIA AND ITS MEASUREMENTS

Malaria is endemic in over 90 Countries of the world[1] and nearly 40% of the human population is at risk of contracting the disease (Price & Nosten, 2001). It is still one of the major causes of human mortality being responsible for 1.5 to 2.7 millions of deaths *per* year (Butler, 2004) of which approximately one million are children under five years of age[1]. Although with a larger distribution in the past, nowadays malaria endemic areas are mainly located in tropical and subtropical regions of the world (Figure I.6.).

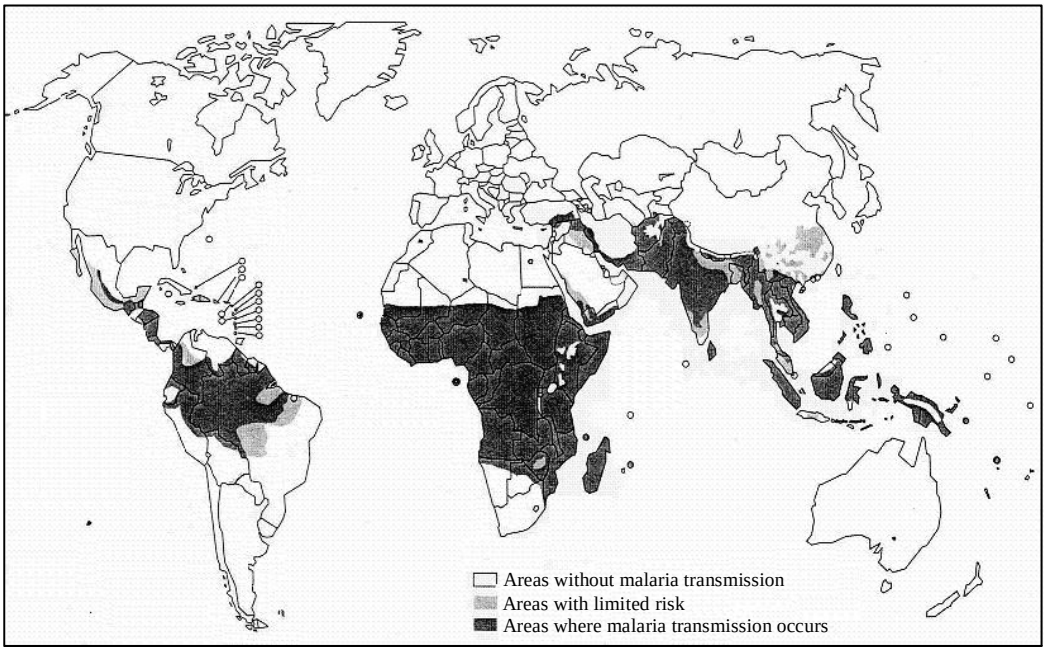


Figure I.6. Malaria world distribution.
Adapted from Gilles, 2002.

[1] www.who.int/malaria/

The measurement of malaria and its components serves mainly to describe and explain the disease distribution as well as to evaluate its control. To achieve the endpoint of these measurements, usually in a form of some epidemiological index, four steps must be taken: (i) the choice of the study subject, a local human and/or vector population; (ii) the design of a sampling scheme including the selection of the collection method; (iii) the implementation of the sampling method chosen and gathering of its data, and finally; (iv) the analysis of results and the calculation of the index. Of these four phases not always distinguishable, step (ii) is particularly sensitive since the choice of the sampling method may dramatically influence the out coming results, as exemplified below.

The most common epidemiological indexes estimated from the human population are: (i) *prevalence*, number of malaria cases in a population at a given time point; (ii) *incidence*, number of new cases detected in a population during a certain period of time; (iii) *mortality rate*, number of deaths due to malaria *per* 100,000 humans *per* year; (iv) *parasite rate*, proportion of people presenting parasites in blood films, and; (v) *spleen rate*, proportion of people of a given population with enlarged spleens (splenomegaly).

Malaria is described as *endemic* when, in a given population, incidence presents similar number of cases over a period of many successive years. It is called *epidemic* when there is a periodic or occasional sharp increase of new cases.

The term *endemicity* refers to the amount or severity of malaria in a certain area or population. The *degrees of endemicity* adopted by the W.H.O. in the 1950's are determined according to the spleen rates of a statistically significant sample of the population under study. These degrees are: (i) hypoendemic, spleen rates in children (with age between 2-9 y) not exceeding 10%; (ii) mesoendemic, spleen rates in children between 11% and 50%; (iii) hyperendemic, spleen rates in children constantly over 50% and in adults over 25%, and; (iv) holoendemic, spleen rates in children constantly over 75%, but low in adults (Snow & Gilles, 2002). A similar classification was proposed by Metselaar & Van Thiel (1959 *fidé* Molineaux, 1988) but using parasite rates, a parameter less subjective to analyse. The boundaries of each degree are the same: (i) hypoendemic, parasite rates in children (with age between 2-9 y) not exceeding 10%; (ii) mesoendemic, between 11% and 50%; (iii) hyperendemic, between 50% and 75%, and; (iv) holoendemic, constantly over 75%.

To characterise malaria transmission two parameters are widely used: the entomological inoculation rate and vectorial capacity of the mosquito population. The *entomological inoculation rate* (EIR) is defined as the number of sporozoite infected bites that one person receives *per* unit of time. This index is calculated as:

$$EIR = mas$$

where, *m* is the number of vectors *per* human; *a*, the number of human blood meals *per* vector *per* day and; s, the fraction of vectors with sporozoites in the salivary glands. As it is easily perceived, the EIR varies with time, vector species and parasite species.

The *vectorial capacity* (C) of a given mosquito population can be defined as the average number of inoculations originating from one case of malaria in a unit of time (usually one day), that the (vector) population would distribute to Man if all the vector females biting the case became infected (Garrett-Jones, 1964a). In places where malaria does not exist, the vectorial capacity will show how receptive to transmission those places may be, that is, what rate of inoculation may be expected to result from the introduction of an imported malaria case (Garrett-Jones & Shidrawi, 1969). This index is estimated according to the formula:

$$C = \frac{ma^2 p^n}{-\ln p}$$

in which, *m* and *a* are the same variables mentioned above; *p* refers to the daily survival probability or proportion of vectors surviving *per* day; and, *n* the incubation period of the parasite inside the vector (extrinsic period of incubation[2]).

Mosquito density *m* is by far the most difficult to obtain precise estimates (MacDonald, 1956). Although usually there is no need to be completely accurate, this estimate should refer to the greatest vector densities occurring over a given period or area (MacDonald, 1956). This parameter is frequently assessed together with *a* under the designation of man biting rate (*ma*). This *ma* may be estimated directly by means of all-day landing catches on human baits (for details see Chapter III) or indirectly from the ratio of indoor resting blood-fed females *per* inhabitant. Although this last method may only be applied where the vector is known to be predominantly endophagic and endophilic and in places where houses are not submitted to residual spraying or other protective measure, it is considered more representative of true incidence of biting contact (Garrett-Jones & Shidrawi, 1969). If a host is not conveniently standing still and waiting to be bitten, a mosquito may be forced to enter several houses before obtaining a blood meal in a quest that may not always be successful. Therefore, the *ma* measured by the direct capture method may be overestimated and it is usually higher when compared with estimates calculated as indoor-resting number of blood fed females *per* sleeper.

[2] Also denoted as n to avoid confusion with *n* as number of observations.

The parameter *a*, designated man biting habit (Garrett-Jones, 1964b) can be assessed independently from *m*. The man biting habit, defined as the mean frequency with which a single vector female bites humans, is estimated as the product of female's mean feeding frequency (*F*) by the human blood index (*HBI*). The feeding frequency represents the number of feeds taken by a female mosquito *per* unit of time (usually *per* day) while *HBI* refers to the proportion of freshly-fed females found to contain human blood (Garrett-Jones, 1964b). The selection of a proper mosquito sampling is again crucial for a proper *HBI* estimate and Garrett-Jones (1964b) offered some warnings concerning the collection of unbiased samples: (i) collections should be performed at a time and a place where blood-fed mosquitoes are likely to be resting; (ii) the resting places should be grouped according to their characteristics into meaningful units (*e.g.* houses, animal shelters, outdoor's shelters). The *HBI*s can be computed as crude estimates that result from the direct application of its definition, or expressed as either a weighted or unweighted means (Garrett-Jones, 1964b, 1980).

With respect to the daily survival rate (*p*), there are several methods for its estimation (Molineaux *et al.*, 1988). It can be computed by direct methods derived from mark-recapture experiments or from the observation of captive specimens. However, both of these methods have their inherent difficulties, as the low rates of recapture for the first or the presence of artificial conditions for the second. When indirectly estimating survival on the basis of age composition of field collected mosquitoes, the estimation of *p* is often made by extracting the i_0^{th} root of the proportion of parous females, where i_0 is the duration in days of the first gonotrophic cycle (Davidson, 1954). This model, that assumes that mosquito adult emergence and death-rate are constant, may be affected by three types of constrains: (i) the existence of adult emergence peaks; (ii) mosquito collection methods that sample preferentially certain age-groups and; (iii) age-dependent mortality rates. An alternative method of computing *p* from the proportion of parous females was presented by Garrett-Jones & Grab (1964) that stressed the importance of knowing the mean difference in age between nulliparous and primiparous females as well the sporogonic period of the parasite. Other model frequently used to determine the *p* value is the one described by Vercruysse (1985) which is also based on vertical age-structure of vector population but only uses three age classes: nulliparous biting females taking their first blood meal, nulliparous females taking a second feed and parous females.

A third parameter has played a central role in epidemiological theory for malaria: the *basic reproduction rate* (R_0). This rate is defined as the potential number of secondary cases originating from one case throughout its duration, assuming all members of the population to be fully sensitive (Molineaux, 1988). This concept defined by Macdonald (1957) and can be mathematically estimated as:

$$R_0 = C / r$$

where C is the vectorial capacity of the Anopheline local population and *r* the recovery rate of the human population.

The R_0 also provides an index of transmission intensity, but opposite to the previous parameters (EIR and C) establishes a threshold criterion. If R_0 is greater than one, the number of people infected by the parasite increases, and if R_0 is less than one, that number declines.

Refinements were introduced to this biological model and more recently R_0 has been described as:

$$R_0 = C * b * c / r$$

where *b* is defined as the infectivity of mosquitoes to humans and *c* the infectivity of humans to mosquitoes (Rogers & Randolph, 2000; Smith *et al.*, 2007).

In a non-endemic malaria area R_0 model may also be applied to predict the possibility of disease introduction. In this case R_0 is computed as the product of vectorial capacity of the mosquito local population by the proportion of mosquitoes that became infected after feeding on an infected (gametocyte carrier) human, *i.e.* mosquito vectorial competence (Alten *et al.*, 2007; Smith *et al.*, 2007).

I.2. MALARIA IN EUROPE

I.2.1. ORIGINS

The origin of malaria as a disease of Man is a controversial issue. To the best of our knowledge, it seems that three of the malaria parasites, *P. malariae*, *P. ovale* and *P. vivax*, underwent lateral transfers from other primates to humans. *Plasmodium falciparum* could be an ancient human parasite (Joy *et al.*, 2003) the divergence of which from its closest relative (*P. reichenowi*) ran in parallel with the divergence of hominids and chimpanzees (Escalante & Ayala, 1994).

The polyphyly of the genus *Plasmodium* is generally accepted with *P. falciparum*/*P. reichenowi* forming a monophyletic group separated from the other species (Escalante *et al.*, 1998). Although most evidences indicate that *P. falciparum* have its origins in Africa, as an avian parasite (Escalante *et al.*, 1998; Rathore *et al.*, 2001; Joy *et al.*, 2003), the hypotheses of a mammalian ancestor is not completely discarded (Wiersch *et al.*, 2005). The other malaria parasites are considered to have developed from zoonotic simian Plasmodiids in tropical forests of southeastern Asia (Poolsuwan, 1995). However, the Asian origin of *P. vivax* from a

species parasitic to macaques (Escalante *et al.*, 2005, Cornejo & Escalante, 2006) is challenged by the less advocated hypothesis of humans having acquired *P. vivax* from New World monkeys (Mu *et al.*, 2005).

I.2.2. HISTORICAL RECORDS

All species of human malaria and respective vectors were probably absent from Europe during the Ice Ages of the Quaternary. The adverse environmental conditions of these periods would not have allowed the survival of *P. falciparum* and the scarcity of humans would have yielded the transmission of the other three species difficult to be accomplished (Bruce-Chwatt & Zulueta, 1980a). As to mosquitoes, climate conditions during the last Pleistocene glaciation, although compatible with the presence of today's northern *A. maculipennis* members, would have probably excluded the two most effective European vectors: *An. labranchiae* and *An. sacharovi* (Zulueta, 1973). Malaria transmission could theoretically have occurred during the warmer periods of late Pleistocene and beginning of Holocene but again the small size of human populations during the Upper Palaeolithic and Mesolithic would have delayed the spread of the disease.

With the introduction of agriculture (around 7000 B.C.) more favourable conditions were created for transmission of malaria and one can assume that malaria may exist since the inception of the three riverine civilizations: Mesopotamia, Nile and Indus Valley (Zulueta, 1994). The first written references to a malaria-like disease appears in the works of Hippocratic Corpus, in Greece, in the IV and V centuries B.C., in ancient Indian texts (with no ascertained date) and in Chinese literature of the I millennium B.C. (Sallares *et al.*, 2004). Therefore, there is little doubt that benign tertian and quartan fevers (caused respectively by *P. vivax* and *P. malariae*) were endemic diseases in the Old World around 500 B.C.

The presence of *P. falciparum* in ancient Europe is more problematic to establish. According to Bruce-Chwatt & Zulueta (1980a), although environmental and human conditions during the Neolithic and Bronze Age were adequate for the maintenance of *P. falciparum*, a serious obstacle must have prevented the introduction of the parasite. The authors support their opinion on two assumptions: (i) the absence of both *An. labranchiae* and *An. sacharovi* from Europe until the extensive deforestation of Hellenistic and Roman times, and; (ii) the refractoriness of *An. atroparvus*, the almost exclusive vector of *P. falciparum* by that time. In contrast, Jones (1907 *fidé* Grmek, 1994) and Grmek (1994), based on paleopathological data, including manuscripts of Hippocrates times, supported the hypothesis that *falciparum* malaria was present in Greece since the Mesolithic. This hypothesis has been recently re-enforced by the work of Sallares *et al.* (2004). To these authors, according to

historical documents and archaeological findings, *falciparum* malaria was common in at least some localities in northern Greece, in the V century B.C.

In Italy, the spread of malaria took at least 1500 years, from 500 B.C. to *ca.* 1000 A.D. (Sallares *et al.*, 2004). Literary sources indicate that, around the V century B.C., the disease was already active in Sicily and in southern Italy, where Greek colonies were established since the VIII-VI centuries B.C. It spread to western central Italy during the I and II centuries B.C., but reached the northern areas only during the Medieval period, when *An. sacharovi* colonized the northeastern coast of Italy.

Malaria spread throughout Europe during Roman times by the continuous migration of soldiers, slaves, merchants and administrators (Sallares *et al.*, 2004). However, the process was also dependent on the dispersal of certain species of mosquitoes (Bruce-Chwatt & Zulueta, 1980a), which by their turn were also favoured by navigation and trade as well by man-made environmental changes (*e.g.* deforestation and agriculture activities).

With the fall of the Roman Empire a shift in cultural values has occurred. Although the Medieval times were not devoid of progress, there is an historical hiatus regarding malaria during this period caused by the scarcity of reliable information.

In the XVII and XVIII A.D. centuries, malaria was common in southern Europe as well as in some northern areas, including the Netherlands, the North Sea coast of Germany, Sweden and Finland (Bruce-Chawtt, 1988; Huldén *et al.*, 2005). Although without reliable descriptions of epidemics, the disease must also have been common in eastern Europe since the XV century, as several references to malaria symptoms are present in Polish and Russian folklores (Bruce-Chawtt, 1988).

It was at the beginning of the XVII century that one of the most important events of malaria history took place: the discover of therapeutic effects of the Peruvian bark, cinchona, against intermittent fevers. How this new remedy and the knowledge of its benefits reached Europe is a more or less obscure tale (Boyd, 1949a). The most probable hypotheses is that it was brought by Jesuit priests to Spain where it was used in treatment of Miguel de Barreda, a Professor of Theology at Alcala de Henares, in 1639 (Guerra, 1977 *fidé* Bruce-Chwatt, 1988). However, it was only in 1820 that the main alkaloids of the bark were finally isolated and the chemical process of obtaining quinine and its salts was described by Pelletier and Caventou, two French pharmacists (Bruce-Chwatt, 1988).

In the middle of the XVIII century the first signs of malaria receding appeared in northern Europe. Environmental changes due to the drainage of marshy areas, to novel practices in agriculture and stock breeding as well a better treatment of cases with quinine are the most common explanations for the natural disappearance of malaria from regions such as England (Bruce-Chwatt, 1988; Dobson, 1994). Improvements of social condition with benefits in nutritional status, hygiene, and infant care have provided the human population

with better weapons to fight the disease. The introduction of new crops to provide cattle with winter forage, the increased size and health of the herds, the stabling of cattle and pigs and improvements in house construction have all contributed to the dissociation of humans and mosquitoes (Dobson, 1994).

In the XX century, although declining in northern Europe without any specific control measures, malaria was still an endemic disease in large areas of the southern and eastern parts of the continent. In areas where changes in crop production and stock rearing were absent, malaria did not decline. In Russia, from Black sea to Siberia malaria remained a major public health problem for the entire first half of the XX century, in contrast with other Countries at similar latitudes (Reiter, 2001).

It was the impact of malaria during the First World War and the difficulties in assuring a continuous supply of quinine that stimulated German scientists in the pursuit of new drugs for malaria treatment. In 1924, Schulemann and his colleagues, produced the first synthetic anti-malarial compound, named Plasmochin (pamaquine) and before the Second World War several new, cheap, synthetic drugs were available for malaria therapy (Bruce-Chwatt, 1988). It was also due to the war efforts, but this time during the Second World War, that anti-malarial effects of two other compounds were discovered: cloroquine and amodiaquine. These derivates of 4-aminoquinolines that proved to be two of the most outstanding anti-malarial compounds remained as the best therapeutic and suppressive drugs for over 25 years (Bruce-Chwatt, 1988).

At the beginning of the Second World War another major discovery took place in Switzerland. In 1939, Muller and Wiesmann have testified the amazing insecticide proprieties of a synthetic compound, dichlorodiphenyl-trichloroethane, to which was given the abbreviated name of DDT (Bruce-Chwatt, 1988). This compound with its four extraordinarily characteristics (cheap, long-persistence, highly toxic to insects by contact but with low toxicity to humans) revolutionised malaria control. Before 1944, it was obvious that methods of malaria control by source reduction using larvicides such as kerosene and Paris Green dust, were only feasible in urban centres or limited areas. In contrast, this new insecticide could be applied to vaster regions. In 1944, the first trial with DDT spraying was conducted in the region of Rome, Italy (Bruce-Chwatt, 1988). For the first time a simple, economically sustainable mosquito control method (residual indoor spraying) could be used, especially in rural areas.

In the middle of the XX century several Countries carried-out successful national eradication programmes. In Albania, Italy, Greece and Portugal, these programmes received major stimulus and a generous financial support from the Rockefeller Foundation. In the course of these and other successful post-World War II campaigns it became obvious that the complete elimination of vectors was not a *sine qua non* condition for the cessation of malaria

transmission. By 1975, it was clear that Europe was free from indigenous malaria (WHO, 2006). For the first time in historical times, endemic malaria disappeared from Europe.

I.2.3. PREDICTING THE FUTURE: MALARIA AND CLIMATE CHANGE

The perception that malaria was eradicated from Europe has changed rapidly over the past two decades. In recent years, from residual foci that remained in East Europe the disease has re-emerged as a result of massive population movements or political/economic instability. Of the 52 member states of the WHO European Region, the number of Countries affected by malaria has increased from 3 to 10. At present, malaria continues to pose a challenge in Countries like Armenia, Azerbaijan, Georgia, Kyrgyzstan, Tajikistan, Turkey, Turkmenistan and Uzbekistan, most of them suffering from recent or current malaria epidemics (WHO, 2001a; 2006).

The number of imported cases is also rising especially in West-European Countries (France, Germany, UK, and Italy) due to the continuous increase in international travel and immigration (Legros & Danis, 1998). It has been the concern of many that this increased number of parasite carriers in malaria free-zones coupled with the impact of climate changes may contribute to the re-emergence of malaria in Countries were the disease has been eradicated decades ago (Jelinek *et al.*, 2002; WHO, 2002). In fact, several cases of autochthonous malaria have been reported from Countries as Italy (Baldari *et al.*, 1998), Germany (Kruger *et al.*, 2001), Spain (Cuadros *et al.*, 2002) and France (Doudier *et al.*, 2007).

The relationship between climate and malaria transmission is well documented specially regarding the effect of temperature on adult mosquito survival and parasite development inside the vector. However, the effect of current climate changes on the capacity of mosquitoes from temperate regions to transmit human Plasmodia is not fully understood.

Addressing the biological significance of the effect of climate on mosquito populations and on malaria transmission is a difficult task due to the complex interactions between all the partakers, *i.e.*, humans, mosquitoes, parasites and climate variables. Although all biological parameters involved in malaria transmission are directly or indirectly climate-sensitive, the same type of change may have opposite effects on two different variables. High temperatures tend to decrease the extrinsic incubation period and increase feeding frequency that should promote transmission. However, accelerated biting and egg laying may diminish females survival rate and thus limit transmission rate. Disease transmission may be affected in different ways depending not on the increase or decrease of a certain climate variable but rather on the amplitude of its variation. Rainfall can enhance transmission through the

creation of new breeding sites which will increase mosquito densities. However, heavy rains can have a flushing effect, cleansing those places of larvae. The same type of change may have antagonistic results depending on the location, *i.e.* on the vector-host populations it is affecting. Hot weather with low humidity usually tends to reduce mosquito survival. In areas where malaria vectors are endophilic and adapted to hot/dry conditions, mosquito females seek shelter inside dwellings during severe droughts. While waiting for adequate conditions they continue to blood feed without developing eggs but still maintaining or even increasing parasite transmission due to their high survival rate.

Due to the difficulties in predicting the true biological influence of rain or temperature in vector-borne diseases, the effect of global warming on malaria has been actively debated. However, it is generally accepted that climate will have a major role on the spread and incidence increase of these diseases as well on vector distribution and abundance (*e.g.* Martens *et al.*, 1999). The first places where this effect may be felt will likely be at the edges of the disease distribution, as in the highlands of East Africa where malaria is known to be limited by the low temperatures. The increase of malaria incidence in those regions since the end of the 1970's have been claimed to be already a present manifestation of climate change (Patz *et al.*, 2002; Zhou *et al.*, 2004; Zhou *et al.*, 2005; Patz & Olson, 2006; Pascual *et al.*, 2006). However, the role of climate as the main cause for disease epidemics has been subject of considerable discussion between malaria epidemiologists (Brower, 2001; Crabb, 2002; Alsop, 2007). Several other mechanisms have also been hypothesised as the most probable cause: (i) increased travel from malaria endemic areas to the highlands; (ii) degradation of the healthcare infrastructures; (iii) anti-malarial drug resistance, and; (iv) increased local transmission due to land-use changes (Hay *et al.*, 2002a; Hay *et al.*, 2002b; Hay *et al.*, 2005). Due, in part, to the varying quality of epidemiological data across sites in Africa, as well to difficulties in assessing long-term socio-demographic and biological data, the subject has been under a dispute during the last decade. Since the complexity of the disease and the interplay of climate, vector bionomics and human action defy any simplistic analysis, the true input of climate change will probably never be truly validated.

Another possibility of using climate-sensitive variables of malaria has been exploited by those involved in the elaboration of early warning mechanisms for detection of malaria epidemics. The malaria early warning systems (MEWS) were considered by W.H.O. (WHO, 2001b; 2004) as a core component of epidemic risk management. The objectives of MEWS are: (i) to early detect an epidemic through case surveillance; (ii) to provide early warning of its emergence based on monitoring meteorological conditions, and; (iii) to establish long-range predictions using seasonal climate forecasts (WHO, 2001b). Ultimately, MEWS aim to maximise the time during which decision makers can plan and implement prevention and control strategies to face effectively a malaria epidemics. MEWS are still in the phase of

development but its true benefits are questioned (Hay *et al.*, 2003) since it has been difficult to translate the results of scientific studies on climate-malaria interactions into robust models useful for operational use (Cox & Abeku, 2007). Furthermore, even if such a model is obtained the practical aspects of its implementation will probably represent other substantial difficulties related to health systems issues (Thomson & Connor, 2001; Cox & Abeku, 2007).

The same type of uncertainty is present when assessing the risk of the re-emergence of malaria in Western European Countries due to climate changes. Although world-wide predictive models have already been published (*e.g.* Rogers & Randolph, 2000) these do not focus on Europe at a regional level. For the European region some preliminary work has already been done but mainly regarding the malaria vectors distribution (Kuhn *et al.*, 2002).

According to studies of climate change impact on human health, Portugal is considered as a negligible to a low transmission risk Country regarding malaria re-emergence (Calheiros & Casimiro *et al.*, 2002; Casimiro *et al.*, 2006; Miranda & Moita, 2006). In these studies disease transmission risk was qualitatively classified according to parasite prevalence and vector abundance. The latter was assessed as the vector survival/activity periods estimated as the percentage of days *per* year having favourable temperature threshold limits. Four different scenarios were hypothesised depending on the climate model (current or changed according to regional climate models PROMES and HadRM2) and assuming the current status of vector and parasite prevalence or the introduction of a new parasite-infected vector population (Casimiro *et al.*, 2006; Miranda & Moita, 2006). Under current climate conditions transmission risk of *P. falciparum* and *P. vivax* were considered negligible and very low, respectively. Under the same climate scenario but with the possibility of introduction of a (new) parasite-infected vector population, transmission risk rises to low levels. No change should be observed in malaria transmission risk even under different climate scenarios if no change will happen in vector and parasite prevalence. However, in the same conditions, risk may increase to medium levels if a new population of mosquitoes infected with Plasmodia were introduced (Casimiro *et al.*, 2006; Miranda & Moita, 2006).

In these preliminary studies there is a strong evidence for a link between climate and malaria vectors in the same manner that it is clear the close interaction between climate and malaria prevalence in endemic areas. But the possible effects of the predicted climate change on mosquito abundance as well as on other determinants of vector capacity and competence for each of the former European malaria vectors needs to be further investigated.

I.3. MALARIA VECTORS IN EUROPE

The main vectors of malaria in Europe are member species of the *Anopheles maculipennis* complex. Other Anophelines like *Anopheles superpictus* Grassi, 1899 and *Anopheles claviger* Meigen, 1804 are referred to as secondary vectors in some parts of Europe and species like *Anopheles plumbeus* Stephens, 1828, *Anopheles algeriensis* Theobald, 1903, *Anopheles cinereus hispaniola* Theobald, 1903 and *Anopheles sergentii* (Theobald, 1907) have been associated to sporadic cases of malaria or considered as potential vectors due to their biological or behavioural characteristics (Shute, 1954 *fidé* Snow, 1999; Jetten and Takken, 1994; Marchant *et al.*, 1998).

I.3.1. *ANOPHELES MACULIPENNIS* COMPLEX

I.3.1.1. Historical perspective

The original description of *Anopheles maculipennis s.s.* Meigen, 1818 was published in Gothic German in the beginning of the XIX century. Since 1818 up to the end of the XIX century, *Anopheles maculipennis s.s.* was considered just another mosquito species. However, in 1898, R. Ross described the sporogonic cycle of malaria parasites, inside a mosquito (Gilles, 1993). Soon after, *Anopheles maculipennis* was implicated by the Italian researchers in the transmission of malaria in Europe (Grassi *et al.*, 1899 *fidé* Bruce-Chwatt, 1988) and a series of studies was carried out to better understand the biology of this species.

Several epidemiological studies presented an unexpected result. In certain regions, the distribution areas of the disease were more restricted and not coincident to that of the Anophelines. Furthermore, some places persisted as malaria-free zones in spite of the presence of infected humans, high mosquito densities and ecological and climatic conditions similar to other disease endemic areas. This puzzling subject that was after known as the paradigm of "anophelism without malaria" gave a major impulse to the study of *An. maculipennis*.

During the first thirty years of the XX century, several hypotheses were proposed to explain the malaria absence in areas with high densities of *Anopheles*. Although it was clear that not all of the *Anopheles* species were vectors of malaria, it was confirmed that in both malarious and non-malarious regions with high densities of Anophelines, the species present was *An. maculipennis* (Stepphens & Christophers, 1902, *fidé* Fantini, 1994). Sergent & Sergent (1903) testified for the first time the existence of morphological differences between specimens captured in malaria-free zones of France (Paris) and those of endemic areas of Algeria. On the other hand, for Roubaud (1918) the absence of malaria in Paris could be

explained by some kind of dissociation between mosquitoes and humans. In the following years Roubaud developed this idea, concluding that the dissociation factor relied in the differences observed in *An. maculipennis* host preferences. These differences were the outcome of a slow transformation in the biology of the mosquito (Roubaud, 1920; 1921; 1928). As result of this transformation two physiologic races emerged: one zoophagic (blood feeding mainly in animals), living in malaria-free areas and another anthropophagic (feeding in humans) distributed throughout paludic regions. The two races could be distinguished by the maxillary index (mean number of maxillary teeth). Populations feeding mainly in humans would present a maxillary index inferior to 14 while populations that, by pressure of socio-economic derived conditions, acquired marked zoophilic behaviour would have maxillas with 14-15 or more teeth (Roubaud, 1921; 1928).

Roubaud's theory was soon questioned by Sergent *et al.* (1922), Martini (1924 *fidé* Van Thiel, 1927) and Van Thiel, (1926, *fidé* Hacket *et al.*, 1932). They all concluded that the maxillary index is not a characteristic of "race" but an environmental dependent modification. Adult mosquitoes bred in warmer temperate areas were usually smaller in size when compared with those of cold breeding places, being the maxillary index dependent on the adult mosquito size and not on their feeding habits.

In 1926, Falleroni also divided *An. maculipennis* in two " varieties" (Falleroni, 1926 *fidé* Hackett *et al.*, 1932): one in which the eggs presented a grey surface, few uniformly distributed black spots and small floats (after denominated Falleroni´s grey eggs), and; another with darker eggs, extensive and irregular black areas and big floats (dark eggs "variety"). This was further divided in: (a) banded eggs for those presenting a light grey dorsal exocorion crossed by two dark bands, and: (b) black eggs, uniformly dark (Falleroni, 1926 *fidé* Hackett *et al.*, 1932) (Figure I.7.). The two "varieties" were respectively designated as *Anopheles claviger* (Meigen), 1804 "var." *labranchiae* (grey eggs) and *An. claviger* "var." *messeae* (dark eggs) (Falleroni, 1926 *fidé* Missiroli *et al.*, 1933). The name *basilei* was later suggested for the "variety" with banded eggs considered by that time as the "typical" form of *An. maculipennis* (Falleroni, 1932 *fidé* Bates, 1940).

Contemporary with Roubaud's and Falleroni's work, Van Thiel in 1927 describes a new "type" of *Anopheles maculipennis* based on: (i) the geographical distribution of the malaria in Holland as a disease of brackish areas, and; (ii) in the presupposition that the salinity of the larval breeding sites somehow turned adult mosquito more sensitive to parasite infection. This new "variety" was denoted as *Anopheles maculipennis* "var." *atroparvus* (Van Thiel, 1927) characterised, in comparison with "variety *typicus*", described by Meigen, as being smaller and darker, with a bigger maxillary index during winter, higher tendency to bite humans, starting its hibernation later in the winter and preferring brackish waters to breed.

Dutch authors continued Van Thiel's work, with intensive studies on the morphology and bionomics of local populations (De Buck & Swellengrebel, 1929; De Buck *et al.*, 1930; 1932). However, since both "Dutch races" were susceptible to *Plasmodium spp*. infection, no practical tool was yet available to distinguish *An. maculipennis* from malarious and non malarious regions.

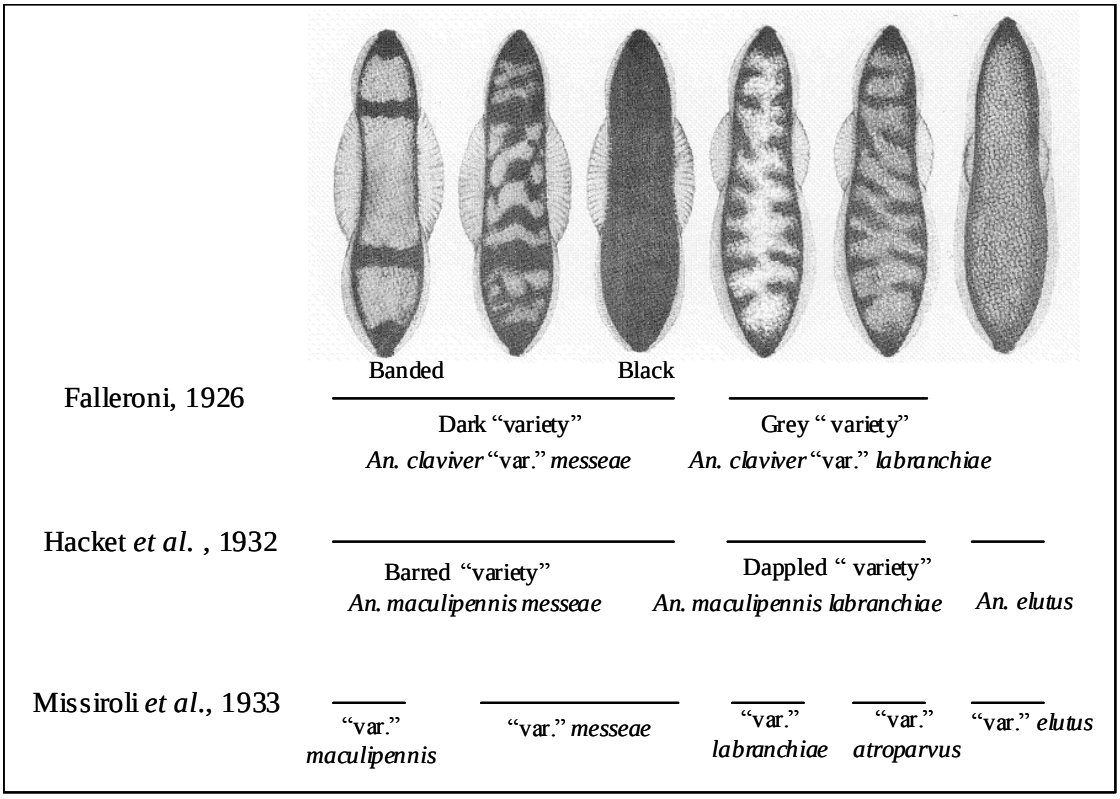


Figure I.7. Egg´s morphology and their designations according to the mentioned authors.

Hackett, *et al.* (1932), integrating the results of the above mentioned authors and acknowledging the importance of the egg exocorion patterns as an identification key-character, divided *An. maculipennis* into two different "races" according to four types of eggs: *An. maculipennis messeae* and *An. maculipennis labranchiae*. The first "race" (Falleroni's dark eggs "variety") would lay barred eggs, irregularly pigmented in bars and angular patches, but always presenting two transverse dark bars located distal to the end of the floats. The latter would be relatively large and long. *Anopheles maculipennis labranchiae* ("variety" grey eggs, according to Falleroni), would present uniformly dappled-grey eggs with relatively small floats and it would correspond to Van Thiel's *An. maculipennis atroparvus* because it occurred in brackish marshes and did not undergone into complete hibernation during winter. This "race" was considered having definite affinities with *An. elutus* which, according to the

authors, may represent a rather strongly differentiated race of *An. maculipennis* characterised by completed dark eggs with no or very reduce floats (Figure I.7.).

In 1933, Van Thiel and Missiroli *et al.* following two independent research lines but both using Hackett *et al.* (1932) eggs descriptions and their vast knowledge on *An. maculipennis*, reached the same fundamental conclusions. These were synthesized by Missiroli *et al.* (1933) when designating and characterizing the five "varieties" of *An. maculipennis* as presented in Figure I.7.

For Missiroli *et al.* (1933) the irregular distribution of malaria cases could therefore be explained by the presence of the different "races" of *An. maculipennis*, since not all presented the same ability to transmit the parasite. Although also firmly convinced of the existence of several *An. maculipennis* "races" or "varieties", for Roubaud & Grascen (1933) and Van Thiel (1934) egg features were not efficient morphological characteristics for the identification of *maculipennis* biological entities. But, while Roubaud & Grascen (1933) defended the use of maxillary index as the key-element to identification, Van Thiel stressed the importance of bionomic studies for the accurate comprehension of *An. maculipennis* composition.

It was only in 1935, with the report elaborated by Hackett & Misssiroli (1935) that the validity of exocorion patterns for the identification of the different "races" of *An. maculipennis* was finally established. The authors, acknowledging the existence of *An. maculipennis* "var." *melanoon*, the new member described by Hackett (1934), presented an exhaustive study on the six "varieties" of the species: *atroparvus*, *labranchiae*, *messeae*, *maculipennis*, *elutus* and *melanoon*. The conclusions of Hackett and Missiroli were further reinforced by the publication of a summarised list of "races", differential characters and behaviours and their relation with malaria transmission by an experts committee ("Commissione della Malaria della Societá delle Nazioni") gathered in a meeting in Rome (Christophers *et al.*, 1935). Still in the same year but following another approach, Shute (1935) presented an identification key for *An. maculipennis* "varieties" based on the characteristics of the male genitalia. In this key, the "variety" *melanoon* is not recognized being its validity equally questioned by Roubaud (1934; 1937).

Between 1934 and 1936, five new "varieties" or "biotypes" of *An. maculipennis* were described. Roubaud and collaborators recognized three new "biotypes": *fallax* (Roubaud, 1934) from Normandia, *sicaulti* (Roubaud, 1935) of Morocco and *cambournaci* from Portugal (Roubaud & Treillard, 1936). The latter was characterised as presenting eggs with *atroparvus*-like pattern but with floats of smaller dimensions (Roubaud & Treillard, 1936). Hackett & Lewis (1935) described the "variety" *subalpinus* occurring in Spain, northwest Italy, Albania and Macedonia. Missiroli (1935) identified the new "variety" *pergusae* from the Lake Pergusa, in Sicily.

Maintained apart from all the controversy generated around the *maculipennis* name were the species: (i) *Anopheles lewisi* and *An. selengensis* described by Ludlow in 1920, from collections carried out in Siberia; (ii) *Anopheles alexandraeschingarevi* proposed as a new species by Shingarev in 1928 based on the observation of Russian adult specimens; (iii) *Anopheles elutus* Edwards, 1921 soon recognized as a junior synonym of *An. sacharovi* (Edwards, 1926 *fidé* Bates, 1940); (iv) *Anopheles elutior* Martini, 1931 that being described as a turkmenian variety of *Anopheles elutus*, was also considered synonym of *An. sacharovi* (Bates, 1940), and; (iv) *Anopheles martinius* and *An. relictus* both described by Shingarev in 1926 and 1928, respectively, and also considered to be varieties of *An. sacharovi* (Zhelokhovtzev, 1937 and Edwards, 1932 *fidé* Bates, 1940).

At the end of almost four decades, with the work of Hackett (1934) and the acceptance of *An. maculipennis* as a species formed by several entities different in their ability to act as malaria vectors, the paradox "anophelism without malaria" reached its conclusion. However, the search of new identification characters as well the evaluation of the taxonomic value of those already described did not cease. In the following decade one can find studies on the form of wing venation and scales (Bali, 1936; Ungureanu & Shute, 1947), on larval chetotaxy (Bates, 1939a) and obviously on eggs' exocorion patterns (Shute & Ungureanu, 1938; Lupascu, 1941; Sautet & D'Ortoli, 1944). These morphological studies together with laboratory crossbreeding experiments carried out during the 30´s (De Buck *et al.*, 1934; Corradetti, 1934; 1937; Bates, 1939b), contributed in a decisive way to the evolution of *An. maculipennis* nomenclature and taxonomy.

One of the major revisions of the *An. maculipennis* taxonomy and nomenclature was carried out by Bates (1940) who proposed a new classification of the group Maculipennis. With regard to the Paleartic region this author considered the existence of five different species: (i) *An. maculipennis s.s.*; (ii) *An. messeae* Falleroni, 1926; (iii) *An. melanoon* with two subspecies, (a) *An. melanoon melanoon* Hackett, 1935 and (b) *An. melanoon subalpinus* Hackett & Lewis 1935; (iv) *An. labranchiae* with also two subspecies, (a) *An. labranchiae labranchiae* and (b) *An. labranchiae atroparvus*; and finally *Anopheles sacharovi* Favr, 1903.

However, as in the past, *An. maculipennis s.l.* seemed fated to be controversial. Bounomini & Mariani (1946, 1953), defending the existence of only three Paleartic species (*An. maculipennis*, *An. labranchiae* and *An. sacharovi*) proposed the creation of a new subgenus designated by *Maculipennia* (Bounomini & Mariani, 1953) which would gather all of the known Holartic species, subspecies and "varieties" of the *maculipennis* group. Bates *et al.* (1949) reinforced his opinion with an exhaustive description of the geographical distribution and ecology of his five species. Vargas (1950) although wrongly considering *Anopheles earlei* Vargas, 1943 and *Anopheles freeborni* Aitken, 1939 as Paleartic species, suggested the existence of seven other species in the Old World: *An. atroparvus*, *An.*

labranchiae, *An. maculipennis*, *An. melanoon*, *An. messeae*, *An. sacharovi* and *An. subalpinus*.

Until 1980, several other revisions on *An. maculipennis s.l.* taxonomy and nomenclature were undertaken (Senevet & Andarelli, 1956; Rioux *et al.*, 1959; Guy *et al.*, 1976; White, 1978) some based mainly on thorough morphological observations of each member but others reflecting the equally important contributes of new analytical techniques. With the emergence of the biological concept of species and the recognition of the sibling or cryptic species (Mayr, 1969) *An. maculipennis* systematics suffered another breakthrough. The existence of groups (called complexes) of morphologically indistinguishable species (or nearly identical) that, besides being reproductive communities, are also diverse ecological and genetic units contributed in a decisive way for the search of techniques that would analyse the genetic pool of each species (or a direct expression of it) instead of its morphological traits.

One of those methods was the cytogenetic analysis of larval salivary gland cells. In these cells, as well as in the nurse cells of half-gravid females, the so called giant or polytene chromosomes can be observed. These chromosomes, when stained and microscopically observed, present a specific pattern of dark and light bands according to the degree of chromatin condensation (Alberts *et al.*, 1994). Since the bands can be recognised by their thickness and spacing, chromosome patterns can be reproduced in maps.

Adapting the technique developed for *Drosophila* and *Chironomus*, Frizzi (1947a) described for the first time the cytogenetic pattern of a mosquito species, *An. atroparvus*. Comparing the banding pattern of *An. atroparvus* with that of other members of the *maculipennis* group, Frizzi (1947b, 1953) described the chromosomal characteristics of *Anopheles labranchiae*, *An. elutus*, *An. typicus*, *An. messeae* and *An. subalpinus*. In the subsequent years, the cytogenetic studies of field populations confirmed the applicability of this method as an identification instrument (Canalis *et al.*, 1954, 1956a;b; Rioux *et al.*, 1959; Kitzmiller, 1967) and contributed to the clarification of some less understood aspects of the geographical distribution of certain species (Frizzi, 1956; Rioux & Ruffié, 1957; Postiglione *et al.*, 1970; Novikov & Alekseev, 1989). However, not all of the species of the *maculipennis* group can be identified through their chromosomal banding patterns. The existence of two groups of species presenting the same patterns severely limits the application of this method as a standard identification instrument. Those homosequential groups are *atroparvus-labranchae-sicaulti* (White, 1981; Zulueta *et al.*, 1983) and *maculipennis-subalpinus-melanoon* (Rioux *et al.*, 1959) within each, species discrimination by cytogenetics analysis is impossible to achieve. Nevertheless, in 1978, a new species of the *maculipennis* group, *Anopheles beklemishevi*, was identified by Stegnii & Kabanova (1978) mainly through the observation of polytene chromosomes. However the validity of this name cannot be ascertained. Since the distribution area of this new member includes the type-localities of *An.*

lewisi Ludlow, 1920; *An. selengensis* Ludlow, 1920 and *An. alexandraeshingarevi* Shingarevi, 1928 and no cytogenetic study can be performed with museum conserved specimens, *Anopheles beklemishevi* may only be a junior synonym of one of these previously described species.

In the early 1980's, a second analytical technique, isozyme electrophoresis, contributed to significant changes in *An. maculipennis* taxonomy. In this method, enzymes (catalytic proteins) migrate in a support matrix under the influence of an electrical field (Murphy *et al.*, 1990). Each protein will migrate at its own speed, depending on its shape, size and charge (determined by its amino acid sequence but varying with the pH), the net charge and strength of the electrical field and the viscosity of the matrix. The enzyme position on the matrix is determined by histochemical visualisation.

In 1980, L. Bullini *et al.* and A.P.B. Bullini *et al.*, using the electrophorectic patterns of 27 enzyme loci, proposed the re-elevation of the nominal form *subalpinus* to the species status and considered *An. sicaulti* a "geographic variety" of *An. labranchiae*. These results were further supported by Cianchi *et al.*, (1981), who showed the existence of enzymatic evidences compatible with reproductive isolation between sympatric populations of *subalpinus* and *melanoon*. The *sicaulti* conspecific status with *labranchiae* was also confirmed by de Zulueta *et al.* (1983), using morphological, geographical, biochemical, chromosomal and genetic data. Based on the results of the multilocus genetic analysis undertaken during this decade, new identification keys using biochemical characteristics were elaborated for the southwestern European species of *An. maculipennis* complex (Bullini *et al.*,1980a;b; Cianchi *et al.*,1981).

Recently, new molecular methods based on the amplification of the nuclear ribosomal spacer ITS2, (Internal Transcribed Spacer 2) have been developed for *maculipennis* species identification. The first method, described by Proft *et al*., (1999), is a diagnostic Polymerase Chain Reaction (PCR) using species-specfic ITS2 primers that, according to each species, generates amplified products of different lengths. This method allows the identification of six sibling species: *An. atroparvus, labranchiae, maculipennis, messeae, melanoon, sacharov*i. Romi *et al*. (2000), using the interspecfic differences in the ITS2 sequences and the relative mobility of heteroduplexes formed between a known ITS2 sequence and the unknown DNA, distinguished all six species mentioned above plus *An. beklemishevi*. In 2002, Linton *et al.* (2002a) designed a new PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) assay also based on ITS2 differential sequences, which allows the rapid identification of *An. atroparvus, maculipennis, messeae, melanoon/subalpinus, sacharov*i and of the two new members of the complex, not yet formally denoted by that time. In 2005, a third new member was recognized on the basis of ITS2 sequencing (Gordeev *et al.*, 2005).

I.3.1.2.Taxonomy and nomenclature

The last major revision on taxonomy and nomenclature of *Anopheles maculipennis* complex was made by White, in 1978. Nine sibling species were recognized: *Anopheles atroparvus*; *Anopheles beklemishevi*; *Anopheles labranchiae*; *Anopheles maculipennis*; *Anopheles martinius*; *Anopheles melanoon* (with its alternative egg morphotype, *subalpinus*); *Anopheles messeae*; *Anopheles sacharovi* and *Anopheles sicaulti*.

Based on the evidences available, White proposed the resurrection of two species (*martinius* and *sicaulti*) and the suppression as possible synonyms of *beklemishevi* and *messeae*, of three *nomina dubia*, *alexandraeshingarevi*, *lewisi* and *selengesis*. This last suggestion was ignored and according to Supplements of the Catalog of mosquitoes of the World (Ward, 1984; Gaffican & Ward, 1985; Ward, 1992) *selengensis* is actually considered synonymical to *An. lewisi*, which maintains its species status, and *alexandraeshingarev*i a junior synonym of *An. maculipennis*. Hence, according to the World Catalog of Mosquitoes and its supplements (Knight & Stone, 1977; Knight, 1978; Ward, 1984; Gaffican & Ward, 1985; Ward, 1992) the *Anopheles maculipennis* complex is composed by 11 Paleartic species (Table I.1.).

Table I.1. *Anopheles maculipennis* complex.
Paleartic species nomenclature according to the Catalog of mosquitoes of the World (Knight & Stone, 1977) and supplements (Knight 1978; Ward, 1984; Gaffigan & Ward, 1985; Ward, 1992).

Species name	Synonyms
Anopheles atroparvus Van Thiel, 1927	*falax* Roubaud, 1935 *cambournaci* Roubaud & Treillard, 1936
Anopheles beklemishevi Stegnii & Kabanova, 1976	
Anopheles labranchiae Falleroni, 1926	*pergusae* Missiroli, 1935
Anopheles lewisi Ludlow, 1920	*selengensis* Ludlow, 1920
Anopheles maculipennis Meigen, 1818	*typicus* Hackett & Missiroli, 1935 *basilei* Falleroni, 1932 *alexandraeshingarevi* Shingarevi, 1928
Anopheles martinius Shingarev, 1926	*elutior* Martini, 1931 *relictus* Shingarev, 1928
Anopheles melanoon Hackett, 1934 *Anopheles messeae* Falleroni, 1926	
Anopheles sacharovi Favre, 1903	*elutus* Edwards, 1921
Anopheles sicaulti Roubaud, 1935 *Anopheles subalpinus* Hackett & Lewis, 1935	

Integrated molecular and morphological approaches have recently contributed to a reappraisal of *An. maculipennis* complex systematics. Based on egg morphology and DNA

sequence analysis obtained by PCR amplification of ITS2, a full characterisation of *An. subalpinus* and *An. melanoon* was once more undertaken with results suggesting their conspecificity (Linton *et al*., 2002b).

In addition, three new members of the complex were also detected by ITS2 sequence analysis. *Anopheles persiensis*, the first new species of Culicid to be characterized mainly on the basis of DNA evidence, was described from specimens collected in the northern coastal provinces of the Caspian Sea, Gilan and Mazandaran, in Iran (Sedaghat *et al.*, 2003). It is genetically related to *Anopheles martinius*, with which it shares 93.2% of ITS2 sequence identity but differs from it by presenting eggs with floaters. In Romania, from the coastal region and plains adjacent to the Danube river, another new species, genetically and morphologically very similar and to *An. messeae*, was described by Nicolescu *et al.* (2004). This species, denominated *Anopheles daciae*, is sympatric with *An. messeae* and can be distinguished by five fixed variable sites in ITS2 sequence and by some characteristics of the egg deck tubercles. It also presents smaller egg size. The third species, *Anopheles artemievi*, in terms of morphology is twin with *An. sacharovi* and *An. martinius* (Gordeev *et al*., 2005), but genetically is more similar to *An. maculipennis* presenting 91 % of homology with it. The type-locality is Alga, in the Batkensk region of Kyrgyzstan.

In conclusion, and considering that the findings of the last five years are valid contributes, the *Anopheles maculipennis* complex is currently constituted by 13 Paleartic members.

I.3.1.3. Geographic distribution of Western-European species

Like most of the biological diversity of the Paleartic region, the distribution of European mosquitoes must have been highly affected by the Wűrm glaciation. The effects of the climatic changes on the distribution and prevalence of plants and higher animals is well known but regarding mosquitoes, due to the lack of fossils, these can only be inferred. Since at that time southern Europe had flora and fauna similar to today's northern Europe, it can be assumed that the same is true for the *An. maculipennis* complex (Bruce-Chwatt & Zulueta, 1980a). Thus, species like *An. maculipennis*, *An. messeae* and *An. atroparvus* could have been found in the south of the continent (Bruce-Chwatt & Zulueta, 1980a) but according to de Zulueta (1973) climate conditions were inadequate to the survival of *An. labranchiae* and *An. sacharovi* that would have been confined to North Africa and West Asia, respectively.

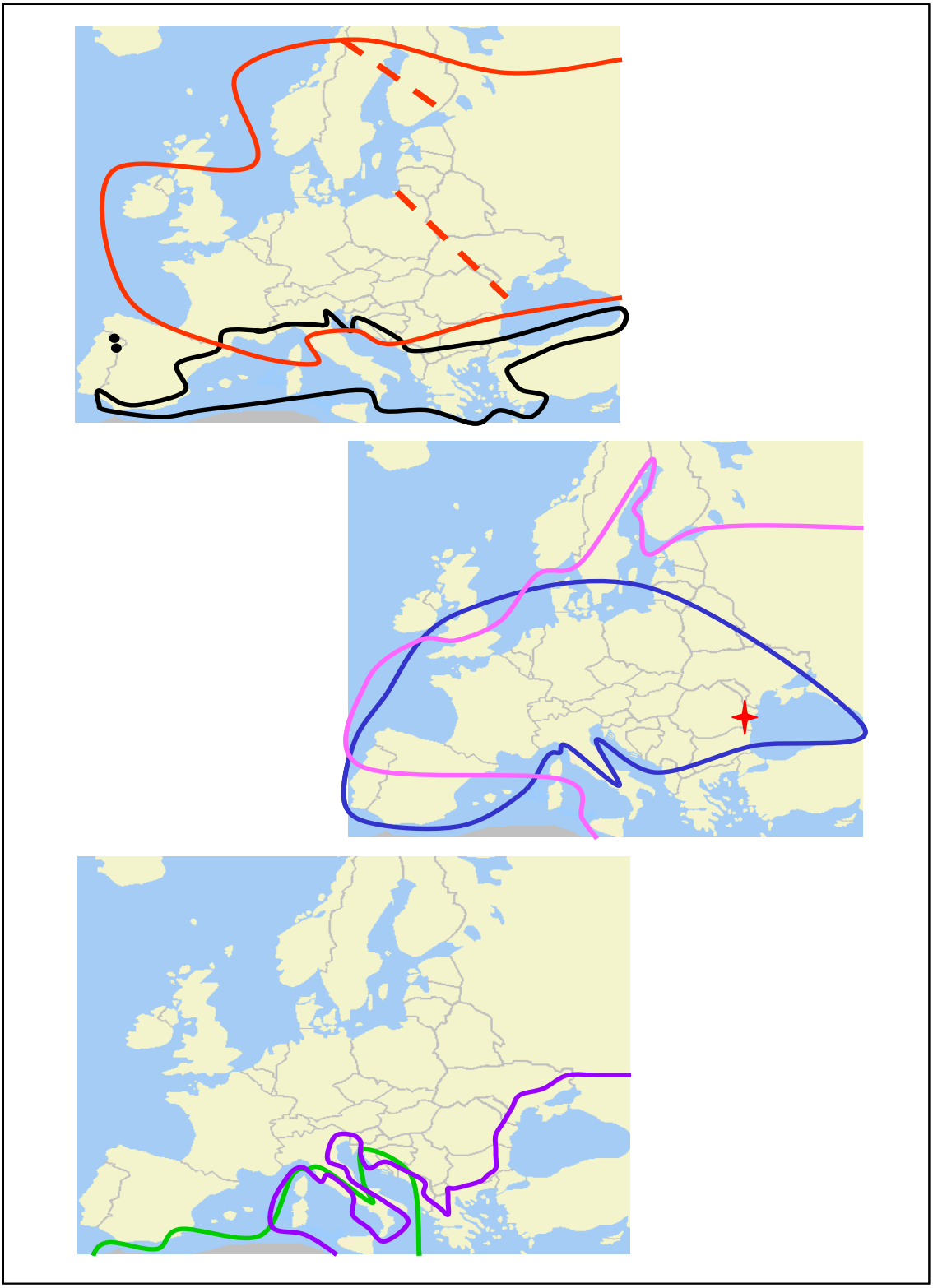


Figure I.8. Geographic distribution of the Western-European members of the *Anopheles maculipennis* complex. Based on the works of: Ribeiro *et al.*, 1980a; Ramos *et al.*, 1982; Jetten & Takken, 1994; Nicolescu *et al.*, 2004. Black line and dots: *An. melanoon* distribution. Solid red line: distribution of *An. messeae* and *An. beklemishevi*. Interrupted red line: possible boundary between *An. messeae* (located southwestern) and *An. beklemishevi* (located northeastern) distributions. Blue line: *An. atroparvus* distribution. Pink line: *An. maculipennis* distribution. Red star: *An. daciae*. Green line: northern boundary of *An. labranchiae* distribution. Violet line: *An. sacharovi* northern boundary.

At the end of the Pleistocene and during early Holocene, species tended to move north. As to *An. labranchiae* and *An. sacharovi*, the introduction of these species into the Aegean area, Italy and Spain probably occurred by nautical dispersal (Bruce-Chwatt & Zulueta, 1980a) during the Hellenistic and Roman times. The increase of navigation and also deforestation and coastal alluviation resulting from extensive agriculture activities must have favoured their spread and establishment into new territories. Although present in a small area between Alicante and Murcia (Collado, 1938) *An. labranchiae*, failed to disperse throughout the southeastern coast of Spain, mainly due to the presence of *An. atroparvus* with which it had to compete for similar ecological niches. This species disappeared from Spain after the malaria control campaigns of the middle of the XX century (Encinas Grandes, 1982).

The present distribution of the Western-European members of *An. mac*ulipennis complex is presented in Figure I.8.

I.4. MALARIA IN PORTUGAL

I.4.1. FIRST RECORDS UNTIL THE 1940´S

There are no records referring to the existence of malaria in the Iberian Peninsula during the classical antiquity (from VIII-VI centuries B.C. to IV-VI centuries A.D.). However, at least the benign tertian and quartan malaria must have existed already, probably spread through the population movements that took place during that period (Bruce-Chwatt & Zulueta, 1980a). As to *falciparum* malaria, although likely to exist during the Caliphate of Cordoba (Bruce-Chwatt & Zulueta, 1980a), it was probably a rare disease in the south of Iberia during the Arab domination (Cambournac, 1942; Bruce-Chwatt & Zulueta, 1977).

Rice should have been introduced in the Iberian Peninsula during the Arab civilization and by the XII century its cultivation was well known in the south of Spain (Sevilla) (Bruce-Chwatt & Zulueta, 1980a). However, no record of an event that could be interpreted like a malaria epidemic was recorded during the conquest of Alentejo and the Algarve (central and south Portugal) to the Moors during the XII and XIII centuries (Cambournac, 1942).

It is only with the beginning of the overseas expeditions that Portugal seems to have been swept by a wave of epidemics (plague, influenza, typhus) that left its marks on the demographics of the Country from the XV to the XVII century. Along with the deforestation for ship building, the *exodus* of people to the overseas territories, their return as carriers of several pathogens and the importation of African slaves may have contributed for the rise of malaria in Portugal (Cambournac, 1942). With new policies to promote rice cultivation implemented during the XVIII century the area of land dedicated to this culture started to increase (Dias, 2001). In fact, the introduction of rice paddies in the area of Comporta is dated

as taking place in the beginning of the XVIII century (Atlantic Company, 1999 *fidé* Faustino, 2006). However, the significant expansion of rice fields only started in the XIX century when again new laws overtaxing the imported rice favoured the increase of its cultivation (Atlantic Company, 1999 *fidé* Faustino, 2006).

In the second half of the XIX century, a debate regarding the impact of rice fields in public health was opened within the medical and scientific community (Cascão, 1993 *fidé* Faustino, 2006), and an Inquiry Commission was formed to evaluate the relationship between rice cultivation and diseases. In the conclusions of the report presented by this Commission in 1860 (Couto, 1860 *fidé* Faustino, 2006), a public health problem of unknown origin was recognized to be associated with the presence of rice fields. But the true relation between rice and malaria was not perceived by the Commission since the role of Anophelines in disease transmission had not yet been established. By the end of the XIX century, infectious diseases, among which malaria was included, were considered one of the main causes of death (*ca.* 44%) in Portugal (Cascão, 1993 and Graça, 1999 *fidé* Faustino, 2006).

In the early years of the XX century the first mosquito checklist of Portugal was published (Sarmento & França, 1901). In 1903, in an extensive work regarding malaria distribution and epidemiology and its relation with rice culture, Ricardo Jorge laid the foundations for the studies later carried out during the control program of 1940´s (Faustino, 2006).

The major step in the investigation of malaria in Portugal took place in 1931 with the implementation of a malaria centre in Benavente, located in the Tagus river valley at the centre of Portugal. The work of Figueira & Landeiro (1931 *fidé* Faustino, 2006) and Landeiro & Cambournac (1933), already developed under the auspices of the Rockefeller Foundation (United States of America), increased the knowledge about malaria prevalence. Those efforts have also permitted the identification and characterization of the four main malaria areas of Portugal: the hydrographic basins of the rivers Douro, Mondego, Tagus and Sado. However, it was Fancisco Cambournac who, over a period of 40 years, most contributed to the knowledge about malaria in Portugal. His monograph on the epidemiology of malaria in Portugal (Cambournac, 1942) was of fundamental importance to malariology in southern Europe (Bruce-Chwatt & Zulueta, 1980a).

I.4.2. MALARIA CONTROL/ERADICATION PROGRAM OF PORTUGAL

Malaria control in Portugal started in 1931 with the public opening of the "Estação Experimental de Combate ao Sezonismo" in Benavente. The first task of those responsible for the station was to evaluate the prevalence and spleen rates of the local population. The

infected patients were then identified and went on with a 28-days treatment with quinine. Drug distribution to each patient was strongly monitorized and patients that interrupted treatment were presented with a house call and persuaded to re-start the therapy. Another task of Benavente centre was the identification of Anopheline breeding places and mosquito abatement through the application of Paris Green. Window and door screens were applied to both the Benavente centre and local hospital, "Hospital da Misericórdia" (Bruce-Chwatt & Zulueta, 1980b).

In 1934, a second centre was implemented in Águas de Moura, in Sado river estuary. This centre, which started with similar objectives to the Benavente station, soon became an educational and training facility for future malariologists. With the visit, in 1937, of a Rockefeller Foundation delegation, by proposal of its delegates, "Águas de Moura" centre was transformed in a research and education institution, giving rise to the "Instituto de Malariologia (IM)" of Portugal (Bruce-Chwatt & Zulueta, 1980b).

The official opening of the IM took place in 1938, the same year of the creation by national authorities of a central organization totally devoted to malaria control, the "Serviços Anti-sezónicos" (Filipe, 2001). In the first year, the IM was directed by Rolla Hill but in 1939, F. Cambournac was nominated Director, remaining in charge of the Institute until 1954 (Borges, 2001).

Francisco Cambournac developed an extensive work on malaria epidemiology in Portugal. Based on his monograph of 1942, one can have a very clear picture of what was the burden of this disease for the Portuguese population. He identified six malarious regions, all associated with the major rivers of Portugal: the Douro, the Mondego, the upper zone of Tagus, the lower zone of Tagus, the Sado and the Guadiana regions (Figure I.9.).

Francisco Cambournac classified the endemicity of malaria in each region according to spleen rates of 6-12 years-old children as: light (2-10% of children with splenomegaly); moderate (10-25%); severe (25-50%) and hyperendemic (more than 50%). Douro, Upper Tagus and Guadiana regions were classified as light to moderate endemic areas while Mondego and Lower Tagus presented levels of light to severe malaria endemicity. The Sado region was the only with hyperendemic malaria, showing parasite prevalence and spleen rates similar to those observed in tropical areas (Cambournac, 1942). This region presented the highest mortality rate mainly because it was also the only one where *P. falciparum* prevalence was higher than other malaria parasites. For the period 1936-40, *P. falciparum* prevalence varied between 34% and 55%, *P. vivax* was responsible for 32-36% of the cases and *P. malariae* for only 2 to 12%. Refering only to Alcácer do Sal, number of clinical cases *per* year varied between 2,469 and 6,763 patients.

Anopheles maculipennis var. *atroparvus* (synonym: *An. atroparvus*) was identified as the only malaria vector in that region. Its favoured breeding places were the extensive rice

paddies present in Sado region that could shelter as many as 20.000 larvae *per* hectare (Cambournac, 1994). *Anopheles atroparvus* morphology, seasonality, behavioural (feeding, resting and copula) traits as well as breeding habits and survival patterns were thoroughly analysed (Cambournac, 1942). There was also a deep concern to characterise malaria according to certain environmental conditions. An extensive description of the vegetation and climatic variables in each region was undertaken in order to identify which local characteristics would contribute more to malaria transmission.

Figure I.9. The malarious regions of Portugal (Cambournac, 1942). In pink: the Douro region. In yellow: the Mondego. In violet: the upper zone of Tagus. In blue: the lower zone of Tagus. In red: the Sado. In green: Guadiana region. Light-pink and light-green: areas where spleen rates are inferior to 10% and that do not belong to a specific endemic region.

It was based on all this fundamental work that several control measures were implemented. In the beginning, malaria control was based on the efforts of four anti-malaria stations (Montemor-o-Velho, Benavente, Idanha-a-nova and Alcácer do Sal) and four medical centres under the supervision of "Serviços Anti-sezónicos" (Bruce-Chwatt & Zulueta, 1980b). In 1945, this organization was re-organized, expanded and made independent from central government. The new "Serviços de Higiene Rural e Defesa Anti-Sezónica", with headquarters in Lisbon, was composed by 10 delegations and 52 dispensaries spread all over the country. Throughout this time the IM remain the centre for malaria research and teaching/training giving all the technical and scientific support for the implementation of control measures (Bruce-Chwatt & Zulueta, 1980b).

In 1939, disease notification became mandatory (Bruce-Chwatt & Zulueta, 1980b). Besides the identification and treatment of malaria patients, first with quinine and after with

cloroquine and pirimetamine (Bruce-Chwatt & Zulueta, 1980b), several anti-vector activities were undertaken in a remarkable example of integrated control. Larval abatement included the introduction of predatory *Gambusia* fish and intermittent irrigation of rice-fields (Borges, 2001). The use of protective measures such as window/door screens and bed nets were boosted and, in 1946, DDT spraying was implemented, but only in some selected areas (Bruce-Chwatt & Zulueta, 1980b). Because malaria was intimately associated with rice cultivation, a new tax over rice production was applied to fund anti-malaria actions. Landowners were also forced to provide their workers with better sanitation conditions. Rice cultivation demanded a seasonal influx of workers during spring and summer. People coming all over the country were sheltered in wood and thatch huts standing next to the rice fields, with no sanitation facilities. By the new law, houses with dormitories, kitchens and bathrooms had to be constructed. These should be located at a certain distance from the water collections and provided with fixed window screens and double net doors (Faustino, 2006).

By 1956, the number of endemic cases was already much reduced (*ca*. 150) and the remaining disease foci were small and located only in Sado and Mira margins (Bruce-Chwatt & Zulueta, 1980b). From a control strategy national authorities moved to eradication policies. In 1958, since only four autochthonous cases were reported it was considered that malaria eradication entered in its last phase. DDT spraying was interrupted and epidemiological surveillance was restricted to passive case detection of infected patients (Bruce-Chwatt & Zulueta, 1980b). However, it was a common notion that a surveillance program had to be implemented. It was only in 1964 that this programme was elaborated and implemented with the financial support of the Calouste Gulbenkian Foundation (Portugal). The project was based on active case detection and ended two years later, when it was confirmed that most of the 146 cases of malaria reported in 1965 were identified by passive case detection (Bruce-Chwatt & Zulueta, 1980b).

In November 1973, the World Heath Organization's Expert Committee on Malaria declared that Portugal entered in the official record of areas where malaria had been eradicated (W.H.O., 1974 *fidé* Bruce-Chwatt & Zulueta, 1977).

I.4.3. AFTER THE ERADICATION

Since 1973, only one autochthonous case of malaria was detected in Aljustrel (near Beja, centre of Portugal), in 1975 (Bruce-Chwatt & Zulueta, 1980a, Antunes *et al.*, 1987). Despite the social and political instability of Portugal in 1975-76 and the great influx of both civilians and servicemen repatriated during those years from the former Portuguese territories of Africa, malaria transmission did not re-emerge (Bruce-Chwatt & Zulueta, 1977).

From 1959 up to 1972 the number of imported malaria cases detected raised from 10 to *ca.* 250. In 1974, 903 confirmed cases were associated with the return of thousands of people from endemic areas of Africa. However, in 1977 the number of cases was less than a quarter and from 1977 to 1985, around 50 or fewer cases were reported annually (Antunes *et al.*, 1987). For the decade 1993-2002, the annual number of cases varied between 50 and 85 (Castro *et al.*, 2004). These cases, although in superior number of those recorded in 1977-85, showed no trend of increase (Figure I.10.).

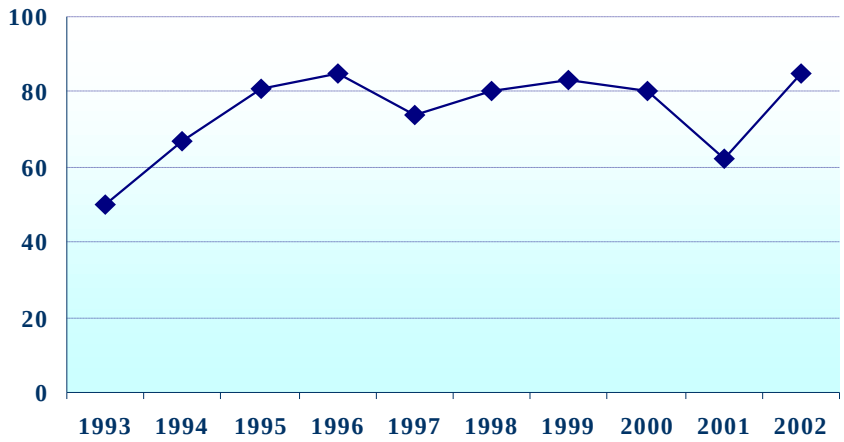


Figure I.10. Number of reported cases of malaria, in Portugal, from 1993-2002.
Adapted from Castro *et al.*, 2004.

I.5. MALARIA VECTORS IN PORTUGAL

There are 38 mosquito species recorded for continental Portugal (Ribeiro & Ramos, 1999); of these, nine are Anophelines and five have been associated with malaria transmission: *An. atroparvus*, *An. claviger*, *An. cinereus hispaniola*, *An. maculipennis* and *An. plumbeus*, (Ribeiro *et al.*, 1988; Jetten & Takken, 1994).

In Portugal, during the malaria endemic times, *An. atroparvus* was the only mosquito species identified as the vector (Cambournac, 1942). This species presents a country-wide distribution and together with *An. maculipennis* and *An. melanoon*, it is one of the three species of *An. maculipennis* complex recorded in the Country (Ribeiro *et al.*, 1988). The distribution of the three *An. maculipennis* sibling species in Portugal is reported in Figure I.8.

Anopheles atroparvus was the subject of detailed studies during the control/eradication period and in the 1970-80´s, and the knowledge of this species was largely enriched by the work of H. Ribeiro and collaborators. Nowadays, *An. atroparvus* is one of the

most common and abundant mosquito species in Portugal (Almeida *et al.*, 2008, in press). It presents an endophagic and endophilic behaviour and prefers to feed on animals rather than on humans (Landeiro & Cambournac, 1933; Cambournac & Hill, 1938; Cambournac, 1942; Ramos *et al.*, 1992). The males tend to form swarms, but copulation can be performed in narrow spaces (Cambournac & Hill, 1940). Although described for the first time as a brackish water breeder, in Portugal it can be both found in fresh water habitats, such as in rock pools or ditches, as well as in marshlands or saltpans (Ribeiro *et al.*, 1977; Ramos *et al.*, 1977/78; Ribeiro *et al.*, 1977/78, Pires *et al.*, 1982; Ribeiro *et al.*, 1985; Ribeiro *et al.*, 1999a). In areas of high densities, it presents an abundance peak during summer but it can survive overwinter as adult (Cambournac, 1942). Females do not undergo complete hibernation and will continue to feed during the cold months (Cambournac, 1942).

Anopheles maculipennis can be found, sympatrically with *An. atroparvus*, in the central-northern part of the Country, north of the mountain system of Montejunto-Serra da Estrela. In Portugal as it was recorded for other Countries, this species can be found in breeding sites located at high altitudes (Postiglione *et al.*, 1973; Ribeiro *et al.*, 1999b), probably because it tolerates moving water and wider ranges of daily temperatures than other species (Jetten & Takken, 1994).

The presence of *An. melanoon* was detected twice (Ribeiro *et al.*, 1980a), although once it was identified as *An. subalpinus* (Ramos *et al.*, 1982). Since then the species was never recorded again. An attempt to introduce *An. melannon* in Portugal through a mass release of larval specimens in the south of Portugal has failed completely. The unsuccessful establishment of this species in Portugal as well as the inability of *An. labranchiae* to spread throughout the south of Spain are considered to be the result of *An. atroparvus* competitive advantage over these two species (Bruce-Chwatt & Zulueta, 1980a).

As regards *An. plumbeus*, although also present throughout the Country, it has a scanty and sparse distribution (Ramos, 1983/84). *Anopheles claviger* was only found in Serra da Arrábida, nearby Setúbal (Ribeiro *et al.*, 1977/78; 1996), and in central and northern parts of Portugal (Ribeiro *et al.*, 1992; 1999a,b; 2002). *Anopheles cinereus hispaniola* was recorded only once from a locality in Douro basin (Ribeiro *et al.*, 1980a). Given the rarity of the three species, these must have had little or no importance as vectors when malaria was endemic in Portugal.

Chapter II

OBJECTIVES

II.1. MALARIA AND CLIMATE CHANGES

Malaria is a preventable and curable disease but it remains one of the most important health problems in the world, mostly in tropical Countries, causing more than one million deaths and up to 500 millions clinical cases per year[3].

In recent years, like other vector borne diseases, malaria has (re)-emerged and spread in several Countries of the European Region with unpredictable health and socio-economical consequences. Most of these localized epidemics are linked to human-induced changes, often resulting from mass population movements and political and economical instability. In Western-European Countries the risk of malaria re-emergence under current environmental and social conditions is considered minimal. Although the number of imported cases has been rising in the last decade, the socio-economic standards of those Countries and effective healthcare infrastructures has allowed for the prompt treatment of parasite carriers as well as promoting human practices that reduce vector-host contact. Thus, only few autochthonous cases are currently reported from malaria-free places where former malaria vectors are still present in densities compatible with disease transmission. However, the recent outbreaks observed in Eastern-European Countries are a constant reminder that this situation may reverse (WHO, 2001a; 2006). Furthermore, if the predicted global climate change reported by the Intergovernmental Panel of Climate Change (IPCC, 2001[4]) will cause large increases in mosquito vectorial capacity, malaria re-emergence in Europe could become possible (Alten *et al.*, 2007; Takken *et al.*, 2007).

One factor that might have restrained the spread of imported parasites is the recognized refractoriness of *An. atroparvus* to tropical strains of *P. falciparum*. This lack of adaptation between the most widespread former malaria vector in Europe and current *Plasmodium falciparum* strains may also be altered if environmental conditions change. It is known that the genetic basis of mosquito resistance to parasite infection has an important epidemiological role in disease transmission. Recent studies have highlighted the importance of non-genetic factors in the expression of mosquito resistance to malaria parasites (Lambrechts *et al.* 2006). These studies concluded that environmental variation can significantly reduce the importance of genes in determining the resistance of mosquitoes to parasites and thus greatly modifying the outcome of infection.

[3] www.who.int/malaria/
[4] www.ipcc.ch/

II.2. COMPORTA AS A STUDY REGION

The region of Comporta presents a unique setting to assess the vector capacity and competence of *An. atroparvus* from Portugal. It was a former malaria hyperendemic region where, opposite of what was recorded for the rest of the Country, *P. falciparum* was the most prevalent malaria parasite. It is a semi-rural area with vast numbers of mosquito breeding sites, as rice-fields represent most of the land use. Tourism is the second largest economical activity and thus a seasonal influx of tourists occurs every summer, the best climate season for malaria transmission. The recent malaria history of the region is well documented and reasonable good climate data are available since 1981.

II.3. OBJECTIVES

According to several climate models (Miranda & Moita 2006), the percentage of days *per* year with temperature values favourable to both *Anopheles* survival and Plasmodia development will increase. The predicted increase of the period of days adequate for *Anopheles* survival may be considered relatively modest, varying between 10% and 16% (Miranda & Moita, 2006). However, the period during which *Plasmodium* species can develop may experiment a significant extension depending on the climate scenario used. The percentage of days favourable to *P. vivax* may increase 23% to 67%, while for *P. falciparum* it may rise 20% to 55%.

To assess how global change driven factors may be linked to the risk of introduction and/or spread of malaria in Europe it is necessary to characterise the current status of its former vectors. By studying the bionomics and vectorial competence of present-day populations, it is possible to identify and study factors that might trigger disease emergence as well as to provide entomological data to be used in the identification of environmental induced changes of epidemiological significance. Desirably, predictive models of disease emergence and dissemination may be elaborated, identifying hot-spots, key factors of risk and useful indicators for monitoring. The use of such tools in risk assessment, early warning, surveillance and monitoring, will be of major importance in supporting public health decision and policy making. Aiming to contribute to this ultimate goal, this study had the following **objectives**:

1. To optimise tools for *Anopheles atroparvus* identification and sampling.

Specifically:

a) To develop a molecular identification key for *An. maculipennis* sibling species of Portugal.

b) To select and optimise mosquito collection methods for *An. atroparvus* bioecological studies.

2. To estimate *Anopheles atroparvus* vectorial capacity towards malaria and analyse other bioecological parameters with relevance to the (re)introduction of the disease.

Specifically:

a) To determine the species composition of *An. maculipennis* complex in Comporta region.
b) To determine *An. atroparvus* abundance, population structure and seasonality patterns.
c) To determine possible relations between *An. atroparvus* abundance and some meteorological parameters as:
    - Relative humidity at 9 UTC;
    - Daily precipitation;
    - Mean daily temperature.
d) To estimate entomological parameters of medical importance such as:
    - Parity and insemination rates;
    - Blood meal preferences and human blood index;
    - Females biting activity and man biting rates;
    - Duration of gonotrophic cycle and feeding frequency.

3. To determine *Anopheles atroparvus* vector competence for tropical strains of *Plasmodium falciparum.*

Specifically:

a) To establish of a colony of *An. atroparvus* with low levels of inbreeding.
b) To carry-out artificial infection of *An. atroparvus* specimens with different strains of *P. falciparum* under different temperature conditions and mosquito feeding regimens.

Results and specific methodologies applied to the development of the three main objectives are presented in the Chapters IV to VI.

Chapter III

MATERIAL AND METHODS

III.1. STUDY AREA

Entomological surveys were carried out in a former malaria region of Alentejo Province, located in the left margin of Sado River, south of the city of Setúbal and Tróia peninsula and north of Alcácer do Sal (Appendix). For the selection of the study area, several criteria were taken into account:

i. *Its malaria history*: According to Cambournac (1942) the hydrographic basin of Sado River was the only malaria hyperendemic area of Portugal. In Alcácer do Sal, between 1936-1940, the spleen rates, measured in children of 6-12 years-old, reached 54.30 %. The Sado area was also the only Portuguese malaria region where *Plasmodium falciparum* was the predominant species, with a prevalence as high as 70%. *Plasmodium vivax* was responsible for *ca*. 25% of the malaria cases and *P. malariae* prevalence usually did not exceeded 10% of the infections (Cambournac, 1942).

ii. *The existence of large number of potential mosquito breeding sites*: The region, located between Sado River and the Atlantic Ocean is a coastal zone in the vicinity of an estuary, with several types of habitats well known for their high mosquito breeding capacities (*e.g.* marshlands). It is a rural area (INE, 2003[5]) where rice cultivation is the predominant agricultural activity. Paddy fields, a common breeding place for Anophelines (Laird, 1988), represent the majority of land use.

iii. *The presence of a highly mobile human population*: The region and its surroundings are a touristy zone with two major resorts with capacity to accommodate up to 6,000 people (Torralta S.A. and Soltroia S.A. enterprises, personal communication). Furthermore, in the last five-years a residential project for holiday houses and leisure facilities was developed in some of the study localities leading to an additional influx of visitors.

iv. *Its geographical location*: The study area is located *ca*. 60 km south of Lisbon. Access by car through highways and national roads allows frequent visits and an adequate way of transport of the collected mosquitoes to the laboratory.

The study area, referred to as Comporta region, comprises a coastal land strip *ca.* 15-20 km long and 5-10 km wide. Entomological surveys were conducted in six localities: (i) Carrasqueira, Possanco and Comporta that belong to Comporta Parish/ Alcácer do Sal County, and; (ii) Torre, Carvalhal and Pego of Carvalhal Parish/Grândola County (Appendix). The residential areas are situated along a national road, which crosses the study region from north to south, along the coast.

[5] Instituto Nacional de Estatística: http://www.ine.pt

Comporta region is a flatland area with altitudes varying between sea-level and less than 60 m. The region presents a variegate landscape. In the west, in a parallel position with the road and the residential areas, there are extensive areas of rice fields and a system of sand-dunes. The north and northwest part of the study region is a national protected landscape area (RNES[6]). This region is occupied by marshlands, *ca.* 600 ha. of rice fields and some abandoned saltpans. The vegetation of the marshlands is mainly composed by *Spartina maritima* (Curtis) Fernald and several species of the CHENOPODIACEA family as *Arthrocnemum macrostachyum* (Moric.) C. Koch, *Sarcocornia perennis* (Miller) A. J. Scott, *S. fruticosa* (L.) A. J. Scott, *Salicornia ramosissima* J. Woods, *S. nitens* PW Ball & Tutin and *Halimione portucaloides* (L.) Aellen (Ramos *et al.*, 2001, unpublished). Surrounding the saltpans the presence of four other species was recorded: *Inula crithmoides* L., *Polygonum equisetiforme* Sibth. & Sm., *Sueda vera* J. F. Gmelin and *Spergularia marina* (L.) Griseb. (Ramos *et al.*, 2001, unpublished). The south and east areas are mainly occupied by pine forest, *Pinus pinaster* Aiton and *Pinus pinea* L., and some semi-natural agro-forestry systems of cork-oak, *Quercus suber* L.

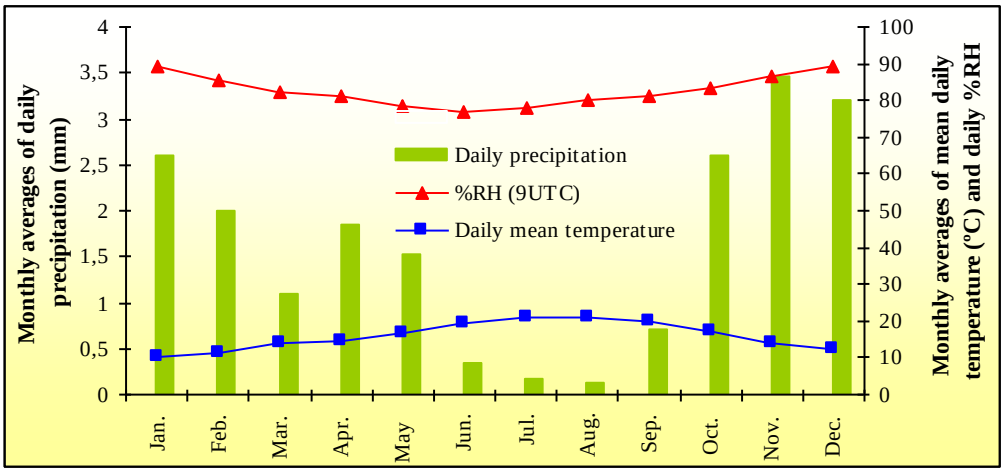


Figure III.1. Climatological series (1981-2000) for Comporta locality.

The climate, according to Köppen Classification System[7] is a moist subtropical mid-latitude climate (type C), subtype Mediterranean with a dry summer and a mild winter (Figure III.1). The monthly averages of mean daily temperatures, for the period 1981-2000, in Comporta locality, varied from 10°C to 21°C and monthly-mean relative humidity at 9 UTC (Coordinated Universal Time) between 76% and 89%. Monthly averages of daily precipitation fluctuated between 0.12 and 3.4 mm of rain.

[6] RNES:Reserva Natural do Estuário do Sado
[7] www.physicalgeography.net/fundamentals/7v.html; koeppen-geiger.vu-wien.ac.at/

According to the 2001 population census (INE, 2001[8]) the Parishes of Comporta and Carvalhal have a total of 2,948 inhabitants, 1,781 (60.4%) males and 1,167 (39.6%) females. Human activities are concentrated in the primary and tertiary sectors of economy.

III.2. MOSQUITO SAMPLING

Mosquito collections were carried out from July 2000 to May 2004, at least once a month, using a variety of sampling methods for adult and immature forms. For each capture a brief enquire was filled containing the following information: location and type of habitat where the collections where made; date; type of collection method used; time of collection; type of hosts present. In larval collections a more detailed form was used with information regarding water parameters (temperature, pH, colour and turbidity) presence of vegetation and sun exposure. All captured specimens were transported in cool boxes to the laboratory for further studies.

Details on the mosquito sampling methods will be presented in the following chapters.

Immature forms sampling with dipper and pipette: Mosquito immature forms were collected using a dipper with a capacity of 400 ml and a 1.5 m handle (Russel & Baisas, 1935 *fidé* Service, 1976). A fine (less than 2 mm wide) mesh screen in one side of the device allowed the drainage of the excess of water without losing the larvae. The number of dips varied according to the size of the breeding site. Specimens captured were transferred with the aid of a pipette to plastic boxes (10x15 cm) containing an appropriate amount of water.

Human baited landing (HB) collections: Due to the presence of high densities of human-biting mosquitoes, HB collections were carried by groups of three persons. One of the team members acted as bait, exposing his/her lower part of the legs, and two collectors, using a torch and a 6-V battery aspirator, captured host-seeking mosquitoes that landed on the bait exposed skin. Every 15 min the bait-person was substituted by one of the collectors. The mosquitoes collected in each hour-period were kept separately in small net cages (8x8 cm). Human baited landing collections were performed outdoors in the vicinity of animal dwellings (HBext), for periods of 3, 12 and 24 h.

CDC miniature light-traps baited with carbon dioxide (CDC-CO_2): A paper recipient containing *ca.* 1 kg of dry ice was attached to the top of each CDC miniature light-traps (Sudia & Chamberlain, 1988). Power was supplied by 6-V rechargeable batteries. Traps were always hung outdoors, around 1 m above the ground, next to animal dwellings, occasionally under sheds.

[8] Instituto Nacional de Estatística: http://www.ine.pt

Resting collections: Outdoor resting (OR) collections were performed using a 12-V battery back-pack aspirator. Indoor resting (IR) captures were performed with 6-V battery aspirators or paper-cup aspirators (Coluzzi & Petrarca, 1973) with the help of a torch. Mosquitoes captured in each collection were maintained in separated tubes or cups. Information regarding number of collectors and time spent in captures was added to the enquiry form.

The *collection effort* spent on each capture was also calculated according to the method used. For CDC-CO_2 traps, collection effort was calculated as the number of traps multiplied by the number of nights during which collections took place. For HB and resting captures, collection effort was determined by the number of collection hours multiplied by the number of collectors/bait-people (see also III.8.1).

III.3. MOSQUITO IDENTIFICATION

III.3.1. MOSQUITO MORPHOLOGICAL IDENTIFICATION

Adult specimens were killed by cold and identified over a chilled table by stereomicroscopical observation of morphological characters. When detailed observation was necessary, specimens were mounted with double pin. Occasionally, dissection and microscopical observation of male genitalia was required for identification. Male abdomens were cut with entomological scissors between the 6^{th} and 7^{th} segments, and the detached portion boiled for *ca.* 5 min in a 67% chloral hydrate solution in a 1:1 mixture of water and formic acid. Genitalia pieces were separated over a slide in a drop of solidifiable formic acid-polymerized vinyl alcohol (FA-PVA) solution (Ribeiro, 1967), mounted in their final position and dried overnight, in an oven, at 37°C. Preparations were made permanent after covered with another drop of mounting medium and a cover slide and dried for a period of 1-2 days at 37°C.

Immature forms of each collection were reared in groups in an insectary with controlled environmental conditions (temperature 26°C ± 1°C; 75-80% relative humidity and 12 h/12 h light/dark photoperiod) until L4 stage or adult emergence. Dead specimens, L4 larvae and moults were collected daily, and preserved in 5 ml plastic tubes, each corresponding to one collection, in a solution of 75% alcohol and 2% glycerine. Specimens were identified under the stereomicroscope and whenever necessary mounted between slide and cover slide with FA-PVA solution for microscopic observation. The same procedure as field collected specimens was followed with laboratory-emerged adults.

Mosquitoes were identified to species or to species complex levels according to Ribeiro & Ramos (1999) identification keys.

III.3.2. *ANOPHELES MACULIPENNIS S.L.* MOLECULAR IDENTIFICATION AND POLYMORPHISM ANALYSIS

Two types of molecular techniques were applied to identify *An. maculipennis* members: (i) nucleotide analysis of Internal Transcribed Spacer 2 (ITS2) of the ribosomal DNA (rDNA) by sequencing of either PCR amplified fragments or cloned products, and; (ii) a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique based on the amplification of the ITS2 region. A third method, Primer Introduced Restriction Analysis-Polymerase Chain Reaction (PIRA-PCR) was used to detect single nucleotide polymorphisms.

III.3.2.1. Sequence analysis of ITS2 region

Sample preparation and storage: Adult specimens chill-killed were dried over filter paper (Whatman n.1) at room temperature and individually preserved in 0.5 ml tubes. Previously, each tube was partially filled with a small amount of silica gel desiccant. A cotton plug was stacked and compressed over the silica gel, in order to prevent contact between the mosquitoes and the desiccant. Tubes with mosquitoes were labelled and kept at room temperature, in plastic bags. Immature forms were preserved in the same glycerinated alcohol solution as previously mentioned (III.3.1.).

DNA extraction: A phenol-chloroform technique described in Ballinger-Crabtree *et al*. (1992) and modified by Donnelly *et al.* (1999) was used to extract genomic DNA from *Anopheles maculipennis s.l.* specimens. Individual whole mosquitoes or parts of a mosquito were transferred to a 1.5 ml tube and homogenised with a pestle grinder in 270 µl of lyses buffer (100 mM Tris-HCl, pH=8.0; 50 mM NaCl; 50 mM EDTA, pH=8.0; 0.15 mM spermine; 0.5 mM spermidine) plus 30 µl of SDS 10% and 5 µl of a 20 mg/ml solution of Proteinase K. Suspensions were incubated overnight at 50ºC. In the following day, 305 µl of buffered phenol-chloroform was added, and tubes were placed in a horizontal agitator for 15 min. After a 15 min centrifugation at 13,000 *g* the aqueous (upper) phase was removed to a clean 1.5 ml tube and 300 µl of chloroform:isoamyl alcohol was added to each tube. The homogenates were gently mixed in a horizontal agitator for 10 min and centrifuged for 10 min at 13,000 *g*. The aqueous phase was transferred to a new 1.5 ml tube and 60 µl of ammonium acetate (10 M) plus 600 µl of absolute ethanol were added to each tube for DNA precipitation. Tubes were gently shaken in the horizontal agitator for 15 min, placed in a freezer at -20°C for 60 min and then centrifuged during 15 min at 13,000 *g*. The supernatant was eliminated and 300 µl of ethanol 70% was added to each pellet. After a 10 min centrifugation at 13,000 *g*, the supernatant was again removed and DNA pellets were dried in an oven overnight at

37°C or in a speed vacuum at 40ºC for 10 min. Dried DNA pellets were eluted in 100 µl of water plus 100 µl of TE buffer (Tris-HCL 10 mM, pH=8; EDTA 1 mM, pH=8.0) and stored at 4ºC or -20ºC, for longer periods of time.

DNA amplification: Polymerase chain reaction amplification of ITS2 region was achieved using the primers of Linton *et al.* (2002c):

mac-ITS2-F: 5'-ATC ACT CGG CTC GTG GAT CG-3';

mac-ITS2-R: 5´-ATG CTT AAA TTT AGG GGG TAG TC- 3'.

Each PCR reaction, with a total volume of 50 µl, contained 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.5 U of *Taq* DNA polymerase. Three brands of enzyme were use: Bio*Taq* DNA polymerase (Bioline®), Go *Taq* flexy DNA polymerase (Promega®) and a proofreading enzyme Pfu DNA polymerase (Promega®). Two microlitres of DNA eluates were added to each amplification reaction. Four positive controls (one for each species) and one negative control (a DNA extraction blank) was included in each set of PCR reactions. Amplifications were performed in a thermal cycler according to the following programme: one cycle at 94°C for 2 min, 34 cycles, each with: DNA denaturation at 94ºC for 30 sec, primer annealing at 53°C for 30 sec and extension at 72ºC for 30 sec. The programme was ended with a final step of extension at 72ºC for 10 min after which reactions were stopped by lowering temperature until 4ºC. Amplified products were preserved at 4°C or at -20°C for longer periods of storage, until further processing.

PCR products visualisation: To monitor the results of the PCR reactions 2 µl of 6X Orange G loading buffer was added to 10 µl of each amplified product and the mixture loaded into a 1.5% agarose gel with ethidium bromide (0.002%) incorporated. Gels were submitted to electrophoresis at 85 V for at least 1 h and bands visualized and photograph in an Eagle Eye® II Still Video System.

DNA direct sequencing: Amplified products were cleaned using a commercially available purification kit (QIAquick® PCR purification kit, Qiagen, Venlo, The Netherlands). No changes were introduced in the manufacture protocols. PCR fragments were sequenced in both directions (forward and reverse) at Stab Vida (Oeiras, Portugal).

DNA cloning and sequencing: PCR products were obtained with a proofreading DNA polymerase following procedures mentioned above. Cloning and transformation was undertaken using the cloning kit TOPO TA Cloning, pCR®2.1-TOPO® (Invitrogen life technologies, product reference K4500-40). LB broth (Sigma®) and LB agar (Sigma®) culture media were prepared with ampicillin (50 µg/ml) according to products protocols. Purification of plasmid DNA was carried out using QIA prep®Miniprep kit and a centrifuge as specified in the product manual.

Small changes were introduced to the cloning kit instructions. For each cloning reaction to transform chemically competent TOPO10 cells, 1 µl of amplified product was

added to 0.5 µl of TOPO®vector, 0.5 µl of salt solution and 0.5 µl of sterile water. Regarding the One Shot® Chemical transformation protocol, the mixture of 2 µl of cloning reaction with the competent cells was incubated on ice during 30 min and only 125 µl of S.O.C. medium, instead of the mentioned 250 µl, was added to the tubes on ice. One hundred microlitres of each transformation was spread in selective LB agar plates previously prepared with 40 µl of a 40 mg/ ml X-Gal solution (Bioline®).

Analysis of the transformants by PCR was performed after plasmid purification using M13 Forward and Reverse primers. One microlitre of plasmid DNA was added to 14 µl of amplification mixture containing 1X PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each primer and 1 U of Go *Taq* flexy DNA polymerase (Promega®). Amplification programme started with one cycle at 94°C for 3 min followed by 35 cycles, each with: DNA denaturation at 94°C for 1 min, primer annealing at 50°C for 30 sec and extension at 72°C for 90 sec. Reactions were stopped at 4°C and PCR products were preserved at 4°C or at -20°C. Amplified products were visualised as previously described.

Sequence analysis: Sequences were edited and aligned using BioEdit version 7.0.0 (Tom Hall Copyright© 1997-2007, Ibis Biosciences[9]). Similarity with sequences available in Genbank was determined using Genbank[10] database search engines.

III.3.2.2. *Species identification by PCR-RFLP*

Anopheles maculipennis s.l. species molecular identification was initially attempted using a primer-specific PCR (Proft *et al.*, 1999) but results were inconsistent and DNA amplification of control mosquitoes was not always achieved. Following a different approach, a new PCR-RFLP protocol was developed in collaboration with Y. Linton (Natural History Museum of London), for the identification of the three *Anopheles maculipennis* sibling species recorded for Portugal (*An. atroparvus*, *An. maculipennis* and *An. melanoon*). This PCR-RFLP technique is based on the amplification of Internal Transcribed Spacer 2 (ITS2) of the ribosomal DNA (rDNA), which presents single nucleotide polymorphisms that vary between species and therefore allows the production of length-specific restriction fragments through the application of selected enzymes. The initial RFLP analysis was further optimised and modified in order to identify a fourth member of the *maculipennis* complex, *An. labranchiae*.

DNA extraction: Due to the large number of specimens to be processed, a simpler technique than the phenol-chloroform method was adopted for the extraction of genomic DNA. Protocols were derived from those described by Collins *et al.* (1987). Individual

[9]http://www.mbio.ncsu.edu/BioEdit/bioedit.html
[10]http://www.ncbi.nlm.nih.gov/sites/entrez

specimens or parts of mosquitoes were placed into a 1.5 ml tube and homogenised with a pestle grinder on 100 µl of lyses buffer (100 mM Tris-HCl, pH=8.0; 80 mM NaCl; 60 mM EDTA, pH=8.0; 160 mM sucrose; SDS 0.5%). Samples were kept at 65°C during 30 min after which 14 µl of potassium acetate (8 M) were added to each tube. Homogenates remained on ice for 30 min. After a 10 min centrifugation at 12,000 *g*, the aqueous (upper) phase was removed to a clean 1.5 ml tube and 200 µl of absolute ethanol were added to each tube. Tubes were kept at –20°C for 60 min. After a centrifugation during 15 min at 12,000 *g*, the supernatant of each sample was eliminated and 200 µl of ethanol 70% were added to each pellet. After another 10 min centrifugation at 12,000 *g*, the supernatant was again discarded and DNA pellets were dried in a speed vacuum at 40°C for 10 min. The DNA pellets were eluted and stored as described above (III.3.2.1.).

DNA amplification and PCR products visualization: A PuReTaq Ready-To-Go™ PCR Beads® (GE HealthCare, Life Sciences) kit was used for the amplification of the whole length of ITS2 using primers mac-ITS2-F and mac-ITS2-R. Twenty-four microlitres of a mixture containing both primers at 0.5 µM and 1 µl of DNA eluates were applied to single wells of the PCR plate. Four positive controls and a DNA extraction blank control were included in each set of PCR reactions. PCR amplification and electrophoresis were performed as already described in section III.3.2.1.

Restriction reactions: Two microlitres of each ITS2 PCR product was added to a 0.5 ml tube containing 1X of restriction enzyme buffer (buffer L, Roche Diagnostics) along with 1.25 U of *Cfo 1* and 1.25 U of *HPA II* (Roche Diagnostics) enzymes, in a total volume of 20 µl. Mixtures were incubated during 3 h, at 37°C, in a thermal cycler. Reactions were stopped at 4°C and products kept at 4°C or at -20°C for longer periods of storage.

Enzyme restriction products visualization: Four microlitres of 6X Orange G loading buffer were added to each restriction tube and mixed with the digested product. Twenty microlitres of the mixture were loaded into a 2% agarose gel with ethidium bromide incorporated (0.002%). After electrophoresis at 85 V for at least 1 h, gels were visualized and photographed in an Eagle Eye® II Still Video System.

III.3.2.3. PIRA-PCR

This technique was used to detect a single nucleotide polymorphism (C/T) at the position 397 of the ITS2 alignment (Chapter IV, Figure IV.2). This method creates an artificial restriction site in PCR fragments, by using a primer close to the mutation of interest designed with a single-base mismatch near to its 3' end. A www-based computer tool was used to screen for appropriate mismatches, to design the primers and to select a suitable restriction enzyme (Ke

et al., 2001). The primers chosen (P397CT-F: 5'- CAA ACG GCG TAC CTC ACC GTA C – 3'; P397CT-R: 5´-GGT CTT GTA TCT CTG CTG CTA TGG CT-3') introduce a mismatch at position 397 which provides the artificial restriction site, 5'-C^TAG-3', recognized by the enzyme *FspBI.*

DNA amplification: The PCR reactions contained 1X PCR buffer, 2.25 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 µM of each primer and 0.5 U of Go *Taq* flexy DNA polymerase (Promega®). One microlitre of DNA eluates were added to each amplification reaction completing a total volume of 20 µl. One negative control (a DNA extraction blank) was included in each set of PCR reactions. Two cloned PCR products, each carrying one of the alternative bases for position 397 (*i.e.* C or T), were used as positive controls. Amplifications were carried with the following cycling programme: one cycle at 94°C for 3 min, 34 cycles, each with: DNA denaturation at 94°C for 45 sec, primer annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The program ended with a final step of extension at 72°C for 10 min. Reactions were stopped by lowering temperature until 4°C and PCR products were preserved at 4°C or at -20°C, until further processing.

PCR products visualization: Four microlitres of amplified product were mixed with 1 µl of 6X Orange G loading buffer and loaded into a 2% agarose gel with ethidium bromide incorporated (0.002%). Electrophoresis and fragments visualization were carried out as described before (III.3.2.2).

Restriction reactions: In a 0.5 ml tube, 5 µl of each PCR product were added to 1X restriction enzyme buffer (buffer Tango^{TM}) and 0.06 U of *FspBI* enzyme (Fermentas, Life Sciences®), in a total volume of 15 µl. Enzymatic digestions were carried out in a thermal cycler during 14 h, at 37°C. Reactions were stopped at 4°C and digested products kept at 4°C or at -20°C.

Enzyme restriction products visualization: Three microlitres of 6X Orange G loading buffer were added to each restriction and 15 µl of the mixture were loaded into a 2% agarose gel with ethidium bromide incorporated (0.002%). Electrophoresis procedures and observation of fragments were performed as already described (III.3.2.2.).

III.4. MOSQUITO BLOOD MEALS IDENTIFICATION

III.4.1. SAMPLE PREPARATION AND STORAGE

All blood meals were obtained from females captured in IR collections. Freshly-fed *An. maculipennis s.l.* females were dissected with sterile needles and their midguts removed and squashed onto Whatman n.1 filter paper. Each paper was identified with a code letter and serial number and dried at room temperature.

Papers with squashed blood meals were packed between clean filter papers and stored in a plastic container with silica gel, at room temperature. The remaining parts of each dissected female were kept in individual tubes for molecular analysis and labelled with the same designation given to the respective blood meal.

Blood samples of potential vertebrate hosts that served as controls were also collected onto Whatman n.1 paper and followed the same procedure as for blood meals.

III.4.2. ELISA FOR BLOOD MEAL IDENTIFICATION

A two-site ELISA derived from Simões *et al.* (1995) protocols was used to identify the blood source of the female meals. Blood meals were tested for the presence of chicken, cow, dog, goat/sheep, horse/donkey, human, pig, rabbit and rat/mouse immunoglobulin G (IgG).

A 2 mm square of each filter paper was cut with the help of scissors and forceps and eluted in 4 ml of PBS-T (0.01 M phosphate buffer, pH=7.4; Tween 20 0.05%), overnight at 4°C. To avoid contamination from different blood meals, scissors and forceps were washed with a hypochlorite solution, rinsed with water and dried with a clean paper after cutting each filter paper.

Flat bottom 96-wells microtiter plates, one for each antibody, were coated with 100 µl of 4µg/ml anti-IgG antibodies (Sigma-Aldrich®) diluted in carbonate-bicarbonate buffer (pH=9.6). Plates were incubated overnight at 4ºC.

In the next morning, anti-IgG antibodies (MoAbs) solutions were removed and plates washed three times with 100 µl of PBS-T. One hundred microliters of a 10% solution of dried skimmed milk in PBS buffer (0.01 M phosphate buffer, pH=7.4) were added to each well as a blocking solution. This was done to all plates with the exception of the ones coated with MoAbs against cow and goat/sheep IgGs. For these plates a 1% solution of human serum in PBS buffer was used as blocking solution.

All plates remained at least 30 min, at room temperature. Blocking buffer was then removed and after another 3 times washing procedure, 100 µl of each filter paper eluate were added to single wells. Four positive controls (homologous blood) and 12 negative controls (heterologous blood) were applied to every plate. After 2 h at 37°C, eluates were eliminated and plates were washed again three times with PBS-T.

A second set of antibodies against the respective coating IgGs but conjugated with a peroxidase enzyme (MoAbs*), were diluted in PBS-T according to the manufacturer instructions (Sigma-Aldrich®). Exceptions were made in case of MoAbs* against cow, horse and goat/sheep IgGs. These antibodies were previously incubated, overnight at 4°C, with 50 volumes of adsorbent serum. Sheep serum was used as adsorbent for MoAbs* anti-IgGs of cow and cow serum for MoAbs* anti-IgGs of horse and goat/sheep. After incubation,

antibodies plus serum were centrifuged at 12,000 *g* during 30 min, at 4°C. The supernatant was then diluted in PBS-T in order to obtain the MoAbs* concentration recommended by the manufacturer. One hundred microlitres of MoAbs* were then applied to each well and plates were incubated 1 h, at room temperature.

Following incubation, MoAbs* were discarded, plates were washed three times with PBS-T buffer (100 µl *per* well *per* wash) and 100 µl of a 0.1% solution of hydroxide peroxide in 5AS (5-aminosalicylic acid**,** Sigma-Aldrich®), were applied to each well. Plates with this enzyme subtract were incubated at room temperature during 20 min after which the enzymatic reaction was stooped with the addition 50 µl of NaOH (4N), *per* well.

Absorvance values were read at 492 nm wave length in an ELISA reader (Anthos 2010 ®, Anthos Labtec Instruments). Cut-off values were calculated for each plate, as the mean plus three times the standard deviation of the negative controls.

III.5. MOSQUITO FECUNDITY STATUS AND PARITY ANALYSIS

III.5.1. SAMPLE PREPARATION AND STORAGE

Indoor resting collected *Anopheles maculipennis s.l.* females were classified according to their gonotrophic condition following Sella's classification (Chapter I, Figure I.4.) as described by Detinova (1963). Females in stage 2 and 3, grouped by gonotrophic stage and collection, were kept frozen in labelled and sealed plastic Petri dishes until ovary dissection.

III.5.2. DISSECTION PROCEDURE, SPERMATHECA AND OVARIES OBSERVATION

Female's dissection started with the removal of abdomens using sterilized needles in order to prevent DNA cross-contamination between specimens. Head and thorax of each individual were put into 0.5 ml tubes for subsequent molecular analysis. Detached abdomens were placed over a slide with a drop of distilled water and dissected under the stereomicroscope. Spermatheca were detached, covered with a drop of water and cover slide, and observed under a microscope (400X magnitude) for the presence of sperm. Ovaries were transferred to a clean slide, placed in a drop of distilled water and dried at room temperature. The same code letter and serial number was attributed to the slides with ovaries and corresponding tubes with the insect head and thorax. Once dried, ovaries were observed under a microscope at 400X and the parity status of the female was determined according to the folding status of ovarian tracheoles (Figure III.2.).

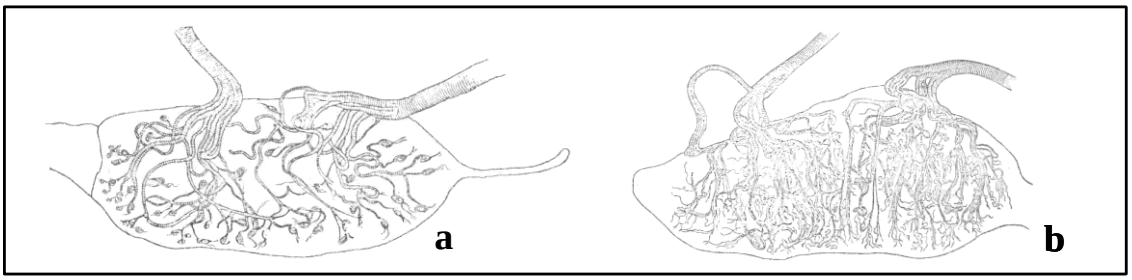


Figure III.2. Ovarian tracheoles coiling status of a nulliparous (a) and parous female (b).
Adapted from Detinova, 1963.

III.6. ANOPHELINES ARTIFICIAL INFECTIONS WITH *PLASMODIUM FALCIPARUM*

Mosquitoes infected with human malaria parasites are considered agents of moderate potential hazard to humans and the environment. All activities carried out with this type of biological material have to be conducted in level 2 biosafety laboratories with special engineering and design features for insect maintenance and containment. Due to the inexistence of such facilities at IHMT, studies for the assessment of *Anopheles atroparvus* vectorial competence to the malaria parasite, *Plasmodium falciparum,* were carried out at the Nijmegen Medical Centre (NMC), Radboud University, The Netherlands. Laboratory procedures regarding artificial infection of mosquitoes were performed by G. van Germert under the co-ordination of A. Luty. Detailed protocols may be found in the literature referenced below.

Two isolates were used to establish *An. atroparvus* vector competence towards *P. falciparum*: the Amsterdam airport strain NF 54 and an Indonesian strain NF 161. Infective gametocytes were produced in a semi-automated cultivation apparatus (Ponnudurai *et al.*, 1982a; 1982b; 1986). Fourteen days after the start of the cultures, parasitized cell suspensions were harvested from the apparatus, washed by centrifugation and mixed with washed uninfected cells suspended in human serum (Feldmann & Ponnudurai, 1989). This mixture was introduced in the mosquito feeders (Ponnudurai *et al.*, 1989) and maintained at 37°C by running heated water. Stretched parafilm was used as feeding membrane. Female mosquitoes were allowed to blood feed for 20 min.

Unfed mosquitoes were eliminated and engorged ones supplied with a 10% fructose solution. When following standard procedures, mosquitoes took only one infective meal and blood-fed females were held at 26°C, in a containment facility, for a period of seven days. Individual mosquitoes were then dissected in a drop of 1% mercurochrome solution and their midguts examined, at the microscope, for oocysts presence (400X magnitude). *Anopheles*

stephensi specimens from the NMC colony referred to as NXK Nij. were used as controls of infection performance. To assess culture infectivity 24 h after feeding, 10 mosquitoes of each cage/feeder were dissected and their disrupted midguts incubated with anti-25 kDa sexual stage-specific monoclonal antibodies conjugated with flurescein isothiocynatein in Evans Blue solution (Ponnudurai *et al.*, 1989). After centrifugation the pellet was resuspended in PBS and ookinetes were counted in a Bűrker-Tűrk chamber using an ultraviolet microscope (Ponnudurai *et al.*, 1989).

As to mosquito infection parameters, prevalence, unless otherwise stated, refers to the percentage of dissected mosquitoes that presented oocysts in their midguts, while intensity is the mean number of oocysts *per* female, again considering the total number of mosquitoes dissected. The terms ookinete or sporozoite prevalence were used to describe the percentage of mosquitoes dissected that presented each of the mentioned parasite developmental stage.

III.7. METEOROLOGICAL DATA

All climate data was downloaded from the "Instituto da Água, Portugal" website and it is part of the "Sistema Nacional de Informação de Recursos Hidrícos (SNIRH), Portugal" databases[11]. Data refers to: (i) daily records of mean temperature, calculated as the quotient between maximum and minimum daily values; (ii) total precipitation, and; (iii) percentage of relative humidity (%RH) at 9 UTC (Coordinated Universal Time).

III.8. DATA ANALYSIS

III.8.1. MOSQUITO ABUNDANCE

Estimate of species abundance varied according to the different mosquito collection method used. For CDC-CO_2 traps, abundance was calculated as the mean number of specimens of each species collected *per* trap *per* night. Regarding resting collections, results are presented as the number of mosquitoes caught by one person during one hour. These estimates were calculated dividing the total number of mosquitoes captured by the collection effort (n. of collection hours x n. of collectors). For HB collections, mosquito abundances are always expressed as the mean number of mosquitoes landing in one person *per* hour. Daily man biting rates (*ma*), defined as the number of bites *per* person *per* day (Garrett-Jones, 1964b) were estimated based on 12 h HB captures.

[11] http://snirh.pt/

Collection sites productivity regarding IR collections was calculated dividing the number of mosquitoes caught by the collection effort. For comparison between CDC-CO_2 and HBext collections, *CDC-CO_2 traps efficiency* (Δ) was calculated as the number of mosquitoes collected *per* trap divided by the number of mosquitoes captured by a collector in a landing catch in the same location and for the same period of time (Laganier *et al.*, 2003).

To evaluate the abundance of mosquito breeding sites, the following parameters were estimated according to Ribeiro *et al.* (1980b).

General breeding index (GBI): This index is calculated as the quotient between the number of breeding sites with immature Culicids and total number of sites prospected. This index gives a measure of the proportion of water bodies that are used as mosquito breeding places for a given locality or region. It varies between zero when no immature forms of mosquitoes are found and one when all water collections present immature Culicids.

Absolute breeding index (ABI): The percentage of positive breeding sites for a particular species referred to the total number of water bodies observed defines the *ABI*. This index varies between zero, when no immature forms of a certain species are found in the study area (although other species may be found) and 100, when the species is present in all breeding places.

Relative breeding index (RBI): This parameter indicates the abundance of the breeding sites of a certain species in relation to the number of water collections where Culicids were found. Like ABI, it also varies between zero and 100, being 100 when the species under study is present in all mosquito breeding places.

III.8.2. SAMPLE SIZE FOR DETERMINING THE LIKELY FREQUENCY OF A SPECIES PRESENCE

The maximum likely frequency of a species being present in the study area but not being collected in the entomological surveys due to sample size can be computed as described by Post & Millest, (1991), according to the equations:

$T = 1-0.05^{1/N}$, for 95% confidence limit $\qquad$ $T=1-0.01^{1/N}$, for 99% confidence limit

where N is the number of specimens of the sample size.

III.8.3. INDEX OF ASSOCIATION BETWEEN SPECIES

The index **d** (Ribeiro *et al.*, 1980b) estimates the degree of association between two species of mosquitoes. This parameter is calculated as the difference between the theoretical frequency of both species being present in the same breeding site (proportion of sites with species A multiplied by the proportion of sites with species B) and the frequency observed in nature. The statistical significance of this difference was tested by Pearson's Chi-square of 2X2 contingency tables with Yates correction for continuity or by Fisher's exact test. In these tests the rows represent the number of breeding sites positive and negative for one of the species and the columns the same type of data but referring to the second species.

III.8.4. ADULT BIOLOGICAL PARAMETERS

Parity rates and *insemination rates* were calculated, respectively, as the proportion of parous and inseminated females regarding the total number of specimens that were classified according to these two features.

The duration of the *first gonotrophic cycle* (i_0) was estimated as the mean number of days between female's emergence and first oviposition, computed for each group of females. The mean number of days between ovipositions determined the duration of subsequent *gonotrophic cycles* (*i*).

Individual blood feeding frequency was estimated dividing the number of meals taken by a female by the sum of days of her survival. When referring to a group, *blood feeding frequencies* (*F*) were calculated as the quotient between the sum of all female's feeds and the sum of the days they survived.

III.8.5. STATISTICAL METHODS

The statistical procedures described below have been derived from the statistical books of Sokal & Rohlf (1981), Zar (1984) and Kirkwood (1988). Statistical analyses were performed with the softwares Microsoft Excel® for Windows®, SPSS 13.0 for Windows® and Nanostat (1987), with the support of the handbooks of Maroco (2003) and Pereira (2003).

III.8.5.1. Descriptive statistics

The average value of a group of individual observations was represented by the arithmetic mean, denoted in the text by mean ($\overline{X}$). This is calculated as the sum of the observed values, where X_i is the i^{th} observation, divided by the total number of observations, n.

$$\overline{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

The median (Md.) was also used as another measure of the average value. If observations are arranged in increasing order, the median is defined as the mid-value that divides the frequency distribution into an equal number of observations on either side. The median is particularly useful in the case of non-normal distributions, *e.g.* if there are extremely high or low values which may turn the mean unrepresentative. When n is even, the median is calculated as the midpoint between the two middle ones.

Md. = $(n + 1) / 2$ th value of ordered observations

The standard deviation of the mean (s) was applied as a measure of the dispersion of a distribution. It is obtained by the square root of the variance (s^2) and it is defined as:

$$s = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \overline{X})}{n-1}}$$

The skewness and kurtosis are two parameters that describe how a frequency distribution curve varies from normality. A normal distribution, also called Gaussian, is characterised by a bell-shaped, symmetrical curve around the mean with skewness and kurtosis equal to zero. The skewness (Sk.) is a measure of the asymmetry of a distribution and the kurtosis (Ku.) is a measure of the extent to which the observations cluster around a central point. Skewness indicates that one tail of the distribution curve is drawn out more than the other. A curve with a positive kurtosis has more observations near the mean than at the tails. A negative kurtosis indicates that observations cluster less and the curve has longer tails. The sample statistics formulas for calculating skewness and kurtosis are:

$$\text{Sk.} = (1/ns^3)\sum_{i=1}^{n}(X - \overline{X})^3 \qquad \text{and} \qquad \text{Ku.} = (1/ns^4)\sum_{i=1}^{n}(X - \overline{X})^4 - 3$$

III.8.5.2. Contingency table tests

Associations between qualitative or discrete variables on contingency tables were tested using the *Person's Chi-square test*. The chi-square value (X^2) is calculated as:

$$X^2 = \sum \frac{(O-E)^2}{E}$$

where, for each cell of the contingency table, O and E are, respectively, the observed number and the expected value according to the null hypothesis: the distribution of individuals among categories of one variable is independent of their distribution among the categories of the other. To determine E value one may use the following equation:

$$E = \frac{\text{column total x row total}}{\text{overall total}}$$

To ascertain the significance of test, the X^2 value calculated must be compared with those of the Chi-square distribution, for a given significance level and degrees of freedom. The number of degrees of freedom in contingency tables analysis is calculated as:

$$\text{d.f.} = (r-1)\,(c-1)$$

where r is the number of rows and c the number of columns. The null hypothesis is rejected when the calculated X^2 value is higher than the critical value of the Chi-square distribution.

The Pearson's Chi-square test for 2X2 contingency tables may be improved by using the Yates' continuity correction. The use of this correction is justified when both the rows totals and the columns are set in advance or when d.f.=1. For d.f.>1 the Yates correction is not applicable. The Yates correction applied to X^2 formula results in the following equation:

$$X_y^2 = \sum \frac{\left(\left| O-E \right| - \frac{1}{2}\right)^2}{E}$$

For the analysis of 2X2 contingency tables the Fisher's exact probability test is preferable to the Pearson's Chi-square test when sample sizes are small. When the overall total of the table is less than 20 or between 20 and 40 but the smallest of the four expected values is less than 5, the use of the Fisher's exact test is recommended. This test is based on calculating the exact probabilities of the observed table and of all other possible tables that may me obtained with the same row and column totals according to the following formula:

$$P = \frac{e!\, f!\, g!\, h!}{n!\, a!\, b!\, c!\, d!}$$

where notations are the following in a general 2X2 contingency table:

		Variable X		
		Category A	Category B	Total
Variable Y	Category C	a	b	e
	Category D	c	d	f
	Total	g	h	n

The significance of the test is determined by the sum of the probability of the observed table with the probability of the more extreme tables defined as the less probable. In the two tailed hypothesis the considered tables are also the more extreme, but in the opposite directions.

III.8.5.3. Tests for comparison of means

t test: This procedure is used for comparing the means of two samples; the test requires that population distributions are normal and that have equal variances. The null hypothesis (H_0) is that the two samples came from the same population. When samples have unequal sizes the t test can be calculated using the following formula:

$$t = \frac{\left(\overline{X_1} - \overline{X_2}\right) - \left(\mu_1 - \mu_2\right)}{\sqrt{\left[\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}\right]\left(\frac{n_1 + n_2}{n_1 n_2}\right)}}$$

where, $\overline{X}_1$, $\overline{X}_2$ and s_1, s_2 are, respectively the two samples means and standard deviations; μ_1 and μ_2 the population means, and; n_1 and n_2 the number of cases of sample 1 and 2. The

degrees of freedom are calculated as $n_1 + n_2 - 2$. Whenever the absolute value of observed *t* is smaller the critical *t*-value for the pretended significance, H_0 can not be rejected.

III.8.5.4. Non parametric methods

Non parametric tests, also called distribution free methods are efficient in detecting differences when parametric assumptions are not met. Thus, these techniques are particularly useful in cases of non-normality of the data or when homogeneity of variances is not observed. To determine the normality of data and to test homogeneity of variances three techniques were used:

Kolmogorov-Smirnov goodness of fit for normality: This procedure tests the null hypothesis that a sample comes from a population whose members follow a normal distribution. It is based on the absolute values of the maximum difference between the observed cumulative distribution and that expected based on assumption of normality. Kolmogorov-Smirnov goodness of fit employs the sample statistics $\overline{X}$ and *s* as estimates of the population mean and standard deviation parameters and therefore the Lilliefors correction should be used.

Shapiro Wilk test: Serving the same purpose as the Kolmogorov-Smirnov test it is particularly suitable for small samples (< 30).

Levene test for equality of variance: it determines if *k* samples have equal variance. The Levene´s test can be computed using the mean of each sample, the median or the trimmed mean. The test based on the median performs best when the underlying data follow a skewed distribution and the trimmed mean should be applied when data presents a heavily tailed distribution. The use of the mean is more adequate to symmetric, moderate distributions. Although the optimal choice depends on the underlying distribution, the definition based on the median is recommended, as it is the choice that provides good robustness and still retaining good power, *i.e.* the ability to detect unequal variances when the variances are in fact unequal.

Four non parametric tests were used to compare samples:

Wilcoxon's signed-ranks test: is a nonparametric alternative to a paired t-test in which the absolute differences between the related variables are ranked (*i.e.* 1, 2, 3, ...) in ascending order of magnitude. The cases in which the difference between variables is zero are excluded and cases with tied differences are averagely ranked. The ranks are then split into two groups: the group of positive ranks, with the cases in which the value of the first variable exceeded the value of the second variable; and the group of negative ranks, with the cases in which the value of the second variable was higher than the value of the first one. The ranks of each group are summed and if the two variables came from populations with identical

distributions, the sums will be similar. If samples differ, one sum would be much smaller and the other would be much larger than expected. The test determines whether the smaller of the observed sums is smaller than would be expected by chance, by comparing its absolute value with the critical values of the test for a given significance level and the appropriate sample size. Note that the sample size is the number of differences that were ranked and therefore do not include the cases in which the differences between variables were zero. For large samples (>30), a normal approximation can be computed and compared to the critical values of the *t* distribution.

Friedman's test: also called Friedman's analysis of variance, is the equivalent of the signed rank test for blocked data from more than two groups. Within each block (group) the data is ranked and the ranks summed for each of the categories of one variable (treatment variable). A X^2 statistic is then computed as:

$$X^2 = \left[\frac{12}{ab(a+1)} \sum_{i=1}^{a} \left(\sum_{j=1}^{b} R_{ij} \right)^2 \right] - 3b(a+1)$$

where a is the number of categories of the treatment variable, b is the number of blocks and R_{ij} is the rank at treatment i in block j. The value obtained is compared with critical values of the Chi-square distribution.

Mann-Whitney U-test: analogue to the two-sample *t* test it computes, as in the other non parametric tests, not the actual measurements but the ranks of the measurements. Data can be ranked either from the highest to lowest values or *vice-versa*. The Mann-Whitney statistic, denoted by *U*, is calculated as:

$$U = n_1 n_2 + \frac{n_1(n_1+1)}{2} - R_1$$

where, n_1 and n_2 are the number of observations in sample 1 and 2, respectively; and R_1 the sum of the ranks of the observations in sample 1. For a two-tailed test both *U* and *U'* (the analogue value of *U* computed for sample 2) must be calculated and the larger of the two is compared to the critical values of Mann-Whitney *U* distribution for the chosen level of significance and appropriate sample size. If the size of the smallest sample exceeds 20 and the size of the largest sample exceeds 40, a normal approximation may be computed and compared to the *t* distribution critical values.

Kruskal-Wallis test: is often called analysis of variance by ranks and can be used to test nonparametrically for intergroup differences in cases with *k* samples and n_i observations *per* sample. The procedure for ranking data is the same as in the Mann-Whitney test. Kruskal-Wallis test statistic is computed as:

$$H = \frac{12}{N(N+1)}\sum_{i=1}^{k} R_i^2 - 3(N+1)$$

where, n_i is the number of observations, N is the total number of observation in all k groups and R_t is the sum of the ranks of the n_i observations in the group i.
For $k < 5$ and small sample sizes (< 8), results can be compared with critical values of Kruskal-Wallis H distribution. For larger sample sizes or/and $k > 5$, H may be considered to be approximated to χ^2 with k-1 degrees of freedom. Note that, when rejecting H_0 (there are no differences between groups), the Kruskal-Wallis test does not provide the information of which groups differ which other groups. It only indicates that at least one difference among the k groups does exist.

III.8.5.5. Correction for multiple tests

The significance level of a test (γ) equals the probability of doing a type I error, wrongly rejecting the null hypothesis when it is in fact true. This means that if the nominal significance level is set, as in this study, at 0.05, it would be expected to get strictly by chance a significant *P*-value in five out of 100 occasions. When several tests are performed simultaneously the probability of type I errors increases monotonically and thus the significance level should be adjusted. One of the methods for this adjustment is the Bonferroni correction. For one tailed tests, this method consists in dividing α by the number (*n*) of tests performed, *i.e.* γ/*n*. For two tailed tests the correction is given by:

$$\gamma = 1 - (1- \gamma)^{1/n}$$

III.8.5.6. Linear regression analysis

The simplest relationship of a given continuous variable to another is the linear regression. It gives the equation of a straight line that describes how the dependent variable *Y* varies (*i.e.* increases or decreases) with an increase in the independent or explanatory variable *X*.

The simple linear regression equation is:

$$Y = \alpha + \beta X$$

where α is the intercept and β the slope of the line, also called regression coefficient. Both α and β are derived through the criterion of the least squares fit. This considers the vertical distances of the points to the line and defines the best fit line as the one that minimises the sum of the squares of these deviations.

The parameters α and β are derived from the expressions:

$$\beta = \frac{\sum_{i=1}^{n}(X_i - \overline{X})(Y_i - \overline{Y})}{\sum_{i=1}^{n}(X_i - \overline{X})^2} \quad \text{and} \quad \alpha = \overline{Y} - \beta\overline{X}$$

where, n is the number of data points comprising the sample, X_i and Y_i the values of the exploratory and dependent variables and $\overline{X}$ and $\overline{Y}$ their respective means.

The slope β has the same sign as the correlation coefficient, expressing quantitatively the dependence of Y on X. When there is no correlation between the variables, β equals zero.

The values obtained for α and β are sample estimates and finding a functional relationship in the sample (*i.e.* $\beta \neq 0$) does not mean that there is a linear association between X and Y in the whole population (*i.e.* $\boldsymbol{\beta} \neq 0$). For testing the significance of a regression, the null hypothesis (H_0): $\boldsymbol{\beta} = 0$, must be rejected. This may be achieved through an analysis of variance (ANOVA) procedure. The first steps are to calculate the overall variability of the dependent variable and the amount of variability among Y_i values that result from there being a linear regression. The first estimate is called the total sum of squares (*TSS*) and the second is termed the linear regression sum of squares (*RGSS*) and these can be calculated as:

$$TSS = \sum_{i=1}^{n}(Y_i - \overline{Y})^2 \quad \text{and} \quad RGSS = \beta\sum_{i=1}^{n}(X_i - \overline{X})(Y_i - \overline{Y})$$

The residual sum of squares (*RESS*) can be obtained by the difference:

$$RESS = TSS\text{-}RGSS$$

After the calculation of *RGSS* and *RESS* the null hypothesis can tested by determining:

$$F = \frac{\frac{RGSS}{\text{d.f.}RG}}{\frac{RESS}{\text{d.f.}RE}}$$

where, d.f.*RG* are the degrees of freedom associated with the variability among Y_i due to regression which in a simple regression equals one and d.f.*RE* refers to the residual degrees of freedom calculated as: $n - 2$.

The numerator and denominator of equation F are respectively termed as regression mean squares (*RGMS*) and residual mean squares (*REMS*).

The square root of the residual mean square is called the standard error of estimate (*s* est) and indicates the accuracy with which the regression function predicts the dependence of *Y* on *X*. The linear regression model can therefore be expressed by the equation:

$$Y = \alpha + \beta X \pm s \text{ est}$$

The proportion of the total variation on *Y* that is accounted by the regression model is named the coefficient of determination (R square or R^2), which is computed as:

$$R^2 = RGSS/TSS$$

This parameter varies between zero and one. When R^2 equals zero, the accuracy of the fitted regression is null and when $R^2 = 1$, all data points fall exactly on the regression line. In the output of SPSS linear regression analysis there are two other parameters related to R square. The square root of R^2 is termed multiple correlation coefficient (R). It reflects the relationship (correlation) between the observed and the predicted values of the dependent variable. The adjusted R square (R^2a), attempts to correct R square value in order to more closely reflect the goodness of the fit of the model in the population. This parameter is determined by the formula:

$$R^2a = 1-(REMS/TMS)$$

where, *TMS* is the total mean squares, calculated as:

$$TMS = TSS/ n\text{-}1$$

A *t* statistic can also be applied to test significance of the fitted regression line. The value of t can be computed as the difference of the value estimated minus the parameter value hypothesized divided by the standard error of the estimated parameter. The degrees of freedom for this testing procedure are: *n* -2.
For the former H_0: $\beta = 0$, *t* is calculated as:

$$t = (\beta - \beta) / s_\beta$$

where β is equal to zero and s_β, the standard error of β, is calculated as:

$$s_\beta = s\,\text{est} / \sqrt{\sum_{i=1}^{n} (X - \overline{X})^2}$$

For the linear regression model to be used for estimation and prediction of dependent variable values based on changes of the independent variable, two assumptions must be satisfied: (i) the residuals or errors of the model must have a normal distribution with a null mean and a constant variance, and; (ii) the residuals have to be independent. The first condition can be graphically analysed using SPSS linear regression package, by plotting the regression standardised residuals against the regression standardised predicted values.

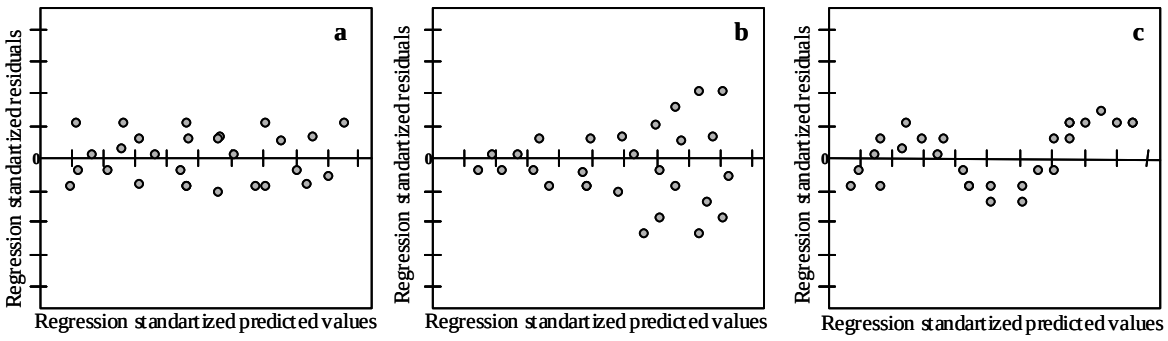


Figure III.3. Graphic analysis of regression residuals.
a: the regression residuals present a random distribution around zero. b: residuals with a non constant variance. c: an example where the relationship between the two variables analysed is non linear. Adapted from Maroco, 2003.

In an ideal situation, the regression residuals present a random distribution around zero (Figure III.3.a). When graphs are similar to Figure III.3.b it can be concluded that the residuals present a non constant variance and when similar to Figure III.3.c that the relationship between *Y* and *X* is non linear.

III.8.5.7. Survival analysis

The statistical significance of observed differences between two survival curves can be assessed by the *log rank test* which is a special application of the Mantel-Haenszel X^2 procedure. In this test, the null hypothesis is that there is no difference between populations in the probability of an event (*e.g.* death) at any time point. For each temporal interval of the life tables, a 2X2 table is constructed to determine if the proportions of deaths are similar. The application of Mantel-Haenszel X^2 to these tables summarizes the interval differences between the two life tables determining the statistical differences between the survival curves.

Chapter IV

OPTIMISATION OF TOOLS FOR *ANOPHELES ATROPARVUS* IDENTIFICATION AND SAMPLING

IV.1. AIMS

Anopheles atroparvus is the most abundant member of *Anopheles maculipennis* complex in Portugal. Although extensively studied before the implementation of the Portuguese malaria eradication campaign during the 1940's, very few concerted studies about this species and its potential role as a vector were undertaken since the eradication of the disease. In the last three decades the only notable exceptions concern the work developed by Ribeiro and collaborators. Studies on Culicid species distribution have provided insights on aspects of *An. atroparvus* bioecology (Ribeiro *et al.*, 1977; Ramos *et al.*, 1977/78; Ribeiro *et al.*, 1977/78; Pires *et al.*, 1982; Ribeiro *et al.*, 1983; Ribeiro *et al.*, 1985; Ramos *et al.*, 1992; Ribeiro *et al.*, 1996; Ribeiro *et al.*, 1999a;b; Ribeiro *et al.*, 2002), and *An. maculipennis* complex members distribution and species identification based on morphological characters (Ribeiro *et al.*, 1980a; Ramos *et al.*, 1982; Ribeiro *et al.*, 1988). With the purpose of implementing a molecular tool for species identification of *An. maculipennis s.l.* specimens and also to determine the most efficient method for sampling *An. atroparvus*, a study, conducted during the period of *ca.* one year, was carried out in the Comporta region. This study, developed within the framework of a project aimed at the identification and characterisation of local mosquito fauna, provided specimens for the molecular work and information for the design of a sampling methodology especially adapted for the bioecological study of *An. atroparvus*.

IV.2. *ANOPHELES MACULIPENNIS* COMPLEX SPECIES MOLECULAR ANALYSIS

IV.2.1. SEQUENCING ANALYSIS OF THE RDNA ITS2

Amplified ITS2 products of seven female mosquitoes to be further used as controls were subjected to direct sequencing: four *An. atroparvus* specimens, three from a long established colony of IHMT and one originated from an egg batch laid by a female captured in Serra da Estrela and identified by egg morphology; one *An. maculipennis*, collected in the same region and identified by the same method; one *An. labranchiae* specimen from Italy, kindly offered by R. Romi and D. Boccolini ("Istituto Superiore di Sanità", Rome, Italy) and one *An. melanoon* female from France, courtesy of D. Fontenille and N. Ponçon ("Institut de Recherche pour le Développement", Montpellier, France). Internal Transcribed Spacer 2 direct sequences were also obtained for other 17 females collected in Comporta region and identified as *An. maculipennis s.l.* Before DNA extraction, all 24 specimens had their

abdomen removed with individual sterile needles to prevent possible contamination with male sperm.

```
                                                                                ↓
AF504248        1   ATCACTCGGC  TCGTGGATCG  ATGAAGACCG  CAGCTAAATG  CGCGTCACAA  TGTGAACTGC
C6 direct           ..........  ..........  ..........  ..........  ..........  ..........
C6 cloned           ..........  ..........  ..........  ..........  ..........  ..........
C15 cloned          ..........  ..........  ..........  ..........  ..........  ..........
C43 cloned          ..........  ..........  ..........  ..........  ..........  ..........

AF504248       61   AGGACACATG  AACACCGATA  AGTTGAACGC  ATATTGCGCA  TCGTGCGACA  CAGCTCGATG
C6 direct           ..........  ..........  ..........  ..........  ..........  ..........
C6 cloned           ..........  ..........  ..........  ..........  ..........  ..........
 C15 cloned         ..........  ..........  ..........  ..........  ..........  ..........
C43 cloned          ..........  ..........  ..........  ..........  ..........  ..........

AF504248      121   TACACATTTT  TGAGTGCCCA  TATTTGATCA  TAACCCAAGC  CAAACGGCGT  ACCTCACCGT
C6 direct           ..........  ..........  ..........  ..........  ..........  ..........
C6 cloned           ..........  ..........  ..........  ..........  ..........  ..........
C15 cloned          ..........  ..........  ..........  ..........  ..........  ..........
C43 cloned          ..........  ..........  ..........  ..........  ..........  ..........

AF504248      181   ACGTGGAGTT  GATGAAAGGG  TCTGGATACG  CCATCCTTTC  TCTTGCATCG  AAGTCGTAGC
C6 direct           ..........  ..........  ..........  ..........  ..........  ..........
C6 cloned           .....A.A..  ......G...  ..........  ..........  ..........  ..........
C15 cloned          .....A.A..  ..........  ..........  ..........  ..........  ..........
C43 cloned          .....A.A..  ......G...  ..........  ..........  ..........  ..........

AF504248      241   GTGTAGCAAC  CCCAGGTTTC  AACTTGCAAA  GTGGCCATGG  GGCTGACACC  TCACCACCAT
C6 direct           ..........  ..........  ..........  ..........  ..........  ..........
C6 cloned           ..........  ..........  ..........  ..........  ..........  ..........
C15 cloned          ..........  ..........  ..........  ..........  ..........  ..........
C43 cloned          ..........  ..........  ..........  ..........  ..........  ..........

AF504248      301   CAGCGTGCTG  TGTAGCGTGT  TCGGCCCAGT  TCGGTCATCG  TGAGGCGTTA  CCTAACGGAG
C6 direct           ..........  ..........  ..........  ..........  ..........  ..........
C6 cloned           ..........  ..........  ..........  ..........  ..........  ..........
C15 cloned          ..........  ..........  ..........  ..........  ..........  ..........
C43 cloned          ..........  ..........  ..........  ..........  ..........  ..........

AF504248      361   AAGCACCAGC  TGCTGCGTGT  ATCTCATGGT  TACCCCCAAC  CATAGCAGCA  GAGATACAAG
C6 direct           ..........  ..........  ..........  ......T...  ..........  ..........
C6 cloned           ..........  ..........  ..........  ......T...  ..........  ..........
C15 cloned          ..........  ..........  ..........  ......T...  ..........  ..........
C43 cloned          ..........  ..........  ..........  ......T...  ..........  ..........
                                                    ↓
AF504248      421   ACCAGCTCCT  AGCAGCGGGA  GCTCATGGGT  CTCAAATAAT  GTGAGACTAC  CCCCTAAATT
C6 direct           ..........  ..........  ..........  ..........  ..........  ..........
C6 cloned           ..........  ..........  ..........  ..........  ..........  ..........
C15 cloned          ..........  ..........  ..........  ..........  ..........  ..........
C43 cloned          ..........  ..........  ..........  ..........  ..........  ..........

AF504248      481   TAAGCAT
C6 direct           .......
C6 cloned           .......
C15 cloned          .......
C43 cloned          .......
```

Figure IV.1. Comparison of a 488 bp fragment of ITS2 generated by this study and the GenBank ITS2 sequence AF504248 (Linton *et al.*, 2002c).

Underlined: primer sequences. ↓: beginning and end of ITS2, according to Linton *et al.* (2002c). C6 direct: ITS2 direct sequence of an *An. atroparvus* colony female amplicons. C6 cloned: ITS2 cloned amplicons sequence of the same *An. atroparvus* colony female. C15, C43 cloned: ITS2 cloned amplicons sequence of *An. atroparvus* females captured in Comporta and Serra da Estrela, respectively.

Seven PCR products, two derived from colony specimens, four from Comporta´s *An. maculipennis s.l.* and one from an *An. atroparvus* female captured in Serra da Estrela, were also cloned and a second set of sequence data generated from cloned fragments. Due to results obtained, direct sequencing of amplified ITS2 fragments of these seven individuals was repeated twice, using a different standard *Taq* DNA polymerase and a proofreading enzyme.

Results of direct sequencing showed absolute homology between *An. labranchiae*, *maculipennis* and *melanoon* ITS2 amplified fragments and GenBank sequences for the respective species. The four *An. atroparvus* control mosquitoes and the 17 *An. maculipennis s.l.* from Comporta showed 99% of sequence homology with *An. atroparvus*. The sequences of the 21 specimens differed from those of *An. atroparvus* ITS2 available in GenBank due to a C→T transition at base 397 (Figure IV.1.). All individuals were found to be heterozygotic for this position by both direct and cloned fragment sequencing. The use of several *Taq* enzymes showed no differences in direct sequence alignments. Cloned products showed higher degree of variability, with three of the seven specimens also presenting two G→A transitions at positions 186 and 188. A fourth transition A→G at site 197 was observed in two of the four females bearing the double G→A substitution (Figure IV.1.).

Another molecular technique was used to confirm the validity of the 397 polymorphism. The PIRA-PCR showed two restriction fragments of sizes compatible with 263 and 236 bp thus corroborating the heterozygosis of all 21 *An. atroparvus* (Figure IV.2.).

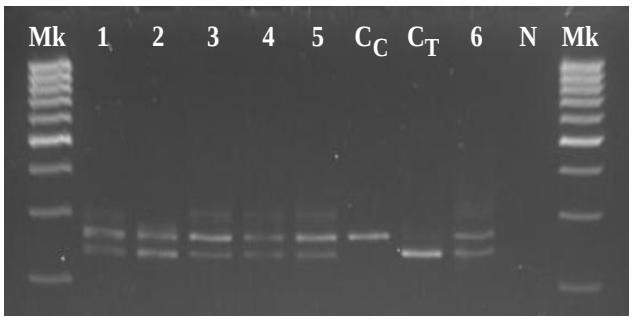


Figure IV.2. An example of PIRA-PCR results.
1 to 5 and 6: *An. atroparvus* specimens. Mk:100 bp marker. N: negative control. C_C: cloned fragment with C base at position 397.C_T: cloned fragment with T base at position 397.

IV.2.2. SPECIES IDENTIFICATION BY PCR-RFLP

A new PCR-RFLP protocol was developed for the identification of the three *Anopheles maculipennis* sibling species recorded in Portugal: *An. atroparvus*, *An. maculipennis* and *An. melanoon*. The technique was modified in order to identify a fourth member of the *maculipennis* complex, *An. labranchiae*. This species, which presence in southeast of Spain was reported until the malaria control campaigns (Encinas Grandes, 1982) is, in terms of geographic distribution, the most likely one to be present in Portugal when compared with the other members of the *An. maculipennis* complex.

IV.2.2.1. Methodological considerations

Enzymatic digestions of ITS2 PCR products may be performed simultaneously, using both enzymes in the same reaction, or in sequential restriction procedures. For mosquitoes collected south of Montejunto-Serra da Estrela mountain system, since the presence of either *An. maculipennis s.s.* or *An. labranchiae* specimens is an improbable event, samples may be processed using first only one restriction enzyme. Each amplified product is digested with only 1.25 U of *Cfo 1*, adjusting the amount of water to a total volume of 20 µl. For those specimens presenting restriction fragments with lengths compatible with *labranchiae/maculipennis s.s.*, identification by means of a second enzymatic digestion with *HPA II* enzyme can then be carried out. For mosquitoes collected in the centre and north parts of Portugal where *An. maculipennis s.s.* is likely to occur the two restriction enzymes may be used in the same reaction.

IV.2.2.2. Results

Forty-three field collected *An. maculipennis s.l.* specimens (40 females and 3 males) were identified as *An. atroparvus* using the PCR-RFLP protocol. All the control mosquitoes, which species identity had been determined by sequence analysis, and also 8 *An. atroparvus* colony females were correctly identified using this protocol.

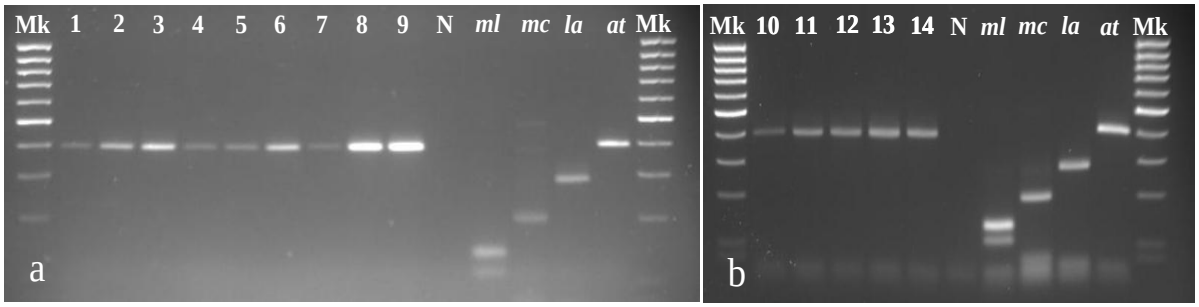


Figure IV.3. A PCR-RFLP identification of immature *Anopheles atroparvus* and adult body parts.
(a): preserved in a 75% alcohol and 2% glycerine. (b): dried with silica gel
1: one egg. 2: five eggs. 3: 10 eggs. 4: one L1 larva. 5: one L2 larva. 6: one L3 larva. 7: one L4 larva. 8: one pupa. 9: head and thorax of adult female. 10: one wing. 11: one leg. 12: head. 13: thorax. 14: abdomen. Mk:100 bp marker. N: negative control. *ml: An. melanoon* (control). *mc*: *An. maculipennis s.s.* (control). *la*: *An. labranchiae* (control). *at*: *An. atroparvus* (control).

The technique allowed the identification of body parts of a single mosquito: head, thorax, abdomen, one wing and one leg; as well as immature forms preserved in a 75% alcohol and 2% glycerine solution. As presented in Figure IV.3, single eggs, larvae, pupae and parts of adult specimens preserved in the alcoholic solution were also correctly identified.

Based on the results obtained, a molecular identification key was elaborated for the identification of the *An. maculipennis* members recorded for Portugal.

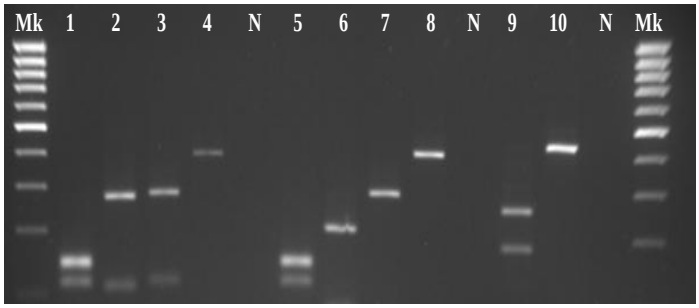


Figure IV.4. *Anopheles maculipennis s.l.* PCR-RFLP patterns using both *Cfo I* and *HPA II* enzymes in the same restriction reaction (lanes 5-8), only Cfo I (lanes 1-4) or only HPA II (lanes 9 and 10).
1: *An. melanoon*. 2: *An. maculipennis s.s.* 3: *An. labranchiae*. 4: *An. atroparvus*. 5: *An. melanoon*. 6: *An. maculipennis s.s.* 7: *An. labranchiae*. 8: *An. atroparvus*.
9:*An. maculipennis s.s.* 10: *An. labranchiae*. MK:100 bp marker. N: negative control.

Molecular Identification key.

1. - ITS2 PCR products incubated with *Cfo 1* (Figure IV.4., lanes 1-4)…… ……..2
   - ITS2 PCR products incubated with both *Cfo 1* and *HPA II* (Figure IV.4., lanes 5-8)…… ….……...……..4

2. - Restriction fragments with 389 bp of length (Figure IV.4., lane 4)……***An. atroparvus***
   - Restriction fragments with 108 and 135 bp of length (Figure IV.4., lane 1)……***An. melanoon***
   - Restriction fragments with other lengths (Figure IV.4., lanes 2-3)……...3

3. - ITS2 PCR products digestion with *HPA II;* fragments with 420 bp (Figure IV.4., lane 10)……***An. labranchiae***
   - ITS2 PCR products digestion with *HPA II;* fragments with 169 and 244 bp (Figure IV.4., lane 9)..***An. maculipennis***

4. - Restriction fragments with 389 bp of length (Figure IV.4., lane 8)……***An. atroparvus***
   - Restriction fragments with with 279 bp (Figure IV.4., lane 7)……..***An. labranchiae***
   - Restriction fragments with 201 bp (Figure IV.4., lane 6)……...***An. maculipennis***
   - Restriction fragments with 108 and 135 bp of length (Figure IV.4., lane 5)……***An. melanoon***

IV.3. SELECTION AND OPTIMISATION OF MOSQUITO COLLECTION METHODS FOR *ANOPHELES ATROPARVUS* BIOECOLOGICAL STUDIES

IV.3.1. METHODOLOGICAL CONSIDERATIONS

Mosquito sampling started in middle July 2000 and was carried out until the end of July 2001. All-night CDC-CO_2 light traps, outdoor HB (HBext) captures and IR collections were performed twice a month, between August and November 2000 and May and July 2001. Collections were conducted only once a month in July 2000 and between December 2000 and April 2001. The IR and all-night CDC-CO_2 collections were carried out in several sites of the six localities (Carrasqueira, Possanco, Comporta, Torre, Carvalhal and Pego, see Appendix). The HBext captures were performed always in the same site located in Comporta, during

three hour periods, centred on the sunset. The IR collections were mainly performed inside of various types of animal dwellings. Only a few IR collections were conducted inside houses due to the presence of window screens and other mosquito defence measures in almost all households of the area. Outdoor resting collections were carried out in July 2000 and 2001 and in May 2001, in all localities. Natural shelters (*e.g.* holes in the ground) and vegetation were the resting places prospected.

To evaluate the possibility of CDC-CO_2 light-traps substituting the HB collections, simultaneous 24 h HBext capture and CDC-CO_2 collections were carried out, in July 2000. The collection sites, located in Comporta, were close to each other (*ca.* 20 m). In both collection methods, mosquitoes captured in each hour period were kept separately for comparison purposes and to determine peaks of biting activity. The CDC-CO_2 trap was re-filled every hour with the same amount of dry ice (*ca.* 1 kg). This procedure was repeated eight times during periods of three hours each, centred at the sunset, between August 2000 and May 2001, in Comporta and Carvalhal.

Sampling of immature mosquitoes took place at the same periods of adult collections. Several types of larval habitats were surveyed: permanent or semi-permanent water collections, such as ponds, ditches, water tanks, rice-fields and marshes; temporary breeding sites, most of them peri-domestic and man-made, as water-storage pots or small tanks, discarded cans and buckets and animal drinking containers. Larval mosquito habitats were grouped into categories (Table IV.6.) based on breeding places descriptions noted during sampling.

IV.3.2. RESULTS

Nine mosquito species or species complexes were recorded in the study area: *An. maculipennis s.l.*; *Culex impudicus* Ficalbi, 1890; *Culex pipiens* Linnaeus, 1758; *Culex theileri* Theobald, 1903; *Culex univittatus* Theobald, 1901; *Culiseta annulata* (Schrank), 1776; *Culiseta longiareolata* Macquart, 1838; *Ochlerotatus caspius s.l.* and *Ochlerotatus detritus s.l.* All species were captured in both adult and immature forms with the exception of *Cx. impudicus* which was only recorded in larval collections. Since no isozyme analysis (Cianchi *et al.*, 1980) was performed with *Oc. caspius s.l.*, and *detritus s.l.* specimens, identification was possible only to species complex level. As regards the *maculipennis* complex, and based on the results of molecular identification procedures previously described, *An. atroparvus* was considered the only species of the complex present in Comporta region.

IV.3.2.1. Adult sampling

Of a total of 44,383 specimens collected in adult sampling, *Culex theileri*, *Oc. caspius s.l.*, *An. atroparvus* and *Cx. pipiens* were the species with the highest numbers of adult mosquitoes collected (Tables IV.1 and IV.2). Two hundred and sixty seven specimens (0.6%) were not identified due to the poor state of preservation.

Table IV.1. Number of mosquitoes, of each species, captured by the different collection methods.

	CDC-CO_2	**HBext**	**IR**		**OR**	**TOTAL**
			Animal shelters	**Houses**		
An. atroparvus	153 (0.345)	64 (0.144)	3,776 (8.508)	0 (0.000)	1 (0.002)	**3,994 (8.999)**
Cx. pipiens	819 (1.845)	49 (0.110)	228 (0.514)	5 (0.011)	8 (0.018)	**1,109 (2.499)**
Cx. theileri	25,596 (57.671)	5,462 (12.307)	858 (1.933)	1 (0.002)	132 (0.297)	**32,049 (72.210)**
Cs. univittatus	295 (0.665)	2 (0.005)	51 (0.115)	1 (0.002)	1 (0.002)	**350 (0.789)**
Cs. annulata	10 (0.023)	0 (0.000)	13 (0.029)	0 (0.000)	0 (0.000)	**23 (0.052)**
Cs. longiareolata	5 (0.011)	1 (0.002)	4 (0.009)	0 (0.000)	2 (0.005)	**12 (0.027)**
Oc. caspius s.l.	3,595 (8.100)	2,661 (5.996)	194 (0.437)	0 (0.000)	97 (0.219)	**6,547 (14.751)**
Oc. detritus s.l.	12 (0.027)	19 (0.043)	0 (0.000)	0 (0.000)	1 (0.002)	**32 (0.072)**
Unidentified specimens	232 (0.523)	6 (0.014)	19 (0.043)	0 (0.000)	10 (0.023)	**267 (0.602)**
TOTAL	**30,717 (69.209)**	**8,264 (18.620)**	**5,143 (11.588)**	**7 (0.016)**	**252 (0.568)**	**44,383 (100.00)**
Collection effort	110 traps	89.0 h	20.7 h	1.0 h	1.3 h	-

In brackets: percentage according to the total number of mosquitoes collected.

Table IV.2. Number of females (F) and males (M) mosquitoes, captured by collection method.

	An. atroparvus	***Cx. pipiens***	***Cx. theileri***	***Oc. caspius s.l.***	**Other species**
CDC-CO_2	142F 11M (3.83)	808F 11M (73.85)	25,316F 280M (79.87)	3,556F 39M (54.91)	288F 34M (77.22)
HBext	64F 0M (1.60)	49F 0M (4.42)	5,461F 1M (17.04)	2,661F 0M (40.64)	22F 0M (5.28)
IR	3,272F 504M (94.54)	223F 10M (21.01)	850F 9M (2.68)	185F 9M (2.96)	45F 24M (16.55)
OR	1F 0M (0.03)	5F 3M (0.72)	40F 92M (0.41)	40F 57M (1.48)	3F 1M (0.96)
TOTAL	3,479F 515M (100.00)	1,085F 24M (100.00)	31,667F 382M (100.00)	6,442F 105M (100.00)	358F 59M (100.00)

In brackets: percentage of total number of mosquitoes (F+M) captured by collection method respectively to the total number of mosquitoes collected of each species.

Culex theileri was the most common mosquito in CDC-CO_2 light-traps and in HBext collections representing 83% (25,596/30,717) and 66% (5,462/8,264), respectively, of the total catches by each method. *Anopheles atroparvus* was the predominant species in IR captures, totalising 74% (3,776/5,150) of all mosquitoes collected.

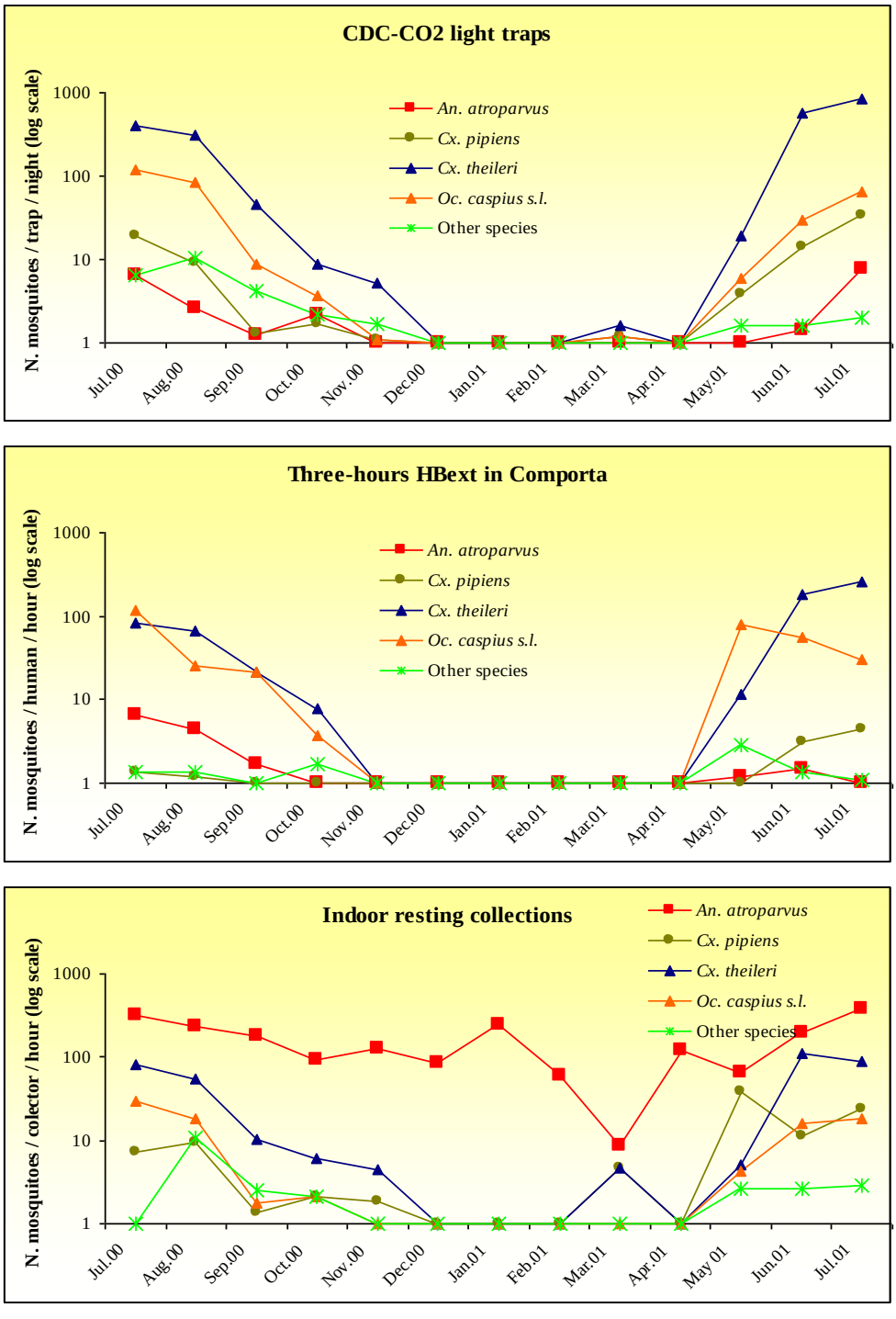


Figure IV.5. Species seasonal variations according to different collection methods, between July 2000 and July 2001.

To determine *Cx. theileri* and *Oc. caspius s.l.* relative abundances and seasonality, all-night CDC-CO_2 and three hours HBext collections proved to be more efficient sampling methods when compared to indoor resting captures (Figure IV.5.). Based on HBext catches, *Culex theileri* showed higher abundance during the month of July while *Oc. caspius s.l.* showed an earlier abundance peak during May-June. Due to the small numbers of *An. atroparvus* captured in CDC-CO_2 and HBext collections, these methods proved to be inefficient to assess this species population dynamics (Figure IV.5.). Indoor resting captures showed to be a more sensitive sampling method, allowing the capture of large numbers of *An. atroparvus* of both sexes (Table IV.2.). This species was present in the adult stage all year round, being most abundant in July. *Anopheles atroparvus* was found resting inside all types of man-made shelters surveyed, from poorly constructed dwellings made of a single wall and a roof to fairly closed animal shelters with a single small opening (Figure V.1., Chapter V). Due to the reduced number of specimens collected, human baited landing collections may be considered the less efficient sampling method to determine *Cx. pipiens* seasonality (Figure IV.5.).

CDC-CO_2 light-traps were unable to reproduce all-day HBext results concerning species biting cycles (Figure IV.6.). Although *Cx. theileri* showed similar biting cycles regardless of collection method used, *Oc. caspius s.l.* presented only one biting peak at dawn in CDC-CO_2 captures. The number of *An. atroparvus* captured in the CDC-CO_2 trap was negligible and did not present a defined pattern of activity. Similar results were obtained for *Cx. pipiens* but in HBext collections. For the comparison of the number of mosquitoes of each species collected by CDC-CO_2 traps and by HBext captures a Chi-square test of 2X2 contingency tables was used. In the test, the rows represented the number of mosquitoes collected by each method and the columns the number of mosquitoes of the species under analysis *versus* the number of mosquitoes of the remaining species. The number of mosquitoes collected was significantly different between the two methods for the species analysed (Table IV.3.). The efficiency of CDC-CO_2 traps (Δ), though able to capture 19.40 times more *Cx. pipiens* specimens than human landing collections, was bellow 0.75 for *An. atroparvus*, *Cx. theileri* and *Oc. caspius s.l.* A Δ of 2.65 for the group formed by the remaining species is explained by the difference in the efficiency of the methods regarding the capture of *Cx. univittatus* specimens (Figure IV.6.). This species, nearly absent in HBext captures (N=1), was collected in significantly higher numbers (N=21, X^2_y =29.25, d.f.=1, P<0.0001) in CDC-CO_2 traps.

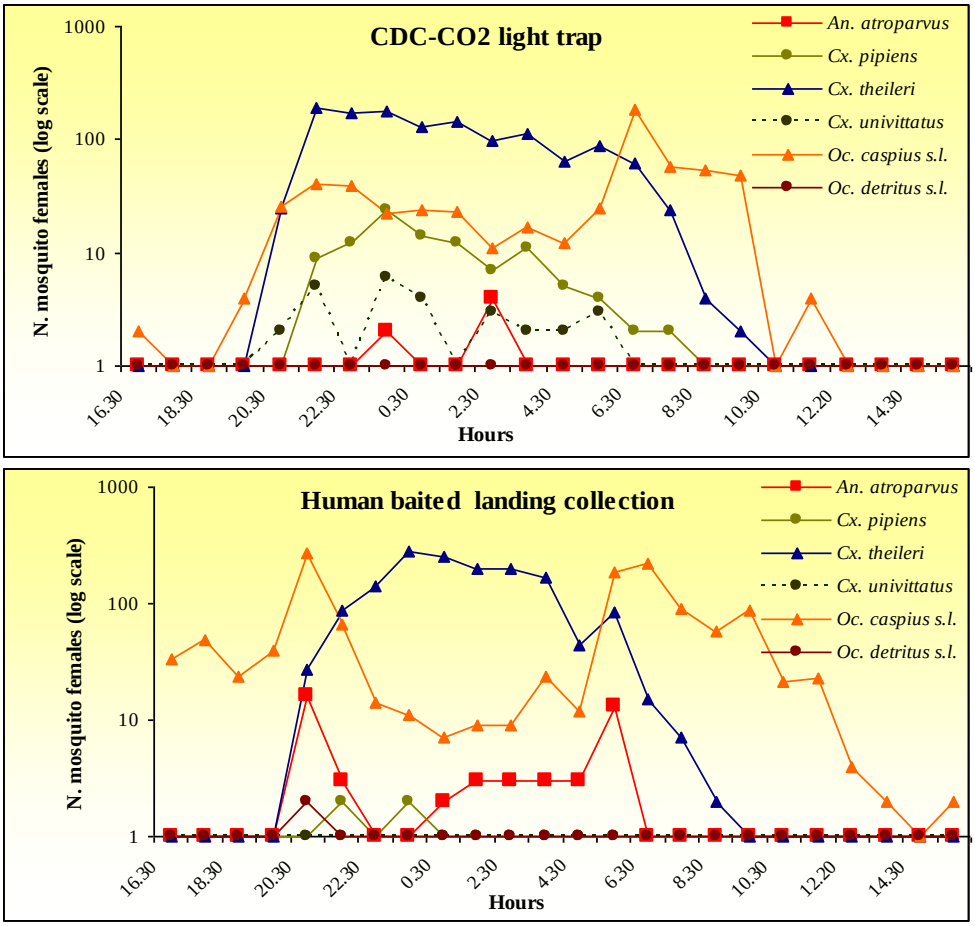


Figure IV.6. Species biting pattern according to collection method performed simultaneously in Comporta, 27th July 2000.

Table IV.3. Number of mosquitoes captured by simultaneously by CDC-CO_2 traps and human baited collections (HBext) in similar location and environment during 3 h periods.

	An. atroparvus		*Cx. pipiens*		*Cx. theileri*		*Oc. caspius s.l.*		Other species	
	CDC-CO_2	**HBext**	**CDC-CO_2**	**HBext**	**CDC-CO_2**	**HBext**	**CDC-CO_2**	**HBext**	**CDC-CO_2**	**HBext**
N	4	58	97	5	1405	1898	736	1839	45	17
X^2_y; d.f.=1	**24.40**		**144.56**		**78.50**		**149.41**		**31.47**	
Δ	0.07		19.40		0.74		0.40		2.65	

N: number of mosquitoes captured. X^2_y**:** Chi-square test of 2X2 contingency tables with Yates correction for continuity. **Δ**:Efficiency of CDC-CO^2 traps. In bold: significant after adjustment by Bonferroni correction.

IV.3.2.2. Larval sampling

Immature mosquito forms were found in 53% (324/613) of the breeding sites prospected (Table IV.4.). Monthly general breeding indexes (GBI) were always above the 0.4, except for

the period of December-January. The highest GBI values were recorded in the months of April and July (Figure IV.7.).

Table IV.4. Number of breeding sites positive for each species between July 2000 and July 2001.

	N	Pos.	*An. atroparvus*	*Cx. impudicus*	*Cx. pipiens*	*Cx. theileri*	*Cx. univittatus*	*Cs. annulata*	*Cs. longiareolata*	*Oc. caspius s.l.*	*Oc. detritus s.l.*
Jul. 00	13	6	0	0	2	4	0	0	0	0	0
Aug. 00	60	26	10	2	6	17	2	0	1	2	0
Sep. 00	82	45	9	2	18	25	5	0	6	0	0
Oct. 00	83	43	9	2	17	16	5	1	5	3	1
Nov. 00	64	33	0	0	23	14	1	2	4	0	0
Dec. 00	15	5	0	0	0	3	0	0	0	2	2
Jan. 01	19	7	0	0	4	1	0	0	0	2	3
Feb. 01	20	11	0	0	3	1	0	1	2	2	4
Mar. 01	19	8	0	0	1	0	0	0	4	0	3
Apr. 01	12	9	0	0	1	0	0	0	1	0	8
May 01	95	49	1	0	23	15	1	1	10	4	9
Jun. 01	99	56	2	0	13	28	0	0	7	16	3
Jul. 01	32	26	0	0	6	21	0	0	2	2	0
TOTAL	**613**	**324**	**31**	**6**	**117**	**145**	**14**	**5**	**42**	**33**	**33**

N: number of potential breeding sites prospected. Pos: number of breeding sites positive for Culicids.

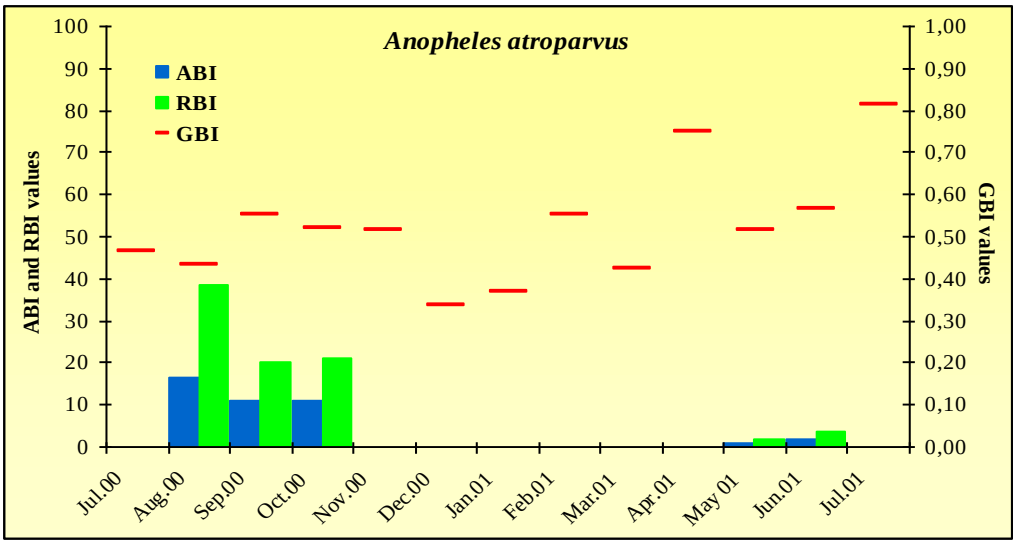


Figure IV.7. *Anopheles atroparvus* monthly ABI, RBI and GBI.

Culex theileri and *Cx. pipiens* were the predominant species in larval sampling (Table IV.4.). *Culex theileri* although not recorded in the collections of March and April was collected in 45% (145/324) of all breeding sites positive for Culicids. *Culex pipiens* was detected all year round with the exception of the month of December and was present in 36% (117/324) of mosquito breeding places. *Culiseta annulata* and *Cx. impudicus* were the less frequent species, collected only in 2% (5/324 and 6/324, respectively) of the mosquito habitats.

Table IV.5. *Anopheles atroparvus* species association regarding breeding sites.

	N	**d**	**Statistics**
An. atroparvus	5	-	-
Cx. impudicus	3	2.43	Fisher's exact test (2-tailed) - **NS**
Cx. pipiens	6	-5.19	X^2_y=3.41 d.f.=1 - **NS**
Cx. theileri	20	6.13	X^2_y =4.57 d.f.=1 - **NS**
Cx. univittatus	5	3.66	Fisher's exact test (2-tailed) - **S**
Cs. annulata	0	-0.48	Fisher's exact test (2-tailed) - **NS**
Cs. longiareolata	0	-4.02	Fisher's exact test (2-tailed) - **NS**
Oc. caspius s.l.	0	-3.16	Fisher's exact test (2-tailed) - **NS**
Oc. detritus s.l.	0	-3.16	Fisher's exact test (2-tailed) - **NS**

N: number of breeding sites where the presence of *An. atroparvus* was detected as the only mosquito species present or in association with another species. **d**: difference between observed and expected frequencies calculated according to Ribeiro *et al.* (1980b). Statistics: Pearson's chi-square test of 2X2 contingency tables with Yates correction for continuity or Fisher's exact test. **S**: significant after adjustment by Bonferroni correction. **NS**:non-significant after adjustment by Bonferroni correction.

Immature forms of *An. atroparvus* were collected between August and October of 2000 and in May-June 2001 (Figure IV.7.). This species always had low monthly absolute breeding indexes (ABI<17) but it was the second and third more frequently found species in August and September-October, with monthly relative breeding indexes (RBI) of 38.5, 20.0-20.9, respectively.

Anopheles atroparvus was the only mosquito species present in 16% (5/31) of all larval habitats (Table IV.5.). This species was more frequently detected with *Cx. theileri* (20/31). It was the only species found together with *Cx. impudicus* in 3 of the 6 breeding sites where the latter was recorded. Only one significant association was found between *An. atroparvus* and the species *Cx. univittatus.* No significant negative **d** value was recorded for the association of *An. atroparvus* with other species.

Table IV.6. Types of potential breeding sites sampled between July 2000 and July 2001 with results referring to presence of Culicids and specimens of *Anopheles atroparvus*.

		Sites prospected		Pos.		Pos. *atroparvus*	
		N	%	N	%	N	%
Marshes		13	2.1	7	53.8	0	0.0
Salt pans		52	8.5	31	59.6	0	0.0
Rice fields		231	37.7	123	53.2	13	41.9
Transient puddles		19	3.1	17	89.5	3	9.7
Ground pools		36	5.9	21	58.3	2	6.5
Ditches		97	15.8	45	46.4	12	38.7
Small streams		4	0.7	1	25.0	0	0.0
Peri-domestic containers	**Size > 0.125 m^3**	125	20.4	58	46.4	1	3.2
	Size < 0.125 m^3	36	5.9	21	58.3	0	0.0
TOTAL		613	100.0	324	-	31	100.0

Pos.-N: number of breeding sites positive for Culicids. Pos.-%: percentage of sites positive for Culicids for each type breeding site. Pos. *atroparvus*-N: number of breeding sites positive for *An. atroparvus.* Pos. *atroparvus*-%: percentage of sites positive for *An. atroparvus* with respect to the total number of positive breeding places for this species.

According to the classification of breeding sites shown in Table IV.6., *An. atroparvus* is more frequently found in rice field paddies and ditches. This species was never recorded in water collections located in the marshlands or in the salt pans, nor in small streams or small man-made peri-domestic containers.

The breeding sites characteristics of the 31 places where the aquatic stages of *An. atroparvus* were detected are presented in Table IV.7. The species was generally found in sunlit breeding sites of brownish and turbid water with vegetation. Water temperature varied between 16.2 and 34.2 ºC and pH values between 5 and 8.

Table IV.7. *Anopheles atroparvus* breeding sites characteristics.

		Number/statistics of breeding sites	Percentage (%)	Total of breeding sites observed
Water colour	**Uncoloured**	4	14.8	27
	Whitish	2	7.4	
	Greenish	5	18.5	
	Brownish	16	59.3	
Water turbidity	**Limpid**	11	40.7	27
	Turbid	16	59.3	
Water temperature (ºC)	**Mean**	22.91		29
	Min.-Max.	16.2-34.2	NA	
	s	3.887		
Water pH	**Mean**	6.22		27
	Min.-Max.	5-8	NA	
	s	0.813		
Sun exposure	**Total**	23	100.0	23
	Partial	0	0	
	Minimum	0	0	
Vegetation	**Absent**	3	13.6	22
	Emerging	13	86.4	
	Floating	6		
	Submerged	7		

NA: Not applied.

IV.4. DISCUSSION AND CONCLUSIONS

In Comporta region, nine mosquito species/species complexes were found. Five of these, *An. atroparvus*, *Cx. theileri*, *Cs. annulata*, *Oc. caspius s.l.* and *Oc. detrirus s.l.*, were already recorded in the area by Ribeiro *et al.* (1983). Three other species, *Cx. impudicus*, *Cx. pipiens* and *Cs. longiareolata*, had been identified in Águas de Moura, a region located 35 km north of Comporta (Cambournac, 1944), on the right margin of Sado river. The remaining species, *Culex univittatus*, has a fairly generalized, but scanty, distribution in the south and central Portugal (Pires *et al.*, 1982; Ribeiro *et al.*, 1988). Therefore its presence in the study area is not surprising.

A single member of the *maculipennis* complex, *An. atroparvus*, was detected in Comporta area, in agreement with what was previously described (Pires *et al.*, 1982; Ribeiro

et al., 1983). Species identification was achieved by a new RFLP technique and sequence analysis of ITS2 PCR products.

All 21 specimens screened showed to be heterozygotic (C/T) for position 397 by both direct and cloned amplicons sequencing. This unusual result was also confirmed by a PIRA-PCR technique. Since no plausible biological reason may explain the existence of only heterozygotic individuals in natural populations and all the *An. atroparvus* sequences published in GenBank are homozygotic for this position, the C→T substitution observed is likely to be an artefact. In all procedures, blank extraction controls never yield PCR products and, thus, contamination with foreigner DNA cannot explain the transition observed. Likewise, the use of different *Taq* polymerases, including a proofreading enzyme, confirms the fidelity of the *in vitro* DNA polymerisation. Furthermore, DNA polymerases tend to induce mutations in a random fashion (Cha & Thilly, 1993) and are not usually associated to specific base substitutions in independent amplifications. Other possible explanations for abnormalities in PCR products are: low number of template molecules (Akbari *et al.*, 2005), PCR jumping (Kraytsberg & Khrapko, 2005), post mortem DNA degradation/damage (Pääbo, 1989) and PCR-induced sequence alterations due to the presence of unidentified components. The first three events are improbable reasons for the observed result because: (i) the region subject to amplification is part of rDNA, which exists in large number of copies *per* cell, (ii) jumping PCR has no effect if template are identical or differ by one mutation (Kraytsberg & Khrapko, 2005), and; (iii) colony specimens were processed soon after death and thus the base transitions also observed in these individuals cannot be explained as a result of DNA oxidative damage. The most plausible explanation relies in the possibility of the C→T being a false sequence specific transition, artificially induced *de novo* during PCR. Unidentified components present in DNA extracts that either directly alter template molecules or reduce the fidelity of the *Taq* polymerises may be responsible for this type of abnormalities (Pusch *et al.*, 2004). It was previously demonstrated that ancient DNA extracts can induce mutations in a non random way (Pusch & Bachmann, 2004) probably due to the presence of multivalent metal ions, as manganese, accumulated during diagenesis. The same mutations can be produced in PCR products of contemporary human DNA when amplification is undertaken in the presence of $MnCl_2$ (Pusch *et al.*, 2004). However, the reason why some nucleotides are more prone to erroneous incorporation than others resulting in sequence specific mutations is not yet understood (Pusch *et al.*, 2004). This phenomenon of extract induced mutations can be a possible explanation for the occurrence of the false C→T transitions. Contamination, with an alien component, of PCR extraction products or of the specimens themselves during capture or preservation procedures may be responsible for the observed artefact. As expected, the number of detected artefacts increases when cloned sequences are analysed (Pääbo & Wilson, 1988; Dunning *et al.*, 1988). In direct sequencing, misincorporations are averaged.

These PCR-induced mutations can only be detected if they are introduced in the early cycles of amplification, when there are still a low number of template molecules. Late misincorporations, present in low number of amplicons and not usually found in direct sequencing, may still be introduced by a vector in transfect bacteria and therefore detected in cloned fragments.

Culex theileri, *Oc. caspius s.l.* and *An. atroparvus* were the most abundant mosquito species in the study area, when considering all adult collection methods. Human baited landing collections and CDC/CO_2 light traps, although responsible for 88% of all adult mosquitoes captured, were less efficient sampling methods for *An. atroparvus*, due to the small numbers of collected specimens (less than 6% of all *atroparvus* adults). Only one *An. atroparvus* specimen was collected in outdoor resting captures. This indispensable technique for sampling exophilic species as *Oc. caspius s.l.* (Rioux, 1958) is not adequate for *An. atroparvus*, which is mainly endophilic and only found in vegetation or in abandoned man-made structures when mosquito densities inside animal shelters are extremely high (Cambournac, 1942; Pires *et al.*,1982). Performing OR collections using a back-pack aspirator had the additional disadvantage of damaging the entomological fauna captured. Blood-fed mosquito females and other more fragile Nematocera tend to be squashed against the cups netting, yielding the identification of these specimens impossible.

Representing 95% of all *An. atroparvus* collected, this species was the major species found in IR captures made inside animal shelters. Besides the capture of large numbers of *An. atroparvus*, this method allowed the collection of specimens all year round. Furthermore, the fact that females in all gonotrophic stages were collected together with males indicates that this method allows sampling of the whole adult population. However, this type of collection was not efficient when performed inside houses. In Comporta region, due to the presence of two highly abundant and aggressive species, *Cx. theileri* and *Oc. caspius s.l.*, local inhabitants are aware of mosquito importance as pest agents. Protective measures to prevent human-mosquito contact, as door and window screens and indoors insecticide diffusers, are common in all households (Teodósio *et al.*, unpublished observations), making IR captures inside houses almost always negative.

Significant differences were found when comparing the number of *An. atroparvus* females trapped in CDC-CO_2 light traps and those captured, simultaneously, by HBext collections. Patterns of *An. atroparvus* females biting activity obtained by these two collection methods were also different. These results rendered the possibility of substituting HBext collections by CDC-CO_2 light traps unfeasible since the latter method did not reproduce the intensity and patterns of human-vector contacts of the standard methodology (HB collections).

In larval sampling, *Cx. theileri* and *Cx. pipiens* were the most frequent species in the 613 breeding places prospected. Although the highest RBI value found for *An. atroparvus* in Comporta region was not significantly lower (χ^2_y=1.78, d.f.=1, *P*=0.182) than the one previously recorded for Alentejo Province (Pires *et al.*, 1982), the presence of this species in the study area was only detected in 9.6% (31/324) of all water bodies positive for Culicids. This species, the sixth more abundant in larval collection, was obviously underrepresented in this type of sampling, by comparison with adult captures. This result, which yielded larval collections an unsuitable method for *An. atroparvus* seasonality studies, may be attributed to the methodology used during collection. Either *An. atroparvus* favourite places to breed were not prospected (*e.g.* in the centre of rice paddies instead of at their margins) or the use of a dipper was not the most efficient method to collect Anopheline larvae. Robert *et al.* (2002), using a net apparatus to collect Anophelines in the rice fields of Madagascar, were able to double the number of captured larvae by comparison to the classical dipping method.

Anopheles atroparvus immature forms were mainly found in breeding sites associated with the rice culture, being detected in paddy fields and ditches used for their inundation or drainage. Such known association (Cambournac & Hill, 1938; Pires *et al.*, 1882), also makes larval sampling an inadequate method for the study of *An. atroparvus* seasonality since rice-fields are flooded only between May-October. The presence of water in the paddies in this period is coincident with the larval seasonal pattern of *An. atroparvus*, with the exception of the month of July, where no *An. atroparvus* larvae were found, in both years. This absence of larvae may be associated with the application of an herbicide (MCPA; active ingredient: 2,4-dichlorophenoxyacetic acid) or with the application of Karate (active ingredient: lambda-cyhalothrin), a product used in some paddies for the control of the rice caterpillar. This pyretroid insecticide is sprayed over the growing rice, by airplane. Sporadically, the product may reach the water surface killing mosquito larvae. If herbicide or Karate applications may affect *An. atroparvus* survival, this does not hold for *Cx. theileri*, as larvae of this species were found in 81% (21/26) of the paddies prospected in July, 2001. This may be due to differences in the insecticide susceptibility of the two species.

Immature stages of *An. atroparvus* are usually found in clean, sunlit, standing brackish and fresh water bodies (Jetten & Takken, 1994). In Portugal, this species presents the same behavioural pattern also being considered a ground breeder (Ramos *et al.*, 1977/78; Pires *et al.*, 1982). It has mainly been found in fresh water habitats, along river margins and rock pools, cement water reservoirs and rice fields, but also in salt works and brackish breeding places at the limits of marshes (Ribeiro *et al.*, 1977; Ramos *et al.*, 1977/78; Ribeiro *et al.*, 1977/78, Pires *et al.*, 1982; Ribeiro *et al.*, 1985; Ribeiro *et al.*, 1999a). In the present study no *An. atroparvus* aquatic forms were recorded in marshes or saltpan water bodies, but all breeding places were located at ground level and included known habitats as rice-fields,

ditches and a single peri-domestic container used for agricultural purposes. All breeding places were well exposed to the sun as described for this species (Pires *et al.*, 1982), though some shade must be provided by the vegetation found in most of them. The pH values ranged from 5 to 8 with an average of 6.2, which is within the range of values recorded for this species (Cambournac & Hill, 1938; Cambournac, 1942; 1944; Ramos *et al.*, 1977/78). Regarding water physical characteristics, in contrast with what is usually described as typical for *An. atroparvus,* this species was frequently found in coloured and slightly turbid to turbid waters. However, most of the breeding sites prospected in this study were rice-fields and ditches, where water tends to be greenish or brownish. Clear and limpid water sites like small rivers or streams accounted only for 0.6% of all sites surveyed, due to its rarity in the area. Thus, concerning these two water parameters, rather than a behavioural trait, results are probably reflecting a characteristic of the study area, where, of all *An. atroparvus* breeding sites, rice fields and ditches are by far the most frequent mosquito larval places.

In continental Portugal, larvae of *An. atroparvus* were found associated with immature forms of mora than half (20/38) of all species recorded (Ribeiro *et al.*, 1977; Ramos *et al.*, 1977/78; Ribeiro *et al.*, 1977/78, Pires *et al.*, 1982; Ribeiro *et al.*, 1985; Ribeiro *et al.*, 1996; Ribeiro *et al.*, 1999a), including some rare ones, as *Cx. lacticintus* Edwards, 1913 or *Uranotaenia unguiculata* Edwards, 1913. This variety of associations is probably a result of *An. atroparvus* wide range of distribution and biological plasticity which allows it to survive in several different types of larval habitats. In this study, significant larval associations were found between *An. atroparvus* and *Cx. univittatus*. This species are mainly fresh-water breeder, frequently found in breeding sites with aquatic vegetation (Senevet & Andarelli, 1959; Ramos *et al.*, 1977/78; Ribeiro *et al.*, 1977/78). Therefore, its association with *An. atroparvus* is not unexpected. This larval association was also found in previous studies, in which, for all the species recorded in Portugal, *Cx. univittatus* was always more frequently found with *An. atroparvus* rather than with other mosquito species (Ramos *et al.*, 1977/78; Ribeiro *et al.*, 1977/78; Pires *et al.*, 1982).

In conclusion, the PCR-RFLP technique implemented proved to be a reliable tool for discriminating the four member of the *maculipennis* complex to be applied in the subsequent bioecological studies of *An. atroparvus.* Sequence analysis revealed a polymormorphic site in ITS2 fragment not described before. Surprisingly, all specimens analysed were heterozygotic for this site. In spite of the attempts this abnormal situation remains to be fully understood.

As in the past, *An. atroparvus* was found to be one of the most abundant mosquito species in the region. However, one cannot exactly compare the abundance levels of this species between present days and those when malaria was an endemic disease since mosquito sampling methodology and result analysis used in this study are not fully comparable with those from previous works (Cambournac, 1942). Based solely on the results of larval

collections it could be concluded that *An. atroparvus* abundance has dramatically decreased over the times, since in no occasion "as many as 20.000 larvae per hectare" (Cambournac, 1942) have been found in our study. However, when analysing the results of adult collections, differences between past and present data are more difficult to be assessed. Although no "40,000 mosquitoes" were ever captured in any month of this study (see Figure V.20., Chapter V), this could have been possible if collections had been done in experimental rabbit houses and if all present mosquitoes had also been collected once every week (Cambournac, 1942) rather than our time-limited (usually 10 min) captures. So, even without any strong evidence, it could be assumed that Anopheline abundances nowadays are not strikingly different from those recorded in the malaria times. As to the differences observed regarding larval abundance, these could be explained by an incorrect sampling methodology (specimens were in the centre of rice fields instead of at their edges) or by the fact that Anophelines are exploiting other breeding sites beside those prospected.

Finally, of all collection methods used, IR captures was found to be most efficient method to assess *An. atroparvus* relative abundance and seasonal population dynamics. Indoor resting captures also presented the additional advantage of permitting the capture of unfed and recently fed females, necessary for parous analysis and determination of blood meal sources. For the assessment of females biting cycle patterns and man biting rates, a second collection method - HBext - must be performed instead of CDC-CO_2 traps.

Chapter V

ANOPHELES ATROPARVUS VECTORIAL CAPACITY

V.1. AIMS

The vectorial capacity (C) concept and formula, elaborated by Garret-Jones (1964b), is still one of the major tools used in the assessment of malaria epidemiology (*e.g.* Afrane *et al.*, 2006; Cano *et al.*, 2006). This so-called "classic formula" has been applied since decades, most probably due to its simplicity, which makes it easily accessible to non-specialists. It is a very straightforward concept, since it uses a relatively small number of variables that are biologically meaningful.

The vectorial capacity, opposite to what happens with the entomological inoculation rate (EIR), is not a function of the sporozoite rate, and therefore is independent of the proportion of humans that are infectious. In this sense, C may be used to describe the transmission potential of a given mosquito population, even in the absence of human *Plasmodium* carriers, and thus, the receptivity of a given area to the (re)emergence of the disease. This concept has been applied to the study of the impact of different climatic scenarios on the transmission of malaria and other vector-borne diseases, since all the variables of C are considered to be environmental-sensitive. The effect of environment/climate in malaria transmission is most evident in temperate zones where suitable conditions for disease transmission are restricted to a few months during the year. Malaria incidence rates usually follow the patterns of mosquito seasonal abundance. This chapter will focus on the results of a study which the main objective was the evaluation of an *Anopheles atroparvus* population transmission potential for malaria through the estimation of its vectorial capacity and the analysis of the effect of mosquito seasonality on this estimate.

V.2. METHODOLOGICAL CONSIDERATIONS

A longitudinal survey took place in three localities of Comporta region, Comporta, Carvalhal and Pego, between June 2001 and May 2004 (Appendix). Mosquito sampling based on indoor resting captures was carried out twice a month. In each locality, two neighbouring, low height (less than 2.1 m) animal shelters were chosen as adult mosquito collection sites (Figure V.1.). A description of these animal facilities is presented in Table V.1. Collections were carried out for periods of approximately 10 min in each collection site.

To evaluate the possibility of more than one member of the *An. maculipennis* complex being present in the study area, between June 2001 and May 2002, 30 females *per* month *per* locality, or the available number of specimens (individually preserved), were identified using the PCR-RFLP procedure described in Chapters III-IV.

All *An. atroparvus* females captured were classified according to Sella's stages, and females in stages 1 and 2 were dissected for the determination of parity rates. The insemination state of these females was analysed only in individuals captured between June 2003 and May 2004.

Table V.1. Information on collection sites from the longitudinal survey of 2001-2004 in Comporta region.

Locality	Code	Georeference and altitude	Type of construction			Host present
			Height (m)	N. of walls	Wall / roof materials	
Comporta	Co1	N 38º 22' 52.4'' W 08º 46' 57.0'' 15.5 m	2.0	3	Wood, iron net / zinc, corrugated cement	S+Ch+D
	Co2		Max.-2.1 Min.-1.7	2	Brick / zinc, corrugated cement	P
Carvalhal	Cr1	N 38º 18' 37,9'' W 08º 45' 06.0'' 7.1 m	Max.-1.7 Min.-1.0	3	Brick , wood / zinc	Rb
	Cr2		Max.-1.7 Min.-0.5	3	Brick, wood, iron net / zinc, wood	Rb+Ch+D
Pego	Pe1	N 38º 17' 45.4'' W 08º 45' 54.4'' 9.7 m	Max.-1.5 Min.-1.0	4	Brick, wood, iron net / corrugated cement	Ch
	Pe2		Max.-1.5 Min.-1.0	4	Brick, wood, , iron net / corrugated cement	Ch

Code: code reference according to Appendix . S: sheep. Ch: chicken. D: duck. P: pig. Rb: rabbit

Figure V.1. Two collections sites in Comporta region.
a: Site Co1 in Comporta locality. b:Site Pe2 in Pego.

The identification of blood meal sources was performed with freshly-fed females collected during periods of July-November of 2000 and May-July 2001, resting inside animal shelters and storage facilities. Captures took place in the three above-mentioned localities and also in Torre and Possanco (Appendix). The most common animals found inside the surveyed shelters were chickens, cows, dogs, ducks, goats, horses, pigeons, pigs, rabbits, sheep and turkeys.

To determine the biting cycle and man biting rates, all night (from 19h30 to 6h30) human landing catches were carried out six times, in the periods of June 2001, and July-

August 2004/2005. Collections were undertaken in Comporta locality in the same site where 2000's HBext collections were carried out (see Chapter IV).

Gonotrophic cycle duration (*i*) and feeding frequency (*F*) estimates are usually determined using laboratory-reared females from wild caught larvae. After emergence, females are allowed to mate and offered a daily opportunity to feed on an appropriated host. However, in the entomological surveys carried out, the number of *An. atroparvus* immature forms captured was always reduced and larvae were sparsely distributed. Therefore, the assessment of *An. atroparvus* gonotrophic cycle duration and feeding frequency was undertaken using females from a long-established colony (15 years old). The influence of sugar feeding in these estimates as well as mortality and oviposition frequency was also investigated. In this study, two groups of 29 and 33 females each were separated after emergence and allowed to mate with older males (2 days old) for a period of 72 h. To both groups was offered a daily opportunity to feed on a host (*Mus musculus* Linnaeus, 1758, CD1 strain) for a period of 30 min. To the second group of females a 10% sucrose solution was also provided between blood meals. All females were kept in individual cages (9 x 9 x 9 cm^3) under the same constant environmental conditions: temperature 26 ºC ± 1ºC; 75-80% relative humidity and 12 h/12 h light/dark periods. The blood meals and ovipositions were recorded daily for each female, until its death. The mammal host was anesthetised with Rompun 2%® (Bayer Healthcare) and Imalgène1000® (Merial) according to the dosages and administration methods recommended (Hedenqvist & Hellebrekers, 2003). Maintenance and manipulation of mammals involved were carried out according to legal dispositions[12].

V.3. MOSQUITO ABUNDANCE, POPULATION STRUCTURE AND SEASONALITY

V.3.1. MOSQUITO COLLECTIONS RESULTS AND ABUNDANCE SPATIAL DIFFERENTIATION

A total of 15,636 mosquitoes, belonging to eight of the species already recorded during the preliminary studies, were collected during the 2001-2004 indoor resting captures (Table V.2.). Thirteen thousand five hundred and twenty one mosquitoes were morphologically identified as *An. maculipennis s.l.* Of these, 483 females were processed by PCR-RFLP analysis and identified as *An. atroparvus.*

[12] Directive for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (86/609/EEC) and the national legislation ("Decreto-Lei nº 92/95" of 12/09; "Decreto-Lei nº 129/ 92" of 06/06; "Portaria nº 1005/92" of 23/10; "Portaria nº 1131/97" of 07/11).

Table V.2. Number of mosquitoes, *per* species, captured during the 2001-2004 survey in IR collections.

	Females	**Males**	**TOTAL**
An. atroparvus	11,538 (73.791)	1,983 (12.682)	**13,521 (86.474)**
Cx. pipiens	736 (4.707)	32 (0.205)	**768 (4.912)**
Cx. theileri	936 (5.986)	34 (0.217)	**970 (6.204)**
Cs. univittatus	142 (0.908)	17 (0.109)	**159 (1.017)**
Cs. annulata	23 (0.147)	0 (0.000)	**23 (0.147)**
Cs. longiareolata	39 (0.249)	7 (0.045)	**46 (0.294)**
Oc. caspius s.l.	122 (0.780)	9 (0.058)	**131 (0.838)**
Oc. detritus s.l.	12 (0.077)	1 (0.006)	**13 (0.083)**
Unidentified specimens	3 (0.019)	2 (0.013)	**5 (0.032)**
TOTAL	**13,551 (86.665)**	**2,085 (13.335)**	**15,636 (100.00)**
N. of collections carried out			**407**
Collection effort			**55.33 h**

N.:number. In brackets: percentage according to the total number of mosquitoes collected.

Table V.3. Locality, date of collection and number of *Anopheles maculipennis s.l.* females, processed by PCR-RFLP.

	2001						**2002**						**Total**
	Jun	**Jul**	**Aug**	**Oct**	**Sep**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	
Comporta	30	30	30	30	30	0	30	12	0	0	0	0	192
Carvalhal	30	30	28	30	4	5	21	13	10	0	4	30	205
Pego	23	30	8	25	0	0	0	0	0	0	0	0	86
Total	83	90	66	85	34	5	51	25	10	0	4	30	483

The origin of these 483 females, presented in Table V.3., covered the three surveyed localities and all the months of the year with the exception of March for which no specimen was available for molecular studies. This methodology was adopted to account for possible geographic or seasonal differences between the complex members. In total, adding the 43 *An. maculipennis s.*l. specimens molecularly analysed in 2001-2002, 526 mosquitoes captured in Comporta region and processed by PCR-RFLP were all identified as *An. atroparvus*. Given this sample size, the maximum likely frequency of other member of the *maculipennis* to be present is 0.6% or 0.9%, according to the respective confidence limits of 95% or 99% (Post & Millest, 1991).

As in the preliminary study, *An. atroparvus*, totalising 86% of all specimens collected, was the most common species found resting inside animal shelters, followed by *Culex theileri* and *Cx. pipiens* representing, respectively, 6% and 5% of the catches.

Table V.4. Productivity of collection sites regarding the total number of mosquitoes and *Anopheles atroparvus* specimens collected.

Locality	Code	N. of collections*	Collection effort (h)	N. of Culicids	N. of *An. atroparvus*	Productivity for Culicids	Productivity for *An. atroparvus*
Comporta	Co1	66	10.35	2,176	1,810	210	175
	Co2	81	2.79	1,726	1,206	619	432
Carvalhal	Cr1	55	9.00	3,172	3,061	352	340
	Cr2	67	10.25	2,606	2,464	254	240
Pego	Pe1	64	10.35	2,808	2,402	271	232
	Pe2	64	10.45	2,354	1,893	225	181
TOTAL		397	53.19	14,842	12,836	-	-

Code: collection sites code references according to Appendix. N.: number.*number of collections considered in this analysis.

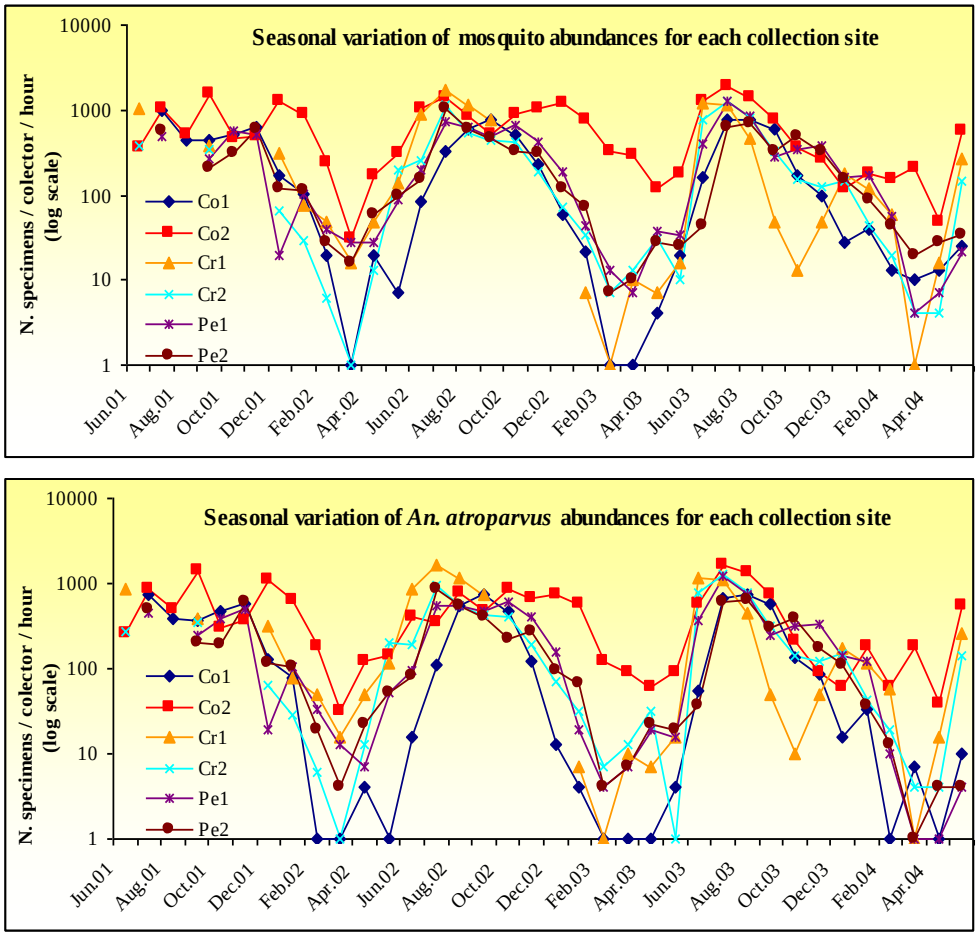


Figure V.2. Seasonal variation of Culicids and *Anopheles atroparvus* abundances for each collection site, between June 2001 and May 2004.

Co1, Co2, Cr1,Cr2, Pe1, Pe2: collection sites, according to Appendix.

The productivity of the six collection sites was different regarding both the total number mosquitoes and the number of *An. atroparvus* specimens collected (Table V.4.). The seasonal patterns of Culicid and *An. atroparvus* abundance, determined for each place (*i.e.* collection site), although presenting the same general trend, showed discrepancies on the longitudinal variation of these estimates (Figure V.2.).

To further investigate these differences, pairwise comparisons of mosquito and *An. atroparvus* abundances between the two collection sites of each locality were carried out using Wilcoxon signed-ranks test (Table V.5.). Overall pairwise comparison across the six places was performed using Friedman test (Table V.6.).

Table V.5. Pairwise comparison between the two collection sites within each locality regarding the abundance (number of specimens by collection effort) of Culicids and *Anopheles atroparvus* (females and males) captured by IR catches.

		Culicids						***An. atroparvus***					
		Comporta		**Carvalhal**		**Pego**		**Comporta**		**Carvalhal**		**Pego**	
Sites		**Co1**	**Co2**	**Cr1**	**Cr2**	**Pe1**	**Pe2**	**Co1**	**Co2**	**Cr1**	**Cr2**	**Pe1**	**Pe2**
Descriptive statistics	***n***	66	66	54	54	64	64	66	66	54	54	64	64
	$\overline{\mathbf{X}}$	221.55	651.23	345.81	266.93	272.42	230.52	184.24	460.10	333.59	253.49	233.81	186.50
	Md.	39.00	480.00	72.00	45.00	117.00	120.00	21.00	360.00	72.00	45.00	101.14	66.33
	s	293.77	562.81	520.82	389.44	320.04	265.16	274.89	446.35	502.20	373.71	298.27	240.50
	Sk.	1.34	1.05	1.77	1.65	1.49	1.50	1.50	1.15	1.81	1.70	1.67	1.52
	Ku.	0.68	0.46	2.70	1.60	1.92	1.85	1.08	0.69	2.95	1.81	2.76	1.45
K-S test*		0.260 d.f.=66 ***P*<0.001**	0.144 d.f.=66 ***P*=0.002**	0.283 d.f.=54 ***P*<0.001**	0.247 d.f.=54 ***P*<0.001**	0.199 d.f.=64 ***P*<0.001**	0.199 d.f.=64 ***P*<0.001**	0.281 d.f.=66 ***P*<0.001**	0.151 d.f.=66 ***P*=0.001**	0.286 d.f.=54 ***P*<0.001**	0.249 d.f.=54 ***P*<0.001**	0.217 d.f.=64 ***P*<0.001**	0.219 d.f.=64 ***P*<0.001**
Levene test**		15.915 $d.f._1$=1 $d.f._2$=130 ***P*<0.001**		0.765 $d.f._1$=1 $d.f._2$=106 *P*=0.384		0.930 $d.f._1$=1 $d.f._2$=126 *P*=0.337		11.356 $d.f._1$=1 $d.f._2$=130 *P*=**0.001**		0.843 $d.f._1$=1 $d.f._2$=106 *P*=0.361		1.169 $d.f._1$=1 $d.f._2$=126 *P*=0.282	
Wilcoxon test***		-6.535[a] ***P*<0.001**		-2.006[b] ***P*=0.045**		-1.605[b] ***P*=0.108**		-6.020[a] ***P*<0.001**		-2.048[b] ***P*=0.041**		-2.024[b] ***P*=0.043**	

n: number of observations. $\overline{\mathbf{X}}$: mean. **Md.**: median. *s*: standard deviation. **Sk.**: skewness. **Ku.**: kurtosis. a: based on negative ranks. b: based on positive ranks. K-S*: Kolmogorov-Smirnov's with Lilliefors significance correction. **: based on median. ***: Wilcoxon signed test, 2-tailed. Underlined: significant at the 0.05 level. Bold: significant at level < 0.01.

Pairwise comparison of *An. atroparvus* abundances confirmed the existence of significant differences between the two collection sites in each locality (Table V.5.). Analysis of Culicid abundance showed similar results with the exception of Pego's collection places, between which no differences were found. Overall pairwise comparison of the six sites showed significant differences regarding both Culicids and *An. atroparvus* abundance (Table

V.6.). This heterogeneity between samples of what would be expected to be the same mosquito population may be a consequence of the variability of resting sites, *e.g.* type of construction or host present. To minimize the effect of this factor in the outcome of the mosquito bionomic studies, it is usually advised to proceed with the highest number of samples possible and to analyse results according to mean values.

Table V.6. Pairwise comparison between the six collection sites regarding the abundance (number of specimens by collection effort) of Culicids and *Anopheles atroparvus* (females and males) captured by IR catches.

		Culicids						***An. atroparvus***					
		Comporta		**Carvalhal**		**Pego**		**Comporta**		**Carvalhal**		**Pego**	
	Sites	**Co1**	**Co2**	**Cr1**	**Cr2**	**Pe1**	**Pe2**	**Co1**	**Co2**	**Cr1**	**Cr2**	**Pe1**	**Pe2**
Descriptive statistics	***n***	52	52	52	52	52	52	52	52	52	52	52	52
	$\overline{X}$	186.40	552.10	186.98	268.64	248.65	216.00	156.63	377.62	324.21	258.14	212.04	174.81
	Md.	27.00	346.50	63.00	45.00	89.14	72.00	6.00	180.00	63.00	45.00	63.00	48.00
	s	290.90	544.95	519.47	394.45	330.17	282.19	278.20	439.43	506.80	379.28	307.46	253.63
	Sk.	1.62	1.34	1.88	1.64	1.73	1.63	1.85	1.62	1.87	1.66	1.93	1.63
	Ku.	1.43	1.10	3.13	1.52	2.60	1.97	2.20	2.07	3.11	1.63	3.56	1.61
K-S test*		0.325 d.f.=52 ***P*<0.001**	0.211 d.f.=52 ***P*<0.001**	0.298 d.f.=52 ***P*<0.001**	0.248 d.f.=52 ***P*<0.001**	0.248 d.f.=52 ***P*<0.001**	0.229 d.f.=52 ***P*<0.001**	0.341 d.f.=52 ***P*<0.001**	0.195 d.f.=52 ***P*=0.001**	0.303 d.f.=52 ***P*<0.001**	0.248 d.f.=52 ***P*<0.001**	0.245 d.f.=52 ***P*<0.001**	0.264 d.f.=52 ***P*<0.001**
Levene test**		2.679 $d.f._1$=5 $d.f._2$=306; $\underline{P=0.022}$						2.057 $d.f._1$=5 $d.f._2$=306; *P*=0.071					
Friedman test		75.642 d.f.=5; ***P*<0.001**						42.705 d.f.=5; ***P*<0.001**					

n: number of observations. $\overline{X}$: mean. **Md.**: median. *s*: standard deviation. **Sk.**: skewness. **Ku.**: kurtosis. K-S*: Kolmogorov-Smirnov's test with Lilliefors significance correction. **: based on median. Underlined: significant at level < 0.05. Bold: significant at level < 0.01.

To determine if there was any spatial differentiation between localities regarding the mosquitoes in general, and the *An. atroparvus* population in particular, a Kruskal-Wallis one-way analysis of variance was carried out for comparison, between localities, of monthly mean abundances (Table V.7.).

Although differences were found regarding the Culicid population, results showed no spatial differentiation between Comporta, Carvalhal and Pego, regarding *An. atroparvus* abundances, in both the analysis of the two genders together or in the analysis of the female population only. Thus, and with respect to *An. atroparvus*, since the samples collected in each locality seemed to be extracted from the same statistical population, the forthcoming

statistical analyses were carried out using the *An. atroparvus* monthly mean abundances, calculated according to results of the catches undertaken in the six collection sites.

Table V.7. Comparison of Culicids and *Anopheles atroparvus* monthly mean abundances, between localities, in IR captures.

		Culicids			***An. atroparvus* (females and males)**			***An. atroparvus* females**		
		Comporta	**Carvalhal**	**Pego**	**Comporta**	**Carvalhal**	**Pego**	**Comporta**	**Carvalhal**	**Pego**
Descriptive statistics	***n***	36	36	36	36	36	36	36	36	36
	$\overline{X}$	458.46	303.64	267.31	343.26	290.40	224.61	316.93	271.94	163.08
	Md.	501.04	147.68	164.25	255.25	147.68	125.25	249.50	143.25	110.79
	s	363.15	379.57	265.29	321.70	362.38	241.71	296.65	340.51	169.65
	Sk.	0.715	1.54	1.04	0.99	1.53	1.13	1.02	1.55	0.98
	Ku.	-0.142	1.77	0.26	0.11	1.64	0.55	0.212	1.72	-0.068
K-S* test		0.174 d.f.=36 ***P*=0.007**	0.270 d.f.=36 ***P*<0.001**	0.167 d.f.=36 ***P*=0.012**	0.170 d.f.=36 ***P*=0.010**	0.266 d.f.=36 ***P*<0.001**	0.184 d.f.=36 ***P*=0.003**	0.165 d.f.=36 <u>*P*=0.014</u>	0.270 d.f.=36 **P<0.001**	0.168 d.f.=36 ***P*=0.011**
Levene test**		1.371 $d.f._1$=2 $d.f._2$=105; *P*=0.258			1.005 $d.f._1$=2 $d.f._2$=105; *P*=0.369			3.084 $d.f._1$=2 $d.f._2$=105; *P*=0.05		
Kruskal-Wallis test		8.592 d.f.=2; <u>*P*=0.014</u>			3.825 d.f.=2; *P*=0.148			5.989 d.f.=2; *P*=0.05		

n: number of observations. $\overline{X}$: mean. **Md.**: median. *s*: standard deviation. **Sk.**: skewness. **Ku.**: kurtosis. K-S*: Kolmogorov-Smirnov's test with Lilliefors significance correction. **: based on median. Underlined: significant at level < 0.05. Bold: significant at level < 0.01.

V.3.2. ANALYSIS OF *ANOPHELES ATROPARVUS* PARITY AND INSEMINATION RATES

A total of 5,969 females, 2,974 (50%) in Sella's stage 1 and 2,995 (50%) in stage 2, were dissected for observation of ovaries (Table V.8.). Of these, 4,795 (80%) were classified as nulliparous or parous according to the tracheoles coiling state. As for the remaining 1,174 (20%) specimens, no classification was achieved due to the ovaries condition that showed development stages beyond Christopher's stage II or were destroyed by the conservation or dissection procedures. No statistical differences were found between specimens in Sella's stage 1 and 2 regarding the monthly percentage of parous females (Table V.9.).

Two thousand three hundred and ninety two *An. atroparvus* females, 1,093 in Sella´s stage 1 and 1,299 in Sella's stage 2, were dissected for spermatheca observation. In 146 (6%) females, the spermatheca was destroyed by either the conservation or dissection procedures. The observation of the remaining 2,246 (94%) individuals revealed the presence of only 46 (2%) virgin females. The insemination percentages according to parity and Sella's stage (1 and 2) are presented in Table V.10. Statistical differences were found between females in the two Sella´s stages (X^2_y=18.38, d.f.=1, *P*<0.001) with fed females presenting an higher percentage of insemination.

Table V.8. *Anopheles atroparvus* female ovaries dissection and parity analysis.

		Females in Sella's stage 1		Females in Sella's stage 2		TOTALS	
		N.	%	N.	%	N.	%
Dissected		2,974	49.8[a]	2,995	50.2[a]	5,969	100
Undetermined		337	11.3[b]	837	27.9[b]	1,174	19.7[a]
Classified	Total	2,637	88.7[b]	2,158	72.1[b]	4,795	80.3[a]
	Nulliparous	1,969	74.7[c]	1,358	62.9[c]	3,327	69.4 [d]
	Parous	668	25.3[c]	800	37.1[c]	1,468	30.6 [d]

N.: number. Dissected: females dissected. Undetermined: females which ovarian condition did not allowed the determination of the parous state. Classified: females classified as nulliparous or parous according to the cooling degree of ovarian tracheoles. [a]: according to the total number of females dissected. [b]: according to the number of dissected females in each Sella's stage. [c]: according to the number of classified females in each Sella's stage. [d]: according to the number of classified females.

Table V.9. Comparison of unfed and freshly fed *Anopheles atroparvus* monthly percentage of parous females.

	Descriptive statistics						K-S*	Levene's test**	t-test (2-tailed)
	n	$\overline{X}$	Md.	*s*	Sk.	Ku.			
Females in Sella's stage 1	35	31.45	25.00	27.07	0.84	0.43	0.123 d.f.=35; *P*=0.200[a]	1.455 d.f.=1 *P*=0.232	-0.455 d.f.=67 *P*=0.651
Females in Sella's stage 2	34	34.58	27.00	30.13	0.01	-1.23	0.147 d.f.=34; *P*=0.061		

n: number of observations. $\overline{X}$: mean. **Md.**: median. *s*: standard deviation. **Sk.**: skewness. **Ku.**: kurtosis. K-S*: Kolmogorov-Smirnov's test with Lilliefors significance correction **: based on mean. [a]: lower bound of the true significance.

Table V.10. Results of *Anopheles atroparvus* female spermatheca dissection and insemination percentages according to parity and Sella´s 1 and 2 stages.

				Classified		TOTALS					
				N.	%	N.			%		
Classified	Virgin females		stg 1	36	78	2,246	46		94	2	
			stg 2	10	22						
	Inseminated females	Nulliparous	stg 1	633	54		2,200	1,172		98	53
			stg 2	539	46						
		Parous	stg 1	260	39			675			31
			stg 2	415	61						
		Undetermined parity	stg 1	104	29			353			16
			stg 2	249	71						
Undetermined insemination status			stg 1	60	41	146			6		
			stg 2	86	59						
Dissected			stg 1	1,093	46	2,392			100		
			stg 2	1,299	54						

Classified: females classified according to the parameter mentioned. Dissected: females dissected. N.: number. stg 1 / 2: females in Sella´s stage 1 or 2 accordingly.

V.3.3. SEASONAL VARIATION OF *ANOPHELES ATROPARVUS* ABUNDANCE, PARITY AND INSEMINATION RATES

The monthly mean abundance rates of *An. atroparvus* females, males and both genders, recorded during the three years survey are presented in Figure V.3. The annual abundance patterns observed during this period were similar to the ones recorded in the preliminary study, with abundance peaks in July-August and the lowest values registered during March-April. *Anopheles atroparvus* females were caught all year round but males tend to disappear in December or January for periods of one to three months, reappearing between February-March.

Parous rates showed an annual pattern with a sharp peak in January-February and a second period of high values between April-June and in August-September, after which rates decline (Figure V.3.). The April-September plateau seems to be formed by the overlap of two other peaks, one in May-June and another in September.

Insemination rates showed little variation during the year with the lowest value (96%) recorded during June (Figure V.3.).

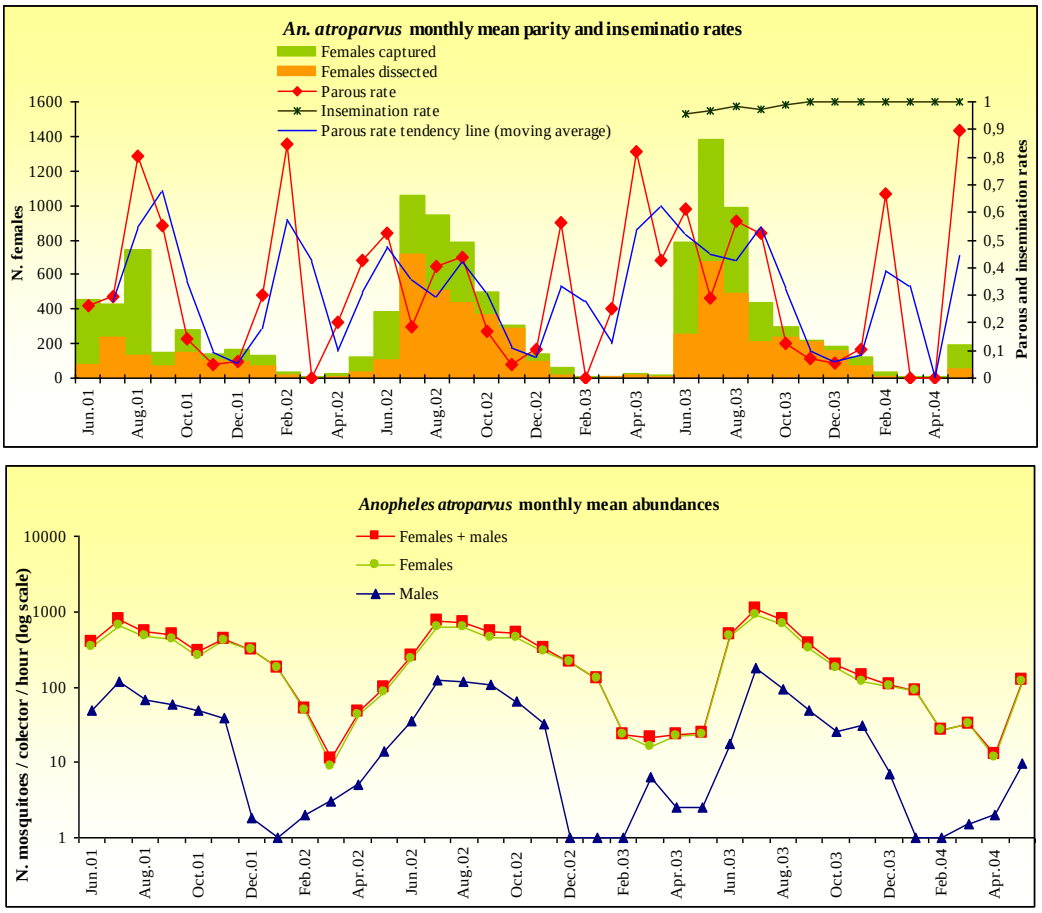


Figure V.3. Seasonal patterns of *Anopheles atroparvus* abundances and parous rates during the period June 2001-May 2004, and insemination rates variation between June 2003 and May 2004.

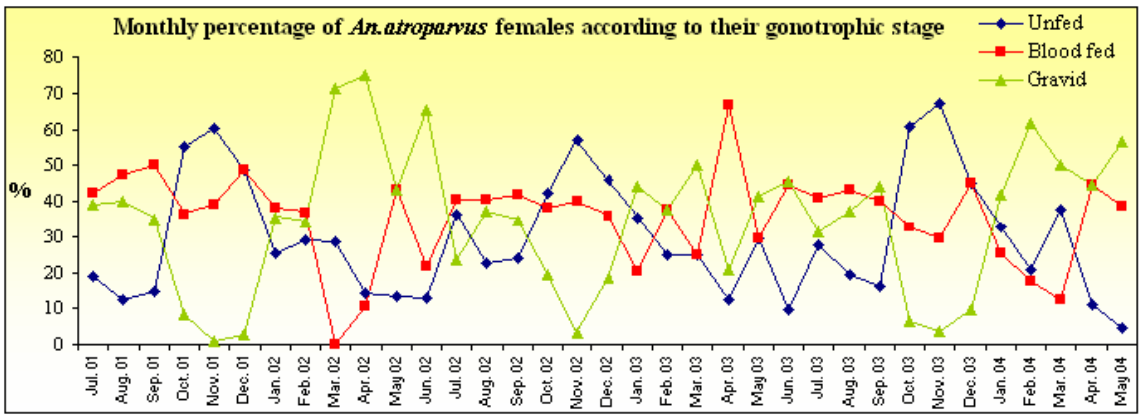


Figure V.4. Seasonal patterns of *Anopheles atroparvus* females in different gonotrophic stages.
%: Percentage computed for the total number of females captured in each month. Unfed: females in Sella´s stage 1. Blood fed: females in Sella´s stage 2-3. Gravid: females in Sella´s stage 4-7.

Females in all gonotrophic stages were captured all year round but the seasonal variation of the different stages did not present a well defined pattern (Figure V.4.). The highest monthly percentages of gravid females, corresponding to mosquitoes in Sella´s stages 4 to 7, were recorded in months February to April, when the percentages of blood fed females (females in Sella´s stage 2-3) were usually low. The lowest percentages of gravid females were recorded in November and coincided with the percentage peaks of unfed females (Sella´s stage 1).

V.3.4. *ANOPHELES ATROPARVUS* ABUNDANCE AND ITS RELATION WITH METEOROLOGICAL PARAMETERS

To detect anomalies in percentage of relative humidity at 9 UTC, daily precipitation and mean daily temperature of the sampling period in comparison to the climatological series of 1981-2000, monthly averages of the three parameters were subtracted to the 20 years mean values of each month (Figure V.5.). The period from November 2001 to March 2002 was found to be the most peculiar, with records of both the lowest and the highest differences in temperature and precipitation in reference to 1981-2000 averages. The winters of 2001-2002 and 2003-2004 where very dry, with precipitation values below average during the months of November to February.

To determine if there was any relation between the three selected meteorological parameters and *An. atroparvus* abundance, scatter plots of the monthly mean number of specimens against the monthly mean values of these variables were constructed and presented in Figure V.6.

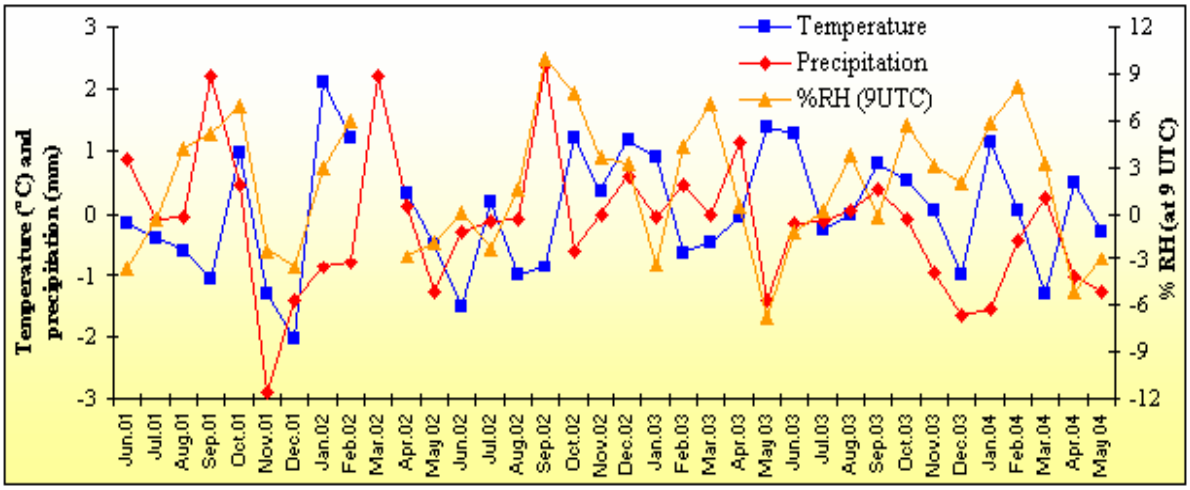


Figure V.5. Anomalies of monthly averages of mean daily temperature, daily precipitation and percentage of relative humidity at 9 UTC for the period June 2001-May 2004, referred to 1981-2000 climatological series.

Based only on graphics observation, *An. atroparvus* monthly mean abundances seemed to be independent from %RH (at 9 UTC) and precipitation. On the other hand, a linear trend was observed when abundance values were plotted against monthly average records of daily mean temperatures (MADMTemp) especially when these were above 15ºC.

Linear regression analyses, between the *An. atroparvus* monthly mean abundances of females, males and both genders and monthly averages of daily mean temperatures of the hottest months (May to October, with MADMTemp above 15ºC) were carried out and the regression equations obtained presented in Figure V.7.

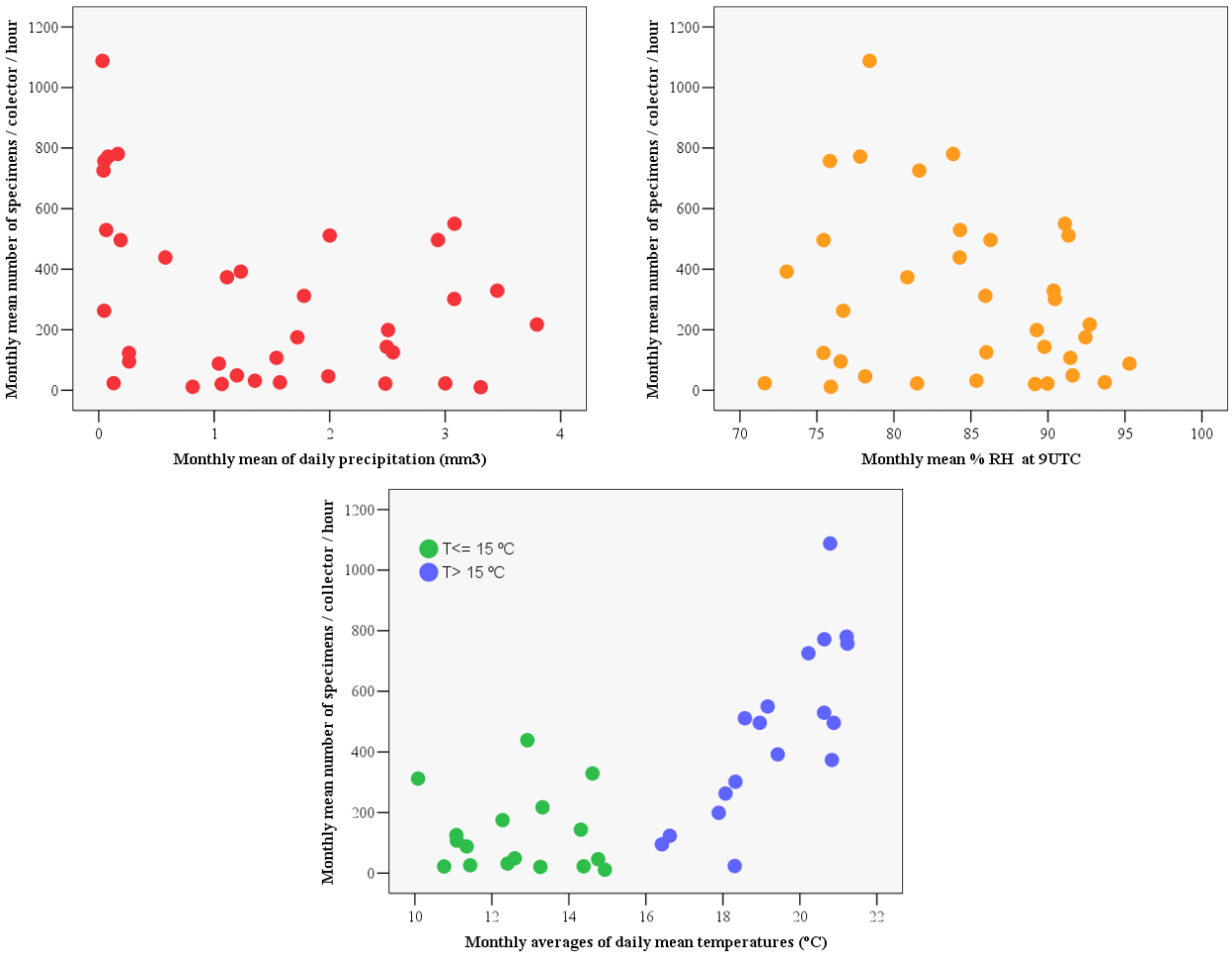


Figure V.6. Scatter plots of *Anopheles atroparvus* monthly mean abundances against the variables mentioned in each graph.

a) $Y = -2339.33 + 145.30MADMTemp$

b) $Y = -2015.08 + 125.21MADMTemp$

c) $Y = -324.25 + 20{,}09MADMTemp$

Figure V.7. Linear regression equations that relate temperature with *Anopheles atroparvus* abundance. MADMTemp: monthly average of daily mean temperatures. a: for *An. atroparvus* (females+males) abundance estimation. b: for *An. atroparvus* female abundance estimation. c: for *An. atroparvus* male abundance estimation.

Coefficients[a] — a

Model		Unstandardized Coefficients B	Unstandardized Coefficients Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	-2339,331	538,086		-4,348	,000
	MADMTemp	145,296	27,737	,795	5,238	,000

a. Dependent Variable: Female + Male monthly mean abundances

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,795[a]	,632	,609	176,329

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	853181,1	1	853181,149	27,441	,000[a]
	Residual	497472,3	16	31092,019		
	Total	1350653	17			

a. Predictors: (Constant), MADMTemp

b. Dependent Variable: Female + Male monthly mean abundances

Coefficients[a] — b

Model		Unstandardized Coefficients B	Unstandardized Coefficients Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	-2015,083	438,099		-4,600	,000
	MADMTemp	125,207	22,583	,811	5,544	,000

a. Dependent Variable: Female monthly mean abundances

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,811[a]	,658	,636	143,564

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	633561,4	1	633561,412	30,740	,000[a]
	Residual	329769,4	16	20610,588		
	Total	963330,8	17			

a. Predictors: (Constant), MADMTemp

b. Dependent Variable: Female monthly mean abundances

Coefficients[a] — c

Model		Unstandardized Coefficients B	Unstandardized Coefficients Std. Error	Standardized Coefficients Beta	t	Sig.
1	(Constant)	-324,248	113,847		-2,848	,012
	MADMTemp	20,089	5,868	,650	3,423	,003

a. Dependent Variable: Male monthly mean abundances

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	,650[a]	,423	,387	37,307

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	16310,443	1	16310,443	11,719	,003[a]
	Residual	22269,357	16	1391,835		
	Total	38579,800	17			

a. Predictors: (Constant), MADMTemp

b. Dependent Variable: Male monthly mean abundances

Figure V.8. SPSS outputs of linear regression analyses between monthly averages of daily mean temperatures of the study period months May to October and *Anopheles atroparvus* monthly mean abundances of females (b), males (c) and both genders (a).

R: multiple correlation coefficients. R square: coefficient of determination. B / (constant): *Y* intersept. B / MADMTemp: regression coefficient. t: t test (two-tailed), d.f.=16. F: ANOVA statistics. Sig.: *P* values.

All the models, according to *t* statistics, were significant ($P<0.05$), thus rejecting the null hypothesis that there is no relationship between the dependent (mosquito abundances) and the independent (MADMTemp) variable. Nevertheless, to analyse the models fit, multiple correlation coefficients (R), coefficients of determination (R square) and adjusted R square were estimated. An ANOVA test was also carried out, to determine if the regression models were significant (Figure V.8.).

Results, presented in Figure V.8., showed that the highest multiple correlation coefficient was found between females monthly mean abundances and MADMTemp, but even for this model the proportion of variation of the dependent variable explained by the regression model (R square) is only 0.66. Similarly, although ANOVA statistics were found significant for all models, the values of the regression sum of the squares estimated were not much higher than those of the residual sum of squares. This indicates that a considerable proportion of the variation of the dependent variables was not accounted for by the models.

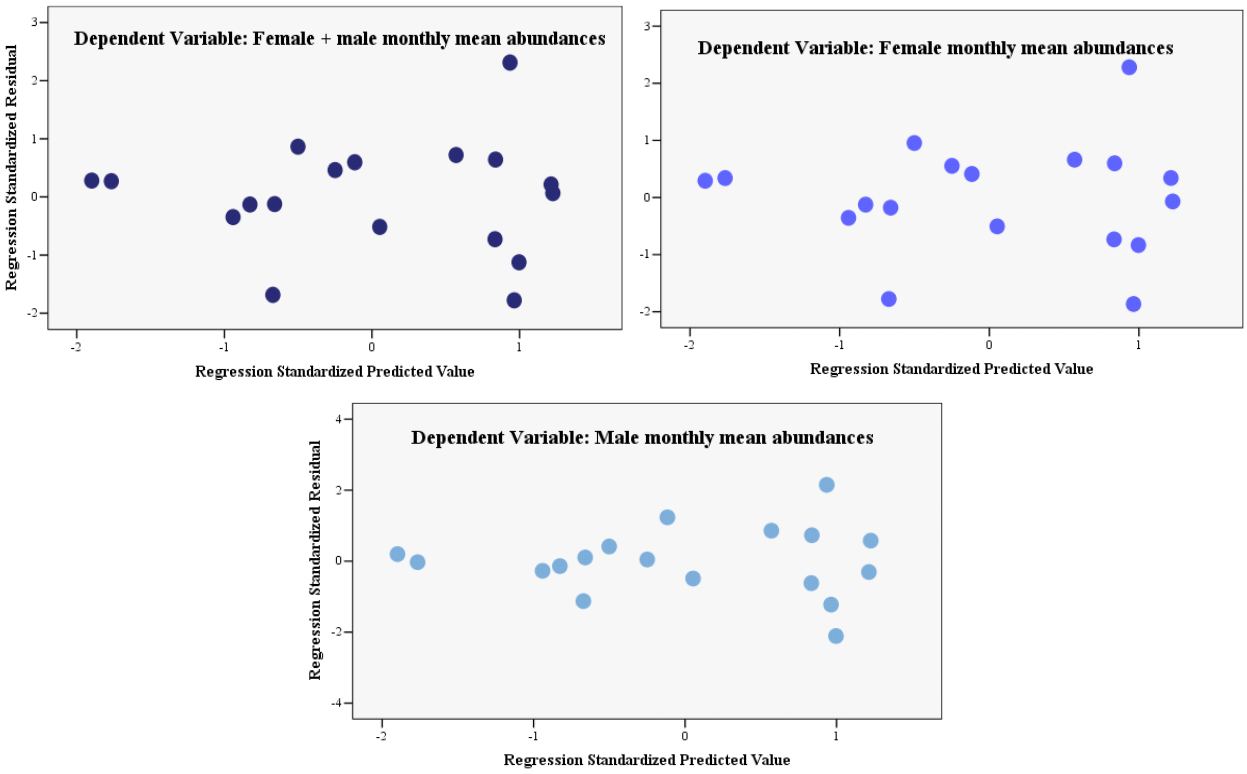


Figure V.9. Scatter plots of regression standardized residuals were against regression standardized predicted values of the mentioned dependent variables.

For validating models, regression standardized residuals were plotted against regression standardized predicted values. The graphics obtained (Figure V.9.) showed that, in all cases, residuals were not randomly distributed around zero. The graphic analysis indicated that the variance of regression residuals was not constant and thus one of the assumptions required for regression models application was not validated.

V.4. *ANOPHELES ATROPARVUS* FEEDING BEHAVIOUR

V.4.1. BLOOD MEAL IDENTIFICATION AND HUMAN BLOOD INDEX

From 671 *An. atroparvus* analysed blood meals, 646 (96%) were obtained from females captured resting inside 59 animal shelters and 25 (4%) from specimens collected in 3 storage houses. Blood fed *An. atroparvus* females were neither collected in outdoor resting captures nor in indoor resting collections performed in households. All 671 blood meals were tested for the presence of the nine IgGs (chicken, cow, dog, goat/sheep, horse/donkey human, pig, rabbit and rat/mouse). Results are showed in Table V.11.

Table V.11. *Anopheles atroparvus* blood meal sources in Comporta region.

	N.	%		N.	%		N.	%
Single feeds	**623**	**92.85**	**Mixed feeds**	**26**	**3.87**			
Chicken	15	2.24	Human+Chicken+Goat	1	0.15			
Cow	45	6.71	Human+Goat	1	0.15			
Dog	7	1.04	Human+Pig	1	0.15			
Goat/sheep	179	26.68	Human+Rabbit	1	0.15			
Horse/donkey	27	4.02	Pig+Cow	2	0.30			
Human	0	0.00	Pig+Dog	1	0.15	**Negative/ feeds in other hosts**	**22**	**3.28**
Pig	227	33.83	Pig+Goat	9	1.34			
Rabbit	123	18.33	Pig+Horse	1	0.15			
Rat/mouse	0	0.00	Pig+Rabbit	3	0.45			
			Rabbit +Chicken	5	0.75			
			Rabbit+Goat	1	0.15			

N.: number of blood meals.%: percentage according to the total number of blood meals analysed.

Twenty two blood meals (3%) were found negative for all the antibodies assayed and 26 (4%) were mixed meals, including a Human/Goat/Chicken triple feed. No rodent blood meals were identified. Pigs were found to be the predominant hosts with 36% of the blood meals positive for the respective IgG, followed by goat/sheep (29%) and rabbits (20%). In 90% of females the blood source agreed with the host present at the collection site.

Only four human feeds were detected and all were mixed meals. A human blood index (*HBI*) of 0.006 was therefore estimated for Comporta region.

V.4.2. BITING ACTIVITY AND MAN BITING RATES

A total of 13,383 mosquitoes were collected in the six all-night HBext collections. Of these, only 52 specimens (0.39%), all females, were identified as *An. atroparvus*, and only two *Culex theileri* individuals were males (0.01%). Seven of the nine species recorded in the study area were collected: *Cx. theileri* and *Ochlerotatus caspius s.l.* represented 65.16% and 33.03% of all catches, respectively; *Cx. pipiens* (1.34%), *Oc. detritus s.l.* (0.04%), *Cx. univittatus* (0.01%) and *Culiseta annulata* (0.01%) totalised the remaining 1.41% of the individuals caught. No specimens of *Cx. impudicus* or *Cs. longiareolata* were captured during these HBext collections.

The biting cycle of *An. atroparvus* and of the remaining species for the six nights of collection are presented Figure V.10. In Figure V.11. are presented the biting cycles of *An. atroparvus* and other species based on the mean number of mosquitoes collected per hour in seven all-night HBext collections performed at Comporta locality, including the one undertaken in July 2000.

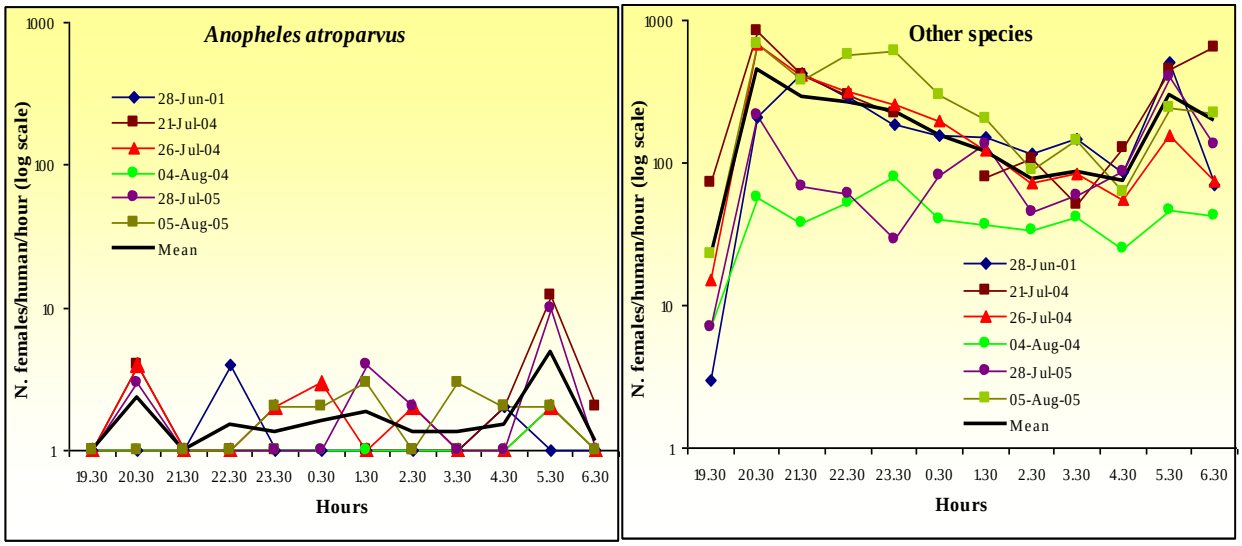


Figure V.10. Biting cycles of *Anopheles atroparvus* and other species recorded in Comporta, at the mentioned dates.

Computing all-night HBext captures, including the one carried out in 2000, an overall biting rate of 2,252 bites *per* person *per* night was recorded for Comporta locality, resulting mainly from the combined biting activity of *Oc. caspius s.l.* and *Cx. theileri* females. *Anopheles atroparvus* seems to be a crepuscular species with two biting peaks, one at dusk,

around 20h30, and the second at dawn, at 5h30 (Figure V.11.). This species, being inactive during the day (see Figure IV.6, Chapter IV), may continue its host seeking activity during the whole night, presenting sometimes a not well defined biting pattern (Figure V.10). For *An. atroparvus* the man biting rate (*ma*), estimated for the months of June-August when abundances are highest, was of 13 bites *per* human *per* night, a value by far lower than the number computed for the group of the remaining species.

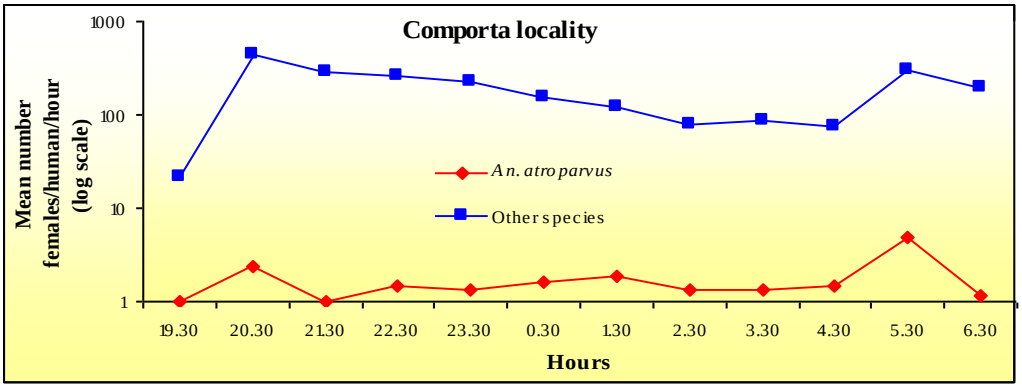


Figure V.11. Biting cycles of *Anopheles atroparvus* and other species recorded in Comporta based on mean number of females collected *per* hour in the seven collections performed in July 2000, June 2001, July-August 2004 and 2005.

V.5. LABORATORY ESTIMATES

V.5.1. DURATION OF GONOTROPHIC CYCLE AND FEEDING FREQUENCY

In the group of 29 females fed only with blood, four died before having any meal and two before laying any egg batch, in spite of having taken a blood meal. In the group of 33 females fed with blood and with access to a 10% sucrose solution, one died without blood feeding and another died without ovipositing.

The mean length, in days, of the first (i_0) and subsequent gonotrophic cycles (i_n), as well as the percentage of parous females and the mean number of egg batches *per* parous females, are presented in Figure V.12. and Table V.12.

Results showed that females deprived of sugar have a first gonotrophic cycle (i_0) significantly longer (Mann-Whitney test, 2-tailed, U=225.00, P=0.019) than those with free access to a 10% sucrose solution. For the mean duration of subsequent cycles no difference was found between the two groups (Mann-Whitney test, 2-tailed, U=8943.00, P=0.971).

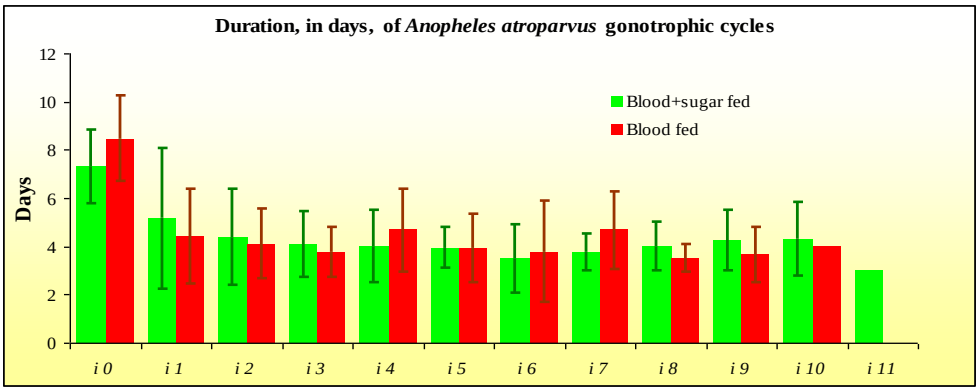


Figure V.12. Gonotrophic cycles (*i*) mean duration, in days, of two groups *Anopheles atroparvus* females subject to different diets.

Error bars represent standard deviation

Note: Columns i 10 (Blood fed group) and i 11 (Blood +sugar group) represent single observations.

Table V.12. Number and percentage of females that laid eggs, mean number of egg batches per parous female, gonotrophic cycles mean duration (in days) and blood feeding frequency of the two groups of females submitted to different food diets.

Diet	N.	N. and (%) parous females	$\overline{X}$ egg batches	i_0 duration		*i* duration		Feeding frequency
				$\overline{X}$	*s*	$\overline{X}$	*s*	
Blood +sugar	33	31 (93.4%)	6.3	7.3	1.5	4.2	1.8	0.37
Blood	29	23 (79.3%)	5.8	8.5	1.8	4.1	1.6	0.57

N.:number of females. i_0: first gonotrophic cycle. *i*: subsequent gonotrophic cycles. $\overline{X}$: mean.

The percentage of females that laid at least one egg batch, although higher in the group of sugar+blood fed females, was not significantly different between diet-groups (X^2_y=1.78, d.f.=1, *P*=0,182). A similar result was obtained for the mean number of ovipositions *per* parous female (Mann-Whitney test, 2-tailed, *U*=323.50, *P*=0.561).

Blood feeding frequency (*F*) was higher in the group fed only with blood (Table V.12.). Females deprived of sugar tend to take a blood meal every two days, whereas sugar fed females take a blood meal every three-days period. These differences were found to be significant when comparing individual feeding frequency between females of each group (Table V.13.).

Table V.13. Comparison of individual feeding frequencies of females submitted to different food diets.

Diet	*n*	Shapiro-Wilk test	Levene test*	t test (2-tailed)
Blood +sugar	32	0.934 d.f.=32; *P*=0.051	0.389 d.f.$_1$=1 d.f.$_2$=55; P=0.535	*t*= 7.164 d.f.=55 ***P*<0.001**
Blood	25	0.933 d.f.=25; *P*=0.100		

n: number of observations. *: based on median.

Anopheles atroparvus females fed only with blood are almost completely synchronous regarding the intake of the first blood meal. Eighty percent (20/25) of all females that took at least one blood meal, fed for the first time in their second day of adult life and the remaining 20% (5/25) in the third day (Figure V.13.). Females that fed on sugar (Figure V.13.), although starting also blood feeding in the second day, showed a more extended distribution with a maximum of 47% of first feeds (15/32) in the fourth day.

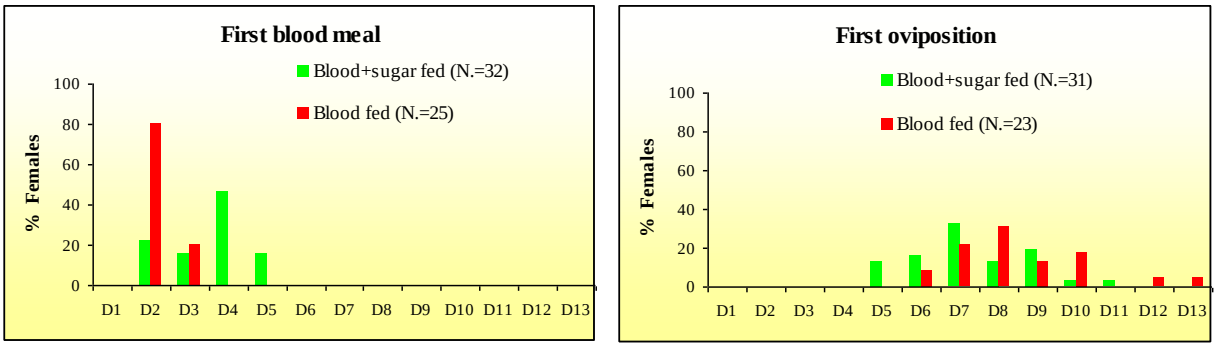


Figure V.13. Daily female percentage that took their first blood meal and laid their first egg batch, considering D_0 as the day of their emergence.
N.:number of females.

Regarding the time of the first oviposition, both groups exhibited similar patterns, with one day of difference (Figure V.13.). This similarity is explained by the fact that, although females that fed on sugar+blood took their first blood meal over a range of several days, 48% (15/31) and 35% (11/31) laid the first batch of eggs three and four days, respectively, after the first blood meal (Figure V.14). On the other hand, females fed only with blood, even if most of them had had their first meal at the same time (Figure V.13.), tended to lay their first egg batches over a longer period of time with a more dispersed distribution (Figure V.14.).

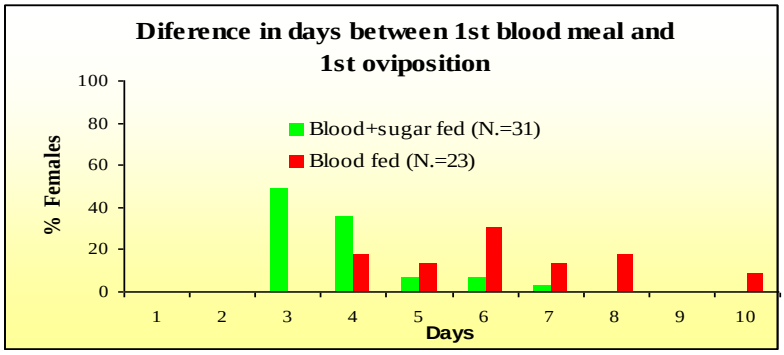


Figure V.14. Female percentage according to the number of days that occurred between the first intake of blood and the following oviposition.
N.:number of females.

The daily percentage of females of both groups that laid eggs and that took a blood meal in each of the 63 days of the entire experiment are presented in Figure V.15. No remarkable difference can be detected between blood fed females and females that fed on the sugar solution, with both groups presenting a decrease in time of the daily percentage of females that oviposited.

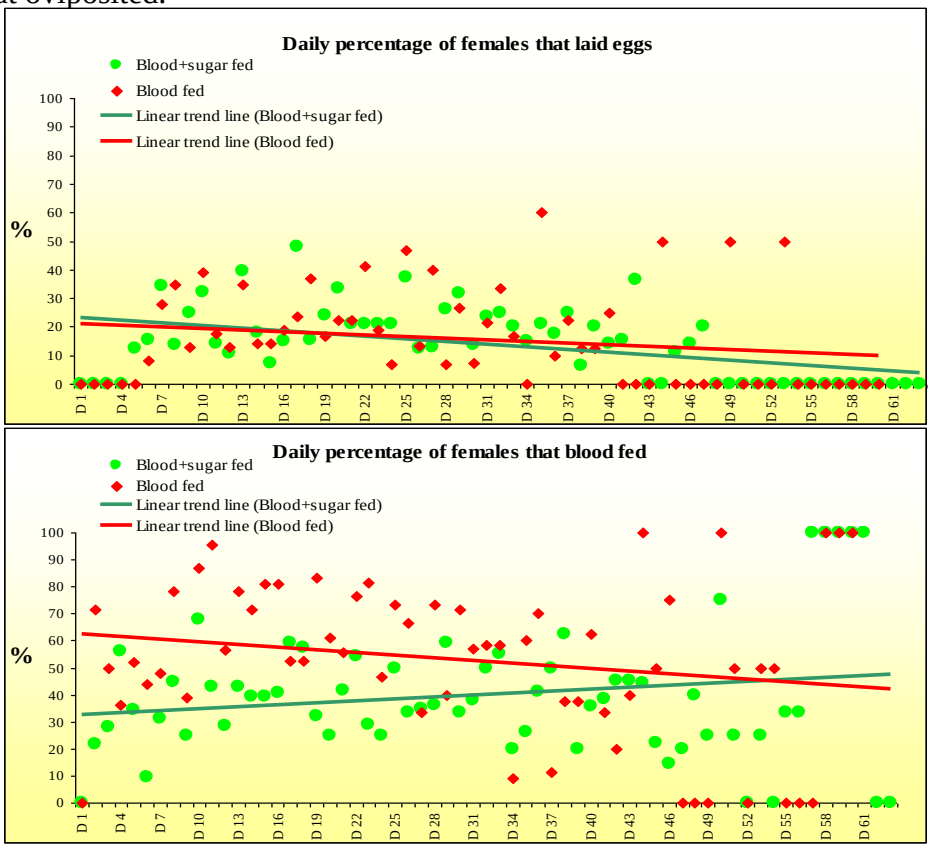


Figure V.15. Daily percentage of *Anopheles atroparvus* females that laid eggs and of those that took a blood meal.

As to the daily percentage of females that took a blood meal, mosquitoes deprived of sugar tend to blood feed more frequently than those with access to the sucrose solution, although this difference tends to decrease as females became older.

Similar results were found when comparing the mean number of blood meals *per* gonotrophic cycle between group-diets (Figure V.16.). Females fed only with blood took, in average, 3.4 feeds to complete the first gonotrophic cycle while females that fed on the sugar solution only made 1.5 blood meals. For the subsequent cycles blood fed females took an average of 2.8 blood meals *per* cycle and females fed also with the sugar solution made, in average, 1.8 blood feeds *per* cycle. The mean number of blood feeds to complete the first gonotrophic cycle, as well to finish the following cycles was found to be significantly higher in the group fed only with blood (for *i0*: Mann-Whitney test, 2-tailed, U=37.00, P<0.001; for *i*: Mann-Whitney test, 2-tailed, U=4458.00, P<0.001).

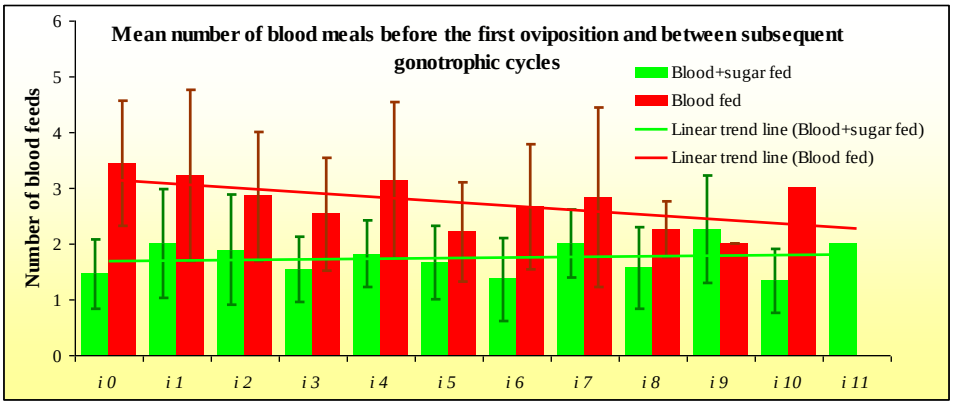


Figure V.16. *Anopheles atroparvus* mean number of blood meals per gonotrophic cycle (i).
Error bars represent standard deviation.
Note: Columns i 10 (Blood fed group) and i 11 (Blood +sugar group) represent single observations.

Table V.14. Comparison of female individual feeding frequencies between group diets of two age categories.

Age	Diet	*n*	Tests for normality	Levene test*	Tests for comparison of means
≤ 11 days old	Blood +sugar	32	*K-S*=0.161 d.f.=32; *P*=0.035	2.833 $d.f._1$=1; $d.f._2$=55 *P*=0.098	*U*=57.000 ***P*<0.001**
	Blood	25	*S-W*=0.832 d.f.=25; ***P*=0.001**		
> 11 days old	Blood +sugar	28	*S-W*=0.929 d.f.=28; *P*=0. 057	1.445 $d.f._1$=1; $d.f._2$=49 *P*=0.235	*t*= 5.678 d.f.=49 ***P*<0.001**
	Blood	23	*S-W*=0.927 d.f.=23; *P*=0.096		

n: number of observations. S-W: Shapiro Wilk statistic. K-S: Kolmogorov-Smirnov statistic with Lilliefors significance correction. *: based on median. U: Mann-Whitney statistic, 2-tailed test. t: t statistic, 2-tailed test. Underlined: significant at level < 0.05. Bold: significant at level < 0.01.

The effect of diet on feeding behaviour early and later in *An. atroparvus* female's life was also analysed comparing the individual feeding frequencies of epidemiological dangerous and non-dangerous female's cohorts. To establish the boundaries of epidemiological importance two scenarios were hypothesised. According to Cambournac (1942) and based on Moshkovsky method (*fidé* Detinova, 1963) the duration of *Plasmodium vivax* sporogonic cycle, the former most prevalent *Plasmodium* species in Portugal and also the one with the shortest extrinsic incubation period, can be estimated as 11 days, at optimal conditions (24ºC). In a worst scenario case, an *An. atroparvus* female may be infected with *P. vivax* during her first blood meal, taken in her first day of life, and become infectious by her 12th day of life. In this case, females younger than 12 days can not be infectious while females with ages of 12 days or more may already be able to transmit malaria. In a second scenario, an extrinsic incubation period of 20 days was considered as a conservative estimate for the duration of *Plasmodium falciparum* (Gary Jr. & Foster, 2001) sporogonic cycle. Based on these

assumptions, individual feeding frequencies of cohorts of females younger and older than 11 and 20 days were compared according to the diet regime and results presented in Tables V.14. and V.15. Regardless of age category, blood feeding frequencies were always significantly higher in females without access to sugar-meals.

Table V.15. Comparison of female individual feeding frequencies between group diets of two age categories.

Age	Diet	*n*	Tests for normality	Levene test*	Tests for comparison of means
≤ 20 days old	Blood +sugar	32	*K-S*=0.136 d.f.=32; *P*=0.138	0.073 $d.f._1$=1; $d.f._2$=55 *P*=0.788	*U*=61.500 ***P*<0.001**
	Blood	25	*S-W*=0.884 d.f.=25; ***P*=0.008**		
> 20 days old	Blood +sugar	24	*S-W*=0.949 d.f.=24; *P*=0.256	0.055 $d.f._1$=1; $d.f._2$=40 *P*=0.817	*U*=82.000 ***P*=0.001**
	Blood	18	*S-W*=0.889 d.f.=18; *P*=0.038		

n: number of observations. S-W: Shapiro Wilk statistic. K-S: Kolmogorov-Smirnov statistic with Lilliefors significance correction. *: based on median. *U*: Mann-Whitney statistic, 2-tailed test. Underlined: significant at level < 0.05. Bold: significant at level < 0.01.

V.5.2. SURVIVAL PATTERNS

The survival patterns of the two diet groups of females showed to be graphically different (Figure V.17). Mosquitoes fed only with blood lived three days less than those with access to sugar meals, and the survival time of 50% of each cohort was reached seven days earlier by the female group deprived of sugar. However, when assessing the statistical significance of observed discrepancies no significant differences were found between the two survival curves (Mantel Haenszel statistic= -1.333, *P*=0.1824).

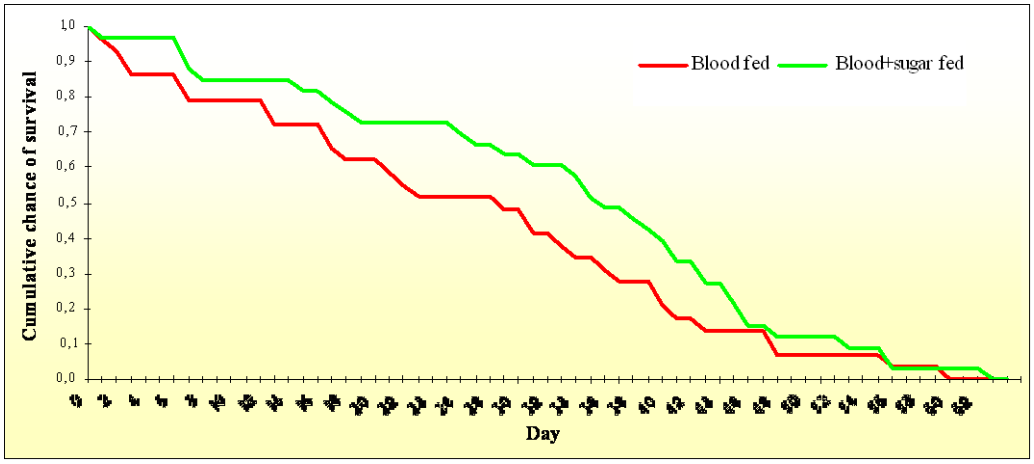


Figure V.17. Daily cumulative chance of survival of groups of females fed with different food-diets. Vertical arrows point to the survival time of 0.5 cumulative chance of survival of each female group.

V.6. VECTORIAL CAPACITY

Different scenarios were hypothesized for computing vectorial capacity estimates (C). Based on longitudinal parity rates calculated from field specimens captured between June 2001 and May 2004, retrospective C values were estimated for i_0 and F values, calculated for female cohorts fed only with blood or also with access to a sugar solution.

Vectorial capacity estimates were also plotted for three different extrinsic incubation periods (n): 11 days, compatible with *P. vivax* development, under optimal conditions (24ºC); 14 days referring to *P. falciparum* sporogonic cycle duration, at 24ºC, and; 20 days, a conservative estimate for *P. falciparum* extrinsic incubation period (Gary Jr. & Foster, 2001). In all calculations, a single human blood index was used (0.006). A maximum (*ma*=38) and a mean (*ma*=13) man biting rates derived from field collections performed in the months of June to August, were also used in C calculations. Results are presented in Figures V.18. and V.19.

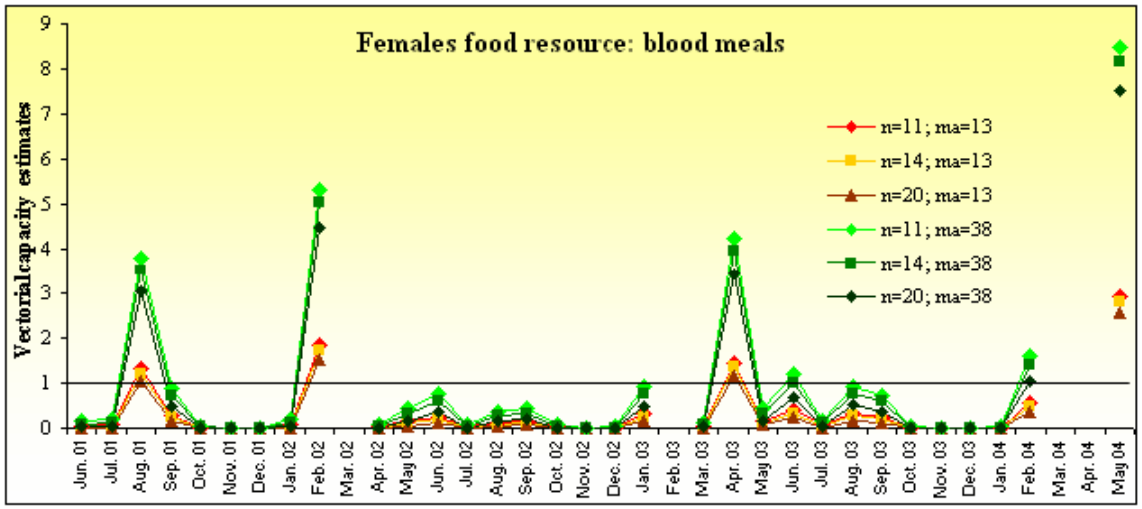


Figure V.18. *Anopheles atroparvus* vectorial capacity estimates (C) calculated for the period of June 2001-May 2004, using i_0 and F values computed for female cohort fed only with blood, for different estimates of n and *ma*.

The highest estimate of C was 8.5, computed for the month of May 2004, considering a sporogonic cycle of 11 days and a man biting rate of 38 bites *per* person *per* day. Values of C where higher for *P. vivax* and for females only fed with blood that presented a higher feeding frequency and a longer first gonotrophic cycle. Vectorial capacity estimates only sporadically cross the threshold of one. This threshold was conventionally chosen as the limit above which one malaria autochthonous case may emerge. This limit was surpassed mainly in the months of late winter and spring and once in August.

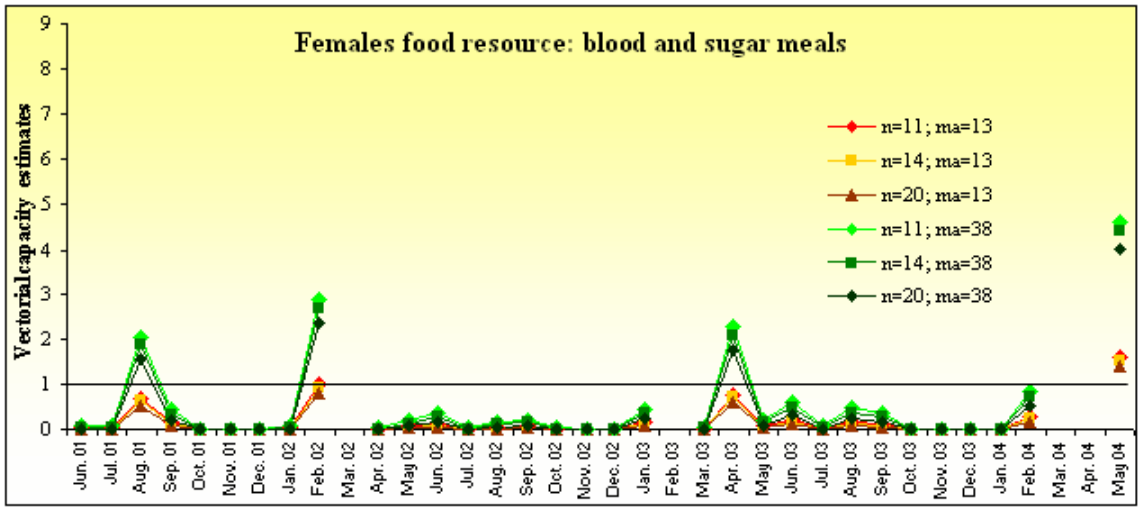


Figure V.19. *Anopheles atroparvus* vectorial capacity estimates (C) calculate for the period of June 2001-May 2004, using i_0 and F values computed for female cohort with access to blood and sugar meals, for different estimates of n and *ma*.

V.7. DISCUSSION AND CONCLUSIONS

Anopheles atroparvus is the only sibling member of *Anopheles maculipennis* complex found in Comporta region. This species, considered to be the most widespread malaria vector in Europe, is present from Ireland and Portugal, in the West, to Iran and Russia, in the East (Jetten & Takken, 1994; Ramsdale & Snow, 2000, Djadid *et al.*, 2007). As to its northern limit, it was recorded in Sweden, up to parallel 57 ºN (Ramsdale & Snow, 2000). This wide distribution is most probably associated with the ecological plasticity early on recognized for this species (Collado, 1938; Rioux, 1958). It can be found from sea-level costal areas to the subalpine heights in France (Rioux, 1958) or at 1800 meters in hilly areas of Portugal (Cambournac, 1942).

The seasonality of this species is as variable as its biotopes. In France, *An. atroparvus* indoor-resting population in the Camargue region, showed the highest abundance rates in October-November (Ponçon *et al.*, 2007), while in Spain, using the same collection method, two abundance peaks were observed, one in May-July and another in October-November (Morales, 1946). In Comporta region, in IR captures, the species showed similar seasonal patterns between collection sites. Statistical analysis showed no spatial differentiation between Comporta, Carvalhal and Pego regarding *An. atroparvus* abundances. This was an expected result due the proximity of the three localities (longest distance: Comporta-Pego, 9.6 km) compatible with *An. atroparvus* flight capacity (Kaufmann & Briegel, 2004) and the inexistence of geographic barriers. In the region, females of this species were collected all

year round but males were not recorded during periods of one to three months, during winter time. The adult seasonal dynamics, with an abundance peak in July, is similar to those described by other authors for this species in Portugal, when malaria was an endemic disease. Two slight differences are worth mentioning. Cambournac (1942) refers to the seasonal pattern of *An. atroparvus* as presenting maximum values of collected specimens between June and August followed by a sudden decrease of captures in September, due to the drainage of the rice paddies. Also according to this author, *An. atroparvus* males only survive until early December being recorded again in early March of the following year (Cambournac & Hill, 1938; Cambournac 1942). In Comporta region, *An. atroparvus* abundances showed a decrease in September but not as striking as referred by Cambournac (1942) (Figure V.20).

As to male seasonality, three different patterns were observed, only one (2003-2004) being identical to what was described by previous authors. Differences found in the shape of seasonality curve may be due to the sampling procedures. While Cambournac (1942) methodology implied the capture of all specimens present in collection sites, in 2001-2004 collections were carried out during periods of 10 min. When mosquitoes are present in very high densities, the number of specimens collected in 10 min depends on abundance but also on the collector performance. Above a certain point, an increase in abundance does not correspond to a proportional increase in captures just because the collector is already operating as fast as possible.

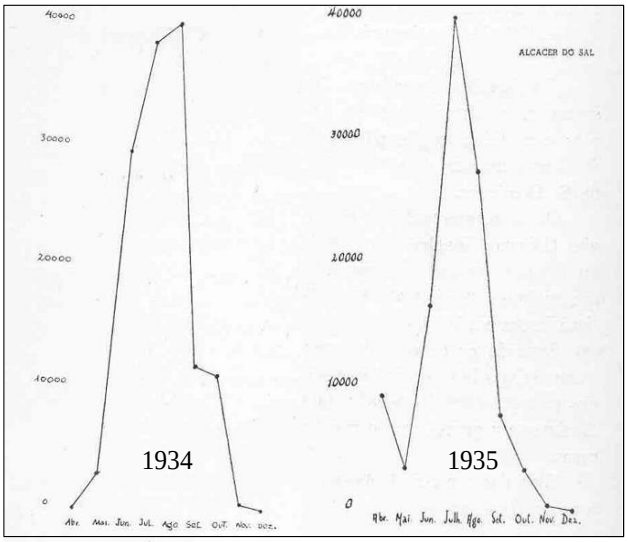


Figure V.20. *Anopheles atroparvus* seasonal variation for the years 1934-1935 in Alcácer do Sal (Cambournac, 1942).

Climate factors may also be responsible for the differences observed. In the years 2001 and 2002, the month of September was humid with values of precipitation and relative humidity above average. These conditions may have favoured the maintenance or appearance of alternative breeding places after the drainage of the rice fields. These breeding sites would sustain *An. atroparvus* larvae development beyond September and the decrease in abundance

would thus be less abrupt. In 2003, humidity and precipitation were near the normal values and the seasonality curve resembles the ones described by Cambournac (1942). Climate factors may also be involved in the discrepancies observed in 2001-2002 seasonal dynamics of *An. atroparvus* males. During this year, with the exception of January, male specimens were collected all year round. Coincidently the winter of 2001-2002 was particularly warm and dry. These factors may have influenced females activity that, favoured by good climate conditions, were able to lay their eggs earlier in the year and thus give rise to an earlier spring generation.

As it happens with other Anophelines (Van der Hurk *et al*., 2000; Minakawa *et al*., 2002) climate factors seem to influence to *An. atroparvus* abundance rates in the same way that are involved in the shaping of other bionomical and epidemiological mosquito characteristics (Bayoh & Lindsay, 2004; Afrane *et al.*, 2005; Devi & Jauhari, 2006; Mabaso *et al.*, 2007). But when statistical analysis was carried out to determine if there was a linear relationship between *An. atroparvus* abundance and meteorological variables like: mean daily temperature, daily precipitation and relative humidity, no relationship of this type was found. It is not easy to establish the nature of the relationships between mosquito activity and ambient temperature or humidity (Clements, 1999). Although linear association between mosquito abundance and temperature, precipitation (Guimarães *et al*., 2001; Fisher & Schweigmann, 2004; Stein *et al.*, 2005) or moisture index (Minakawa *et al*., 2002) were recorded for several species, these relationships do not always completely explain seasonal dynamics of a species (Degaetano, 2005). The effect of climate on the abundance pattern of a given mosquito also varies according to the region where collections are undertaken (Scott *et al.*, 2000). Furthermore, *An. atroparvus* is typically an endophilic species. Males and females at all gonotrophic stages were mostly found resting inside animal shelters and hardly any specimen of this species was captured in outdoor resting collections. Each shelter presents its own microclimatic characteristics and all of them differ from the outdoor environment in terms of ambient conditions. To accurately describe the climatic changes suffered by the resting adult population, measurements should have been performed at each collection site. The absence of significant linear correlation between *An. atroparvus* abundance and climate may therefore be explained by an unsuitable assessment of the meteorological variables.

Similar to what was found for English populations (Ramsdale & Wilkes, 1985) almost all sampled resting females in both Sella's stage 1-2, were mated. Only 10 out of 2,246 females were blood fed virgin females which support the idea that in *An. atroparvus* copulation takes place soon after female emergence. Copulation may take place in the open or even inside shelters since male swarms have been observed both outside and inside dwellings, in spaces as small as one meter high (De Buck *et al.*, 1930; Hackett & Missiroli, 1935; Cambournac & Hill, 1940). Furthermore, swarming is not a pre-required condition for *An.*

atroparvus female's fertilization and this species is able to perform copula in spaces of very reduced dimensions (14.0 x 5.5 x 5.0 cm, Harant *et al.*, 1957), which sustains the possibility of mating taking place inside animal shelters where males and females rest.

Unfed to gravid *An. atroparvus* females were found resting inside animal shelters all year round. Portuguese populations were always considered to have a more reduced winter diapauses (Harant *et al.*, 1957) and terms as "semi-hibernation" have also been used to describe female overwintering behaviour (Cambournac, 1942). In Comporta, where monthly averages of mean daily temperatures are usually above 10ºC, *An. atroparvus* does not seem to hibernate. The need to blood feed during the winter months is a behavioural trait long associated with *An. atroparvus* females and used as a diagnostic character (De Buck & Swellengrebel, 1929, De Buck *et al.*, 1930; De Buck *et al.*, 1933), but the presence of both unfed and gravid females indicates that at least part of the population is gonoactive during the cold months. Unlike *An. atroparvus* English populations (Ramsdale & Wilkes, 1985) but similar to *An. labranchiae* from Sicily (D'Alessandro *et al.*, 1971) overwintering parous and nulliparous females are found together and parous rates increase from November to February when population abundance reaches its lowest limit. These parous females are then derived from gonoactive females which, in favourable days, are able to leave from the dwellings, lay their egg batch and, at least part, return to shelters. It is probably from these eggs that the spring generation is originated. The females of this generation would then copulate and give rise to the parous rate peak of May-June. Offspring of these females may be responsible for the second peak of parous females observed in September. Thus, in Comporta region, *An. atroparvus* seems to present parity annual cycles with three generational peaks.

To understand the epidemiology of human and animal diseases for which mosquitoes act as vectors, it is of major importance to know vector-host relationships and, therefore, mosquito host preferences. It was the perception of this reality that led to the foundation of the *An. maculipennis* complex. Roubaud (1921), in order to explain the paradigm "anophelism without malaria", divided the species in two physiological races according to feeding habits. The zoophilic behaviour of certain mosquito populations was then associated with absence or natural disappearance of malaria in some regions of Europe (Missiroli & Hackett, 1927; Hackett & Missiroli, 1931). *Anopheles atroparvus* was always considered to be a species closely associated to cattle which could bite humans under certain environmental conditions (Missiroli *et al.*, 1933, Hackett & Missiroli, 1935). In Comporta region this species was found to feed on mammals of all sizes from rabbits to horses, thus confirming previous results (Landeiro & Cambournac, 1933, Cambournac & Hill, 1938; Bates & Hackett, 1939; Ramos *et al.*, 1992; Cambournac, 1994). In this study pigs were found to be the favoured host, followed by goat/sheep and rabbits. The predominance of pigs as main host is in contradiction with previous studies that indicate, in decreasing order of preference, rabbits,

horses, cattle and pigs as the main animal blood sources for *An. atroparvus* females (Cambournac, 1942; 1994). Feeding patterns are influenced by both mosquito host preference and host availability, defined by their density, defensive behaviour, spatial distribution and size, among other characteristics (Hess *et al.*, 1968; Edman & Webber, 1975). Furthermore, no real host preference may be determined unless an unbiased mosquito sample is obtained together with information for correctly interpreting blood meal analysis results. In this study, due to the presence of defence measures in almost all households, no blood fed females of any species were found resting inside houses (see Chapter IV). All blood meals tested came from females caught only inside animal shelters or storage houses. *Anopheles atroparvus*, apart from being endophilic, is also considered to be endophagic (Cambournac & Hill, 1938), and in 90% of blood meals analysed the blood source agreed with the host present at the collection site. Therefore, *An. atroparvus* apparent selection of pigs, sheep and rabbits as main blood sources may be the result of different collection efforts surveying those animal shelters and not the reflection of this species host preference. Unfortunately, this aspect cannot be clarified because the dwellings inspected usually sheltered more than one type of animal and the number of specimens of each species present was not totalised.

The reported human blood indexes for *An. atroparvus* vary from 0.02 (Ramos *et al.*, 1992) up to 0.6 (Swellengrebel & De Buck, 1938 *fidé* Jetten & Takken, 1994). The low human blood index determined for *An. atroparvus* in this study is consistent with the low man biting rate observed. However, it is lower than previous estimates, obtained when malaria was endemic in Portugal: *HBI*=0.150, N.=325 (Landeiro & Cambournac, 1933), and; *HBI*=0.10, N.=2320 (Cambournac, 1942). Differences may be explained by the blood meal identification technique used, since those reported *HBI*s were obtained by precipitin tests, a technique less sensitive and less specific than ELISA (Burkot *et al.*, 1981). Another reason for the differences found may be due to the nature of the mosquito samples. When estimating *An. atroparvus HBI* from the 1940's data, considering only blood meals of mosquitoes collected inside animal dwellings, differences are less striking or even absent: *HBI*=0.104, N=182, (Landeiro & Cambournac, 1933); *HBI*=0.006, N=998, (Cambournac, 1942). More recent studies (Ramos *et al.*, 1992) carried out in Portugal southeastern populations using ELISA techniques, showed results similar to our data (*HBI*= 0.015; X^2=1.296; *P*=0.255).

Mosquito sugar feeding has been a controversial issue (Gary Jr. & Foster, 2001) seen by some as ubiquitous and essential (Hocking, 1953; Downes, 1958; Yuval, 1992; Gary Jr. & Foster, 2006), while by others as facultative and incidental (Muirhead-Thomson, 1951 *fidé* Hocking, 1953; McCrae *et al.*, 1976; McCrae, 1989; Edman *et al.*, 1992). Physiological and behavioural findings cannot be extrapolated from Culicines to Anophelines, since the underlying physiological mechanisms are different (Briegel, 1990), but as regards the latter the contradictory positions are maintained. Some authors claim that in nature sugar feeding is

a rare event based on the fact that field-collected females neither contain fluids in their esophageal diverticulum nor test positive for fructose (Beier, 1986). On the other hand, other authors state that feeding on carbohydrate sources is part of a normal behaviour in Anopheline species (Laarmar, 1968; Holliday-Hanson *et al.*, 1997). Since it is reasonable to think that females may require both blood and sugar, even on an occasional base or depending on physiological or environmental conditions (Foster, 1995; Holliday-Hanson *et al.*, 1997; Foster & Takken, 2004), the effect of sugar-feeding in *An. atroparvus* feeding and oviposition frequencies, duration of gonotrophic cycles and longevity was then investigated.

In mosquitoes, a higher production of eggs is usually associated to females that fed on blood and sugar sources (Nayar & Sauerman Jr., 1975; Mostowy & Foster, 2004), but in lifetime studies total fecundity and intrinsic rates of growth are frequently higher for female fed only with blood (Scott *et al.*, 1997; Naksathit & Scott, 1998; Costero *et al.*, 1998; Braks *et al.*, 2006). In *An. gambiae*, although daily fecundity was higher for females given blood alone, sugar availability showed no effect in the overall reproductive output, due to the longer longevity of sugar fed females (Gary Jr. & Foster, 2001). As to *An. atroparvus*, by comparing the two female cohorts, one fed only with blood and the other fed with blood but with access to a 10% sucrose solution, results showed no differences between the two groups regarding the percentage of parous females and the mean number of ovipositions *per* parous insect. Unfortunately, the number of eggs was not accounted for and therefore is not possible to state that in *An. atroparvus* sugar availability does not influence overall reproductive output. The number of ovipositions *per* female was similar to those observed by Shute (1936) but far superior to the one presented by Roubaud & Treillard (1937). Although the authors do not refer the females sugar feeding status, the number of egg batches *per* female varied between 2.5 (Roubaud & Treillard, 1937) and 4.3-4.6 (Shute, 1936), while in this study values recorded where 4.6 for blood fed females and 5.9 for those fed with blood + sugar[13].

Feeding on blood alone led to a significant increase in overall feeding frequency as well to a higher daily percentage of fed females. For this cohort, *F* was a similar to Roubaud & Treillard (1937) results (F=0.31) although again no reference is made regarding sugar availability. By contrast to what was observed for *An. gambiae* (Straif & Beier, 1996; Gary Jr. & Foster, 2001) differences in the daily proportion of blood fed females tend to decrease, as females became older. However, feeding frequencies of epidemiological dangerous females, with ages greater than 20 days, deprived of sugar meals, are still significantly higher than those fed both with blood and sugar. The substantial increase in blood feeding due to sugar deprivation is not observed in all Anopheline species. Foster & Eischen (1987) found no differences in blood feeding frequencies of blood fed *An. quadrimaculatus* cohorts when compared to blood+sugar fed females. Thus, the way sugar availability affects Anopheline

[13] Data was re-computed for comparison purposes.

blood feeding behaviour seems to differ between species and, although evidences are sometimes discrepant due to methodological differences, it is probably a species-specific characteristic as stated by Yuval (1992).

Regarding the daily oviposition frequencies, both blood fed and blood+sugar fed females showed a slight reduction with time and no differences were observed between groups. Females deprived of sugar must therefore take more blood meals within each reproductive cycle since the blood feeding frequency of this cohort is higher. This assumption was confirmed with the highest differences between groups recorded for the first gonotrophic cycle. Females fed only with blood took, in average, 3.4 meals to produce their first egg batch while 19 of 31 blood+sugar fed females only required a single blood meal. In this study females deprived of sugar were unable to complete the first oogenesis with only one blood meal, confirming the findings of Fernandes & Briegel (2005) in which *An. atroparvus* females either fed on sugar or took several blood feeds in order to be able to produce eggs. By contrast De Buck & Swellengrebel (1929), in the early studies to establish the so-called "*atroparvus* race", were able to obtain egg batches from females without access to a sugar solution after a single human blood meal. In this case specimens were field collected females during winter season (February) which probably had already blood fed several times prior to the beginning of the experiment. So, it appears that in *An. atroparvus*, as in other Anophelines (Briegel, 1990), additional meals on blood or carbohydrate sources are fundamental, probably to build up maternal protein and caloric and lipid storage, thus compensating for limited teneral reserves gained during larval development.

Feeding patterns during the first gonotrophic cycle was also different according to female's diet. Eighty percent of females deprived of sugar blood fed in the second day after emergence, while females with access to a sugar source reached the 80% threshold of blood feeding only at the fourth day. Similar patterns, also for *An. atroparvus* females, were obtained by Fernandes & Briegel (2005) although with time a delay on reaching the same threshold values, probably due the lower experimental temperature (22ºC).

The length of *An. atroparvus* first gonotrophic cycle was significantly longer for females fed only on blood while no differences were found between the two female cohorts with regard to mean duration of the following cycles. Values of seven and four days for i_0 and i, respectively, are identical to those found by Ramos *et al.* (1992). In this case, freshly fed field females captured in southeastern Portugal were kept in laboratory conditions until oviposition with access to a sugar water solution (personal communication).

Regarding survival curves of the two female groups, cohorts showed different life spans, with blood+sugar females living three days longer, and the 50% survival time was reached seven days earlier by the female group fed on blood alone. However, survival analysis showed no significant differences between groups. These results contrast with those

found for *An. gambiae* in which females with access to a sucrose solution had a longer age-specific survival than those fed only with blood (Straif & Beier, 1996; Gary Jr. & Foster, 2001).

Vectorial capacity (C) has been widely used during the last fifty years in malaria and arbovirus epidemiology. However, in almost all studies, authors refer to its intrinsic limitations and use C as a descriptive and predictive estimate rather than as an absolute measure of the daily rate at which future inoculations arise from a currently infective case. Even in comparative studies, as it happens when accessing the effect of control measures, bias can be introduced at several stages of parameter estimation (Dye, 1990). One of the most frequent problems occurs by the fact that all variables are environment-sensitive and some are determined by temperature. Although temperature values are sometimes available at local meteorological stations, it is rare to have continuous measurements of temperature and humidity at mosquito resting sites. Temperature differences between outside and inside shelters may induce errors in the estimates of parameters required for computing the C value. One evident example is the length of the sporogonic cycle ($\underline{n}$). Since it is more frequently computed as the cumulative difference between daily temperature and a threshold value given for each *Plasmodium* species, a difference of one degree Celsius (from 20ºC to 21ºC), may result in a difference of 5 days in the estimate of $\underline{n}$ in the case of *P. falciparum*. Another problem arises from the fact that each variable can be estimated with several methods, that when compared may not always give the same result. This is particularly evident in the calculation of the gonotrophic cycle, the duration of which usually varies significantly between mark-release-recapture-based studies and laboratory experiments (Reiter, 1996; Santos *et al.*, 2002). The mosquito collection methods adopted may also be crucial. Human blood index (Garret-Jones, 1964b) and parity rates may vary considerably according to the type of sampling performed (McHugh, 1989; Ghavami, 2005). Even in the absence of disease and without parasite-induced effects (Koella *et al.*, 1998), due to the difficulties in avoiding all of these and other biases, caution should be placed in interpreting the epidemiological meaning of vectorial capacity estimates.

In the present study, C was calculated: (i) for three different extrinsic incubation periods; (ii) for mean and maximum man biting rates recorded for *An. atroparvus* Comporta's population in the months of June-August when abundances are highest, and; (iii) using i_0 and F estimates derived from colony studies. Keeping in mind that estimates of C are often biased due to the violation of some basic assumptions (*e.g.* unbiased sample of blood females from different resting sites) and or to the choice of less realistic methods (i_0 and F estimates based on laboratory studies), C was tentatively used as an expression of the worst possible scenario.

The i_0 and F estimates used in this study translate the best mosquito performance, since specimens were free from all sources of stress and with access to unlimited food supply.

Thus, the underestimation of i_0 due to optimal environmental conditions and an excessive value of *F* due to food availability are adequate to the proposed scenario. As to the artificial conditions where experiments were undertaken, the choice of a temperature of 26ºC also seems suitable to the objective. Although a lower experimental temperature may have led to a decrease in the duration of the gonotrophic cycle no significant differences were found when rising ambient temperature from 27ºC to 30ºC (Rúa *et al.*, 2005). In any case, the i_0 value used in C calculation may not be completely devoid of reality since identical estimates were found by Ramos *et al.* (1992) for freshly fed, field captured females.

The daily survival rate was computed as the i_0 root of the parous rate of field collected females and age determination was achieved using Detinova method. This method although simple to carry out, is only applicable in the early stages of the ovarian cycle (up to Sella's stage 2) and the proportion of parous females determined may be affected in three ways (Molineaux *et al.*, 1988). First, gonotrophic maturation goes on after collection and the number of specimens eligible for screening decreases with time. Second, classification of females according to their gonotrophic development is dependent on the observer subjective avaliation. Third, the actual parous rate is likely to be smaller among the females that are eligible for the method than among those that are not, because the method is applicable for a longer fraction of the first cycle than of subsequent cycles. In order to avoid the first pitfall, females after capture were kept always under refrigerated conditions and immediately sacrificed by cold, as soon as they would arrive at the laboratory. To prevent the second issue, females which ovary maturation was difficult to assess, as being in Sella's Stage 2 or 3, were always dissected and observed. If ovaries were still in Christopher's stage II-mid, then ovarian tracheoles were checked to determine their coiling state. The third reason for bias cannot be avoided by any technical procedure and therefore there is no way to confirm that parity rate was identical in eligible and non eligible females.

The difficulties in obtaining representative mosquito samples to determine the human blood index and the best methods to its computation were thoroughly discussed by Garrett-Jones (1964b). By contrast to what is recommended by this author, the *HBI* determined for Comporta region was calculated as the proportion of the whole sample found to contain human blood. This method of deriving the *HBI* is considered to conduct to erroneous and misleading results but it is the only applicable to the data available, since almost all (646 out of 671) blood fed mosquitoes came from the same generic type of resting place, *i.e.* animal shelters.

Based on available information that *An. atroparvus* is typically an endophagic species biting inside animal shelters or in their proximity (Cambournac, 1942), man biting rates were evaluated through human baited landing captures performed in the vicinity of animal shelters. Between the possibility of performing landing catches inside animal shelters or in their

vicinity, the second option was chosen, supported by two arguments. First, if collections were undertaken inside animal shelters, the presence of more favoured hosts would probably deflect the mosquito attraction from the human baits. Second, *An. atroparvus* is a crepuscular species presenting an activity peak around 20h30 when most of the people are outside working in their small parcels of land (43%), or sitting outside enjoying the warm summer late afternoons (65%) (Teodósio *et al.*, unpublished observations). Since mosquito-human contact probably takes place when people engage in these activities it seemed reasonable to perform landing caches outside in order to reproduce a real situation.

Cambournac (1942) conclusions regarding *An. atroparvus* feeding habits were confirmed by the present study. Although having the ability to enter in very well built dwellings, it could also feed outdoors on a host closely located to its resting place. *Anopheles atroparvus* females in order to accomplish a complete gonotrophic cycle need to engorge an amount of blood of about the double of their weight (Detinova, 1963). This results in severe limitation to their flight capacity and therefore ovarian maturation usually takes place near the host. Likely for this reason, the blood source of 90% of female meals analysed agreed with the host present at the collection resting site.

A vectorial capacity threshold of C=1 was surpassed only in the months of August 2001, February 2002, April 2003, and May 2004. In this month C reached nine, which was the maximum number of new daily inoculations that might occur if an infective host would be introduced in the area. This estimate was computed for a sporogonic cycle of 11 days (compatible with *P. vivax* development, under optimal conditions) and the highest man biting rate obtained in this study (38 bites per person per day). This value of C is similar to that obtained for some other malaria vectors (Prakash *et al.*, 2001; Sousa *et al.*, 2001). As to the other values of C above the threshold of one, these occurred in winter/spring months when parous rates were above 0.95 but abundances were at their lowest levels. Furthermore, most of the computed variables were overestimated. Therefore, one can foresee that the receptivity of the area to the re-emergence of the disease is very limited.

Chapter VI

ANOPHELES ATROPARVUS VECTOR COMPETENCE

VI.1. AIMS

During the second half of the XX century endemic malaria was eradicated from all Countries of West Europe. However, the number of imported cases is continuously rising due to the constant increase in international travels and immigration (Legros & Danis, 1998; Jelinek *et al.*, 2002). Airport malaria cases have been reported (*e.g.* Jafari *et al.*, 2002) and sporadic autochthonous transmitted malaria cases have been recorded in the last decades in Countries such as Italy and Germany (Baldari *et al.*, 1998; Kruger *et al.*, 2001). Much has been discussed regarding the effects of global warming in the re-establishment of malaria transmission in Europe. It is generally accepted that, as it happened in the past (Zulueta, 1994), the refractoriness of European vectors to tropical strains of *Plasmodium falciparum* will act as a barrier to the re-introduction of this parasite species in Europe (Jetten & Takken, 1994). This belief is sustained by a series of unsuccessful experiments to infect *Anopheles atroparvus* with several strains of *P. falciparum* carried out after malaria disappearance. Although the artificial infection of European mosquito species with malaria parasites was a common practice in malaria therapy studies (James, 1930/31) it was Shute (1940) that for the first time tried to evaluate the susceptibility of European Anopheline populations after the disappearance of the disease from England. Although susceptible to European strains (Romanian and Italian) English *An. atroparvus* specimens did not get infected when fed on gametocyte carriers of *P. falciparum* strains from India and East and West Africa (Shute, 1940). This mosquito species was also found refractory to Nigerian strains. English specimens transported to Lagos, fed on local malaria patients and maintained under natural ambient conditions failed to develop oocysts while Nigerian *An. gambiae*, used as control, could easily become infected (Zulueta *et al.*, 1975).

These studies on the susceptibility of *An. atroparvus* to tropical strains of human malaria parasites were followed by others using similar methodologies (Table VI.1.). Two small scale experiments were undertaken in Portugal with local *An. atroparvus* (Roque *fidé* Zulueta *et al.*, 1975; Ribeiro *et al.*, 1989). In the first study, 58 females were successfully blood fed on an Angolan patient. Between day five and twelve, 25 specimens were dissected, three presented a single oocyst in their midguts and four a few each (precise number not specified). Other 30 females were examined 12-18 days after feeding but no sporozoites were observed in their salivary glands. In the second study, 48 females blood fed on gametocyte carriers from Angola and Mozambique did not develop any sign of infection.

Table VI.1. Results summary of *Anopheles atroparvus* infections with human malaria parasites.

Plasmodium		**Mosquito origin and sample size***		**Temperature during infections**	**Infection results**	**References**
Species	**Origin**					
falciparum	East/West Africa	England		24ºC and 34ºC	0% oocyst prevalence	Shute, 1940
	India			25ºC and 30ºC		
	Italy	NM		25ºC and 30ºC	50% oocyst prevalence	
	Romania			NM	Positive to oocysts	
malariae	Africa, Europe and South America	England 45		NM	40% sporozoite prevalence	Shute & Maryon, 1955
malariae strain Vs	Romania	Romania 59		19ºC to 30ºC	19% sporozoite prevalence	Constantinescu & Negulici, 1967
malariae strain Vs	Romania	Romania 2,747		19ºC to 22ºC	97% infection rate	Lupascu *et al.*,1968
falciparum	Kenya	Italy	20	24ºC to 28ºC	0% oocyst prevalence	Ramsdale & Coluzzi, 1975
	Nigeria		177	22ºC to 30ºC	1.7% oocyst prevalence 0.03 oocyst intensity	
falciparum	Nigeria	England NM		Nigerian ambient condition	0% oocyst prevalence	Shute fidé Zulueta *et al.*, 1975
falciparum	Angola	Portugal 55		25ºC to 29ºC	3 females with 1 oocyst and 4 with a few each 0% sporozoite prevalence	Roque fidé Zulueta *et al.*, 1975
vivax	Brazil	USSR NM		25ºC to 29ºC	Positive to sporozoites	Bibikova *et al*., 1977
vivax	Azerbaïdjan	USSR 200		NM	65% sporozoite prevalence	Dzhavadov *et al*., 1977
falciparum	Ce. Afr. R. [a]	Romania	50	25ºC	0% sporozoite prevalence	Teodorescu, 1983
	Nigeria		75			
malariae	Ce. Afr. R. [a]		50	25ºC	0% sporozoite prevalence	
	Gabon		204			
	Madagascar		76			
ovale	Nigeria		132	25ºC	16% sporozoite prevalence	
vivax	Iraq/Turkey		50	25ºC	92% sporozoite prevalence	
	Iraq		65		92% sporozoite prevalence	
	Lao D.R.[b]		50		70% sporozoite prevalence	
	?		73		90% sporozoite prevalence	
falciparum	14 African Countries	URSS	1055	NM	0% oocyst prevalence	Daskova & Rasnicyn, 1982
			895		0% sporozoite prevalence	
malariae	Guinea		10	NM	0% oocyst prevalence	
			21		0% sporozoite prevalence	
ovale	5 African Countries		196	NM	0% oocyst prevalence	
			149		0% sporozoite prevalence	
vivax	India		38	NM	13% oocyst prevalence	
			8		12% sporozoite prevalence	
	Lao D.R.[b]		373	NM	24% oocyst prevalence	
			134		25% sporozoite prevalence	
	Nigeria		33	NM	12% oocyst prevalence	
			41		7% sporozoite prevalence	
	Pakistan		43	NM	7% oocyst prevalence	
			40		5% sporozoite prevalence	
	Yemen		212	NM	25% oocyst prevalence	
			275		15% sporozoite prevalence	
falciparun	Angola and Mozambique	Portugal 56		26ºC	0% oocyst prevalence 0 % sporozoite prevalence	Ribeiro *et al.*, 1989

*: refers to the number of dissected mosquitoes. NM: not mentioned. [a]: Central African Republic; [b]:Lao People's Democratic Republic. ?: unknown origin.

It seems clear that the refractoriness of *An. atroparvus* to African strains of *P. falciparum* is a solid proven fact but until fifty years ago natural populations of this mosquito species were able to sustain malaria transmission in Europe. With the possibility of artificially infecting mosquitoes and being able to select specific parasite strains and to control environmental parameters under which infection is processed, an opportunity occurred to understand what is the actual competence of Portuguese *An. atroparvus* for transmitting *P. falciparum* and which factors may influence parasite development inside the mosquito vector. In this chapter the results of a series of infection experiments with laboratory reared *An. atroparvus* specimens from Portugal will be presented.

VI.2. METHODOLOGICAL CONSIDERATIONS

Experimental infections were conducted at the Nijmegen Medical Centre, The Netherlands, and included specimens from the following colonies of "Instituto de Higiene e Medicina Tropical (IHMT)":

i A 15 years old *An. atroparvus* colony, established with mosquitoes collected in Águas de Moura region, an area located 35 km north of Comporta. After its establishment, no other field specimens were incorporated into the colony which, therefore, is likely to present a high degree of inbreeding.

ii A new *An. atroparvus* colony established for the purpose of this study, formed with field collected females captured in Comporta region.

iii A *An. gambiae* colony originated from specimens of the Suakoko strain from "Universitá di Roma-La Sapienza" (M. Coluzzi/V. Petrarca) and maintained at IHMT since 1996.

iv A *An. stephensi* colony maintained at IHMT since 1995 and originated from specimens of the SDA 500 strain of The Imperial College of London (R. E. Sinden).

The new *An. atroparvus* colony, set up in the summer of 2005, has continually received field specimens with the intention to maintain its genetic pool as similar as possible to the one of the natural population from which it was originated. Indoor resting mosquito collections took place between July and September of 2005 and April and August of 2006. Captures were carried out in six localities: Carrasqueira, Possanco, Comporta, Torre, Carvalhal and Pego (Appendix). The collected specimens were morphologically identified and all blood fed to gravid *An. atroparvus* females, some unfed females and males *An. atroparvus* were separated and introduced in the colony stock. For the maintenance of this colony new protocols for insect laboratory rearing had to be established and optimised.

Specimens were kept under constant environmental conditions of temperature (26ºC ± 1ºC), humidity (75-80% relative humidity) and light (12 h/12 h light/dark periods).

The maintenance and manipulation of all mammals involved was carried out under the specifications of the Directive for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (86/609/EEC) and the national legislation ("Decreto-Lei nº 92/95" of 12/09; "Decreto-Lei nº 129/ 92" of 06/06; "Portaria nº 1005/92" of 23/10; "Portaria nº 1131/97" of 07/11) and performed by certificated personnel.

Mosquitoes to be artificially infected with *P. falciparum* were sent to the Nijmegen Medical Centre by express mail, in adapted boxes, following the security policy establish by the mail transporter and properly conditioned with food and ambient humidity in order to survive the journey.

Besides the standard procedure mentioned in Chapter III, several other infection protocols were tested. These varied mainly in the number of infected and non infected blood meals taken by the females and in the room temperature at the time of infection.

VI.3. COLONY ESTABLISHMENT AND MAINTENANCE PROTOCOLS

The colony was established with a total of 9,209 specimens collected during the two years period (Tables VI.2. and VI.3.). The majority of these specimens were females (93%), of which 83 % were in Sella's gonotrophic stages 2 to 7, and 10% were unfed females. Males totalized 7% of all specimens.

Table VI.2. Number of *Anopheles atroparvus* specimens, according to gender and date of collection, used in the establishment of the colony.

		N. females	N. males	Total
2005	**July**	160	58	**218**
	August	3,374	252	**3,626**
	September	403	30	**433**
Total 2005		3,937	340	**4,277**
2006	**April**	9	2	**11**
	May	418	22	**440**
	June	1,878	29	**1,907**
	July	1,789	100	**1,889**
	August	528	157	**685**
Total 2006		4,622	310	**4,932**

Table VI.3. Number of *Anopheles atroparvus* specimens used in the establishment of the colony, according to locality of collection, gender and gonotrophic stage of development.

		N. of females			N. of males	TOTAL
		Unfed	Blood fed to gravid	Total		
Comporta Parish	**Carrasqueira**	88	1,164	1,252	35	**1,287**
	Comporta	60	155	215	84	**299**
	Possanco	2	324	326	44	**370**
Carvalhal Parish	**Carvalhal**	193	1,784	1,977	75	**2,052**
	Pego	370	998	1,368	174	**1,542**
	Torre	162	3,259	3,421	238	**3,659**
TOTAL		875	7,684	8,559	650	**9,209**

Table VI.4. Weekly schedule of activities carried out for *Anopheles atroparvus* colony maintenance.

	Stock-cage	Emergence-cage	Larvae trays
Daily activities (Monday to Friday)	1. Removal of the Petri dishes with the egg batches and replacement by clean ones. 2. Substitution of the sugar-solution containers. 3. Removal of dead mosquitoes from the bottom of the cage with the aid 6-V battery aspirator.	1. Transfer of adults to stock-cage. 2. Collection and counting of dead and alive pupae and dead adults. 3. Counting of the number of emerged adults.	1. Collection and counting of live pupae and transfer to the emergence-cage tray. 2. Collection of dead larvae/pupae and removal of excessive food accumulated at the bottom of the trays. 3. Starting of new trays with the eggs batches laid by females of the stock-cage in Petri dishes.
Tuesday and Friday extra activities	Mosquito blood feeding.		Removal from the trays of advanced 2^{nd} instar larvae to 4^{th} instar larvae, with the aid of a tea strainer, and transfer into clean trays.

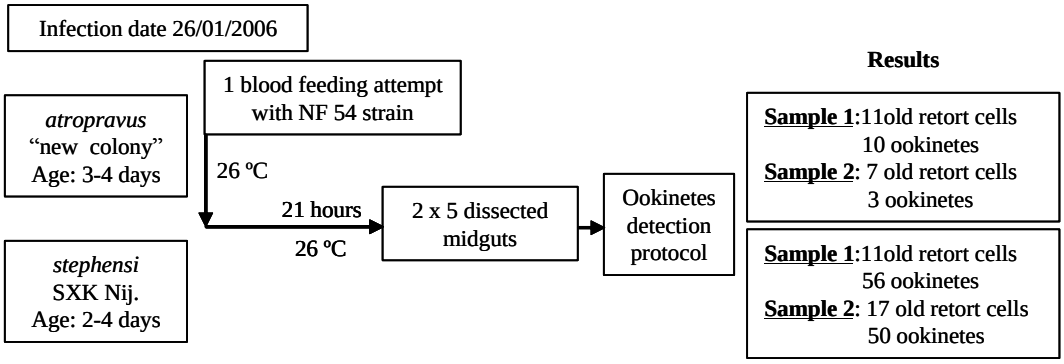
The protocol for insect colony maintenance was largely adapted from routine procedures established for other IHMT mosquito colonies. Optimization focused mainly in host selection and feeding calendar. Adult mosquitoes were kept in two different cages: (i) stock-cage, to where newly emerged mosquitoes were transferred and where copulation, feeding and ovipositon took place and; (ii) emergence-cage, where the pupae trays were kept and where adult emergence occurred. In both cages, a small container with 10% sugar solution was always available. In the stock-cage a Petri dish (9 cm of diameter) with water was also placed to serve as an oviposition site. Larvae were bred in 27 x 18 x 7 cm^3 trays. The

number of larvae *per* tray was not counted, being the larval density and amount of water and food supplied to each tray decided by the operator, based on personal experience. In all procedures tap water was used. Before to be used, the water was left in open containers, at room temperature, during at least 48 h. Larvae food was made of a 1:1 mixture of fish-food (Tetra Menu®, Tetra GmbH, Melle, Germany) and cookies (Bolacha Maria®, DIA S.A., Getafe, Spain) grinded with a pestle. This powdered food was dispersed over the water surface of each tray, in very little amounts, twice a day (in the morning and at the end of the working-day). Adult food consisted in a 10% sugar solution. Blood meals were undertaken on four to six *Mus musculus* Linnaeus, 1758 (CD1 strain), anesthetised with Rompun 2%® (Bayer Healthcare) and Imalgène1000® (Merial) according to the dosages and administration methods recommended (Hedenqvist & Hellebrekers, 2003). A weekly schedule of tasks performed is presented in Table VI.4.

VI.4. ARTIFICIAL INFECTION OF ANOPHELINES WITH DIFFERENT STAINS OF *PLASMODIUM FALCIPARUM*

Three hundred and fifty females from the long established *An. atroparvus* IHMT colony, 75 females of *An. gambiae*, 66 females of *An. stephensi* and 1,857 females of the recent colony of *An. atroparvus* were delivered to Nijmegen for infection experiments. Females sent were two to four days old and all had the chance to mate, although insemination status was not assessed. Ookinete observation was only performed once. The terms "old colony" and "new colony" refer, respectively, to the long and recently established *An. atroparvus* colonies and "NR" stands for unrecorded number of fed females.

VI.4.1. OOKINETE ANALYSIS



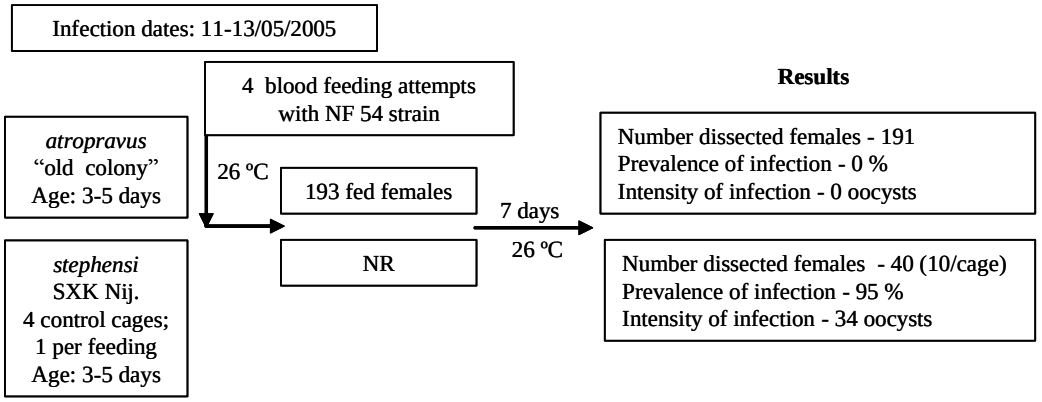
Midguts of *An. atroparvus* showed a smaller number of ookinetes and a higher ratio old retort cells/ookinetes when compared with *An. stephensi* SXK Nij. females. Blood meals were also smaller, and in *An. atroparvus* Sample 2 stomachs were almost empty. *Anopheles atroparvus* slides showed less red intact cells than control slides.

VI.4.2. ARTIFICIAL INFECTIONS

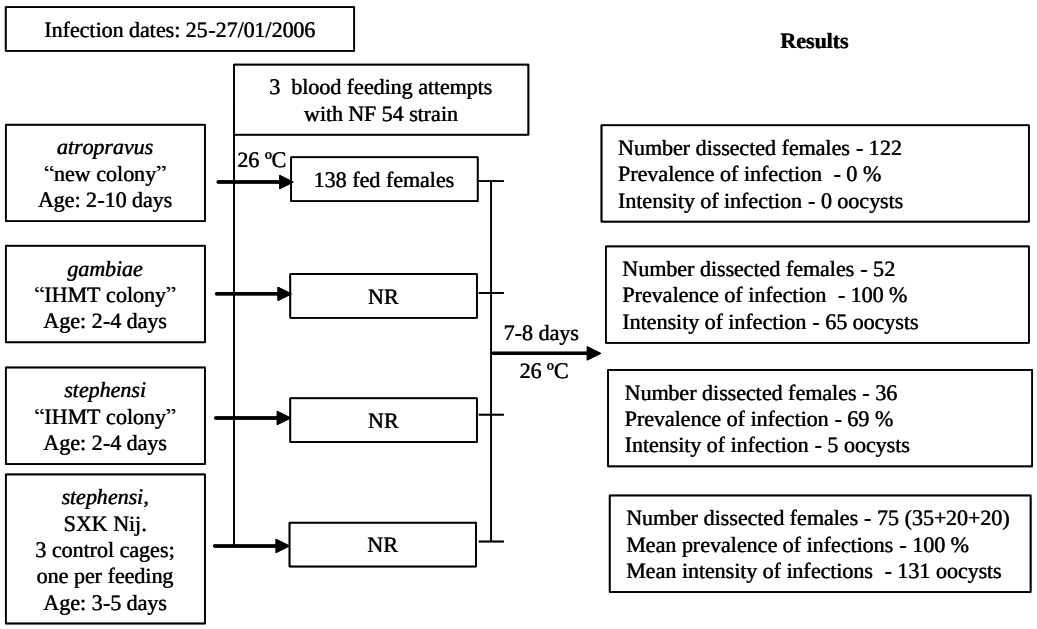
The infection procedures tested and their respective results are shown in the following Diagrams:

i. Infection of specimens with *P. falciparum* NF 54 strain – Standard procedures.

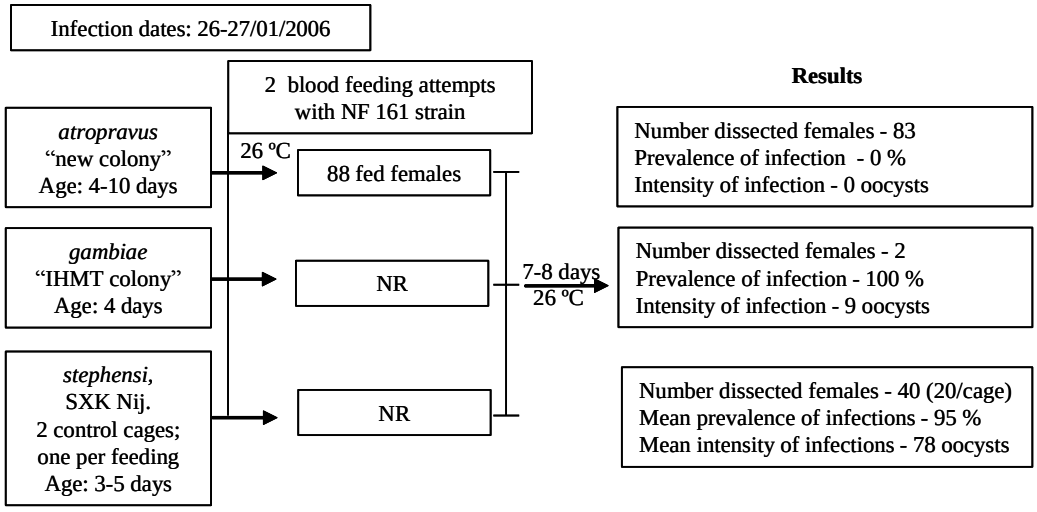
a.



b.

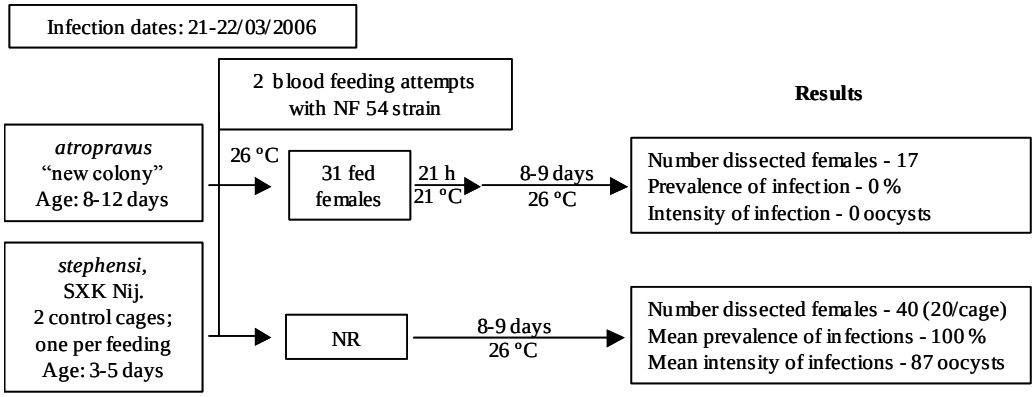


ii. Infection of specimens with *P. falciparum* NF 161 strain – Standard procedures.

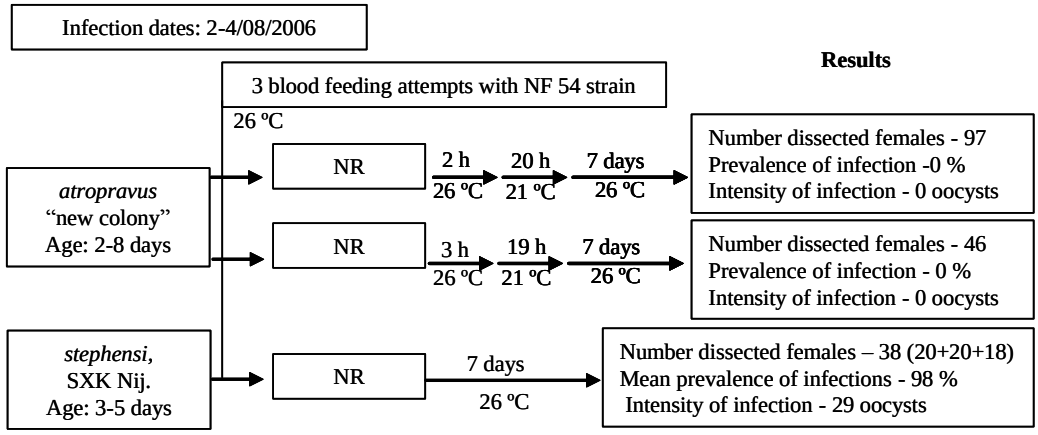


iii. Infection of specimens with *P. falciparum* NF 54 strain – Single blood meal; variation of room temperature of infection.

a.

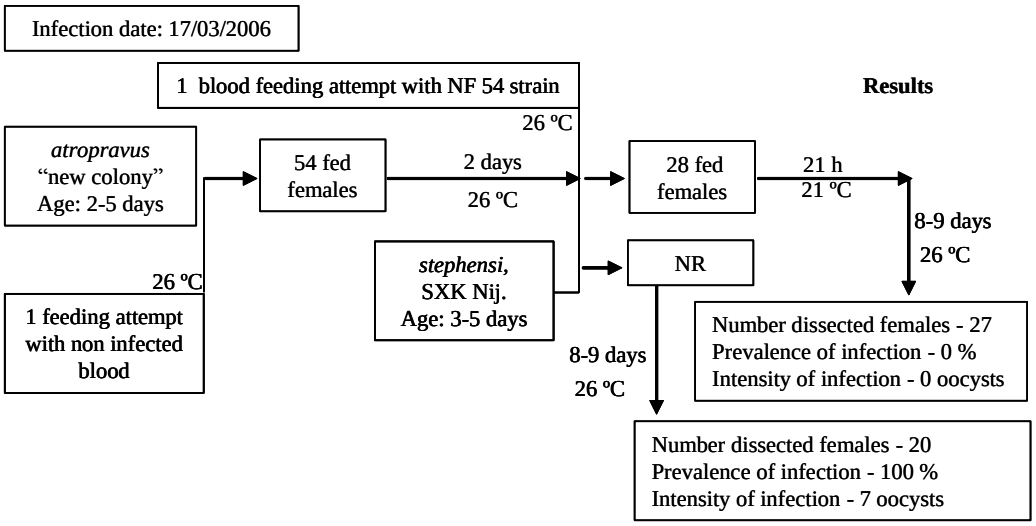


b.

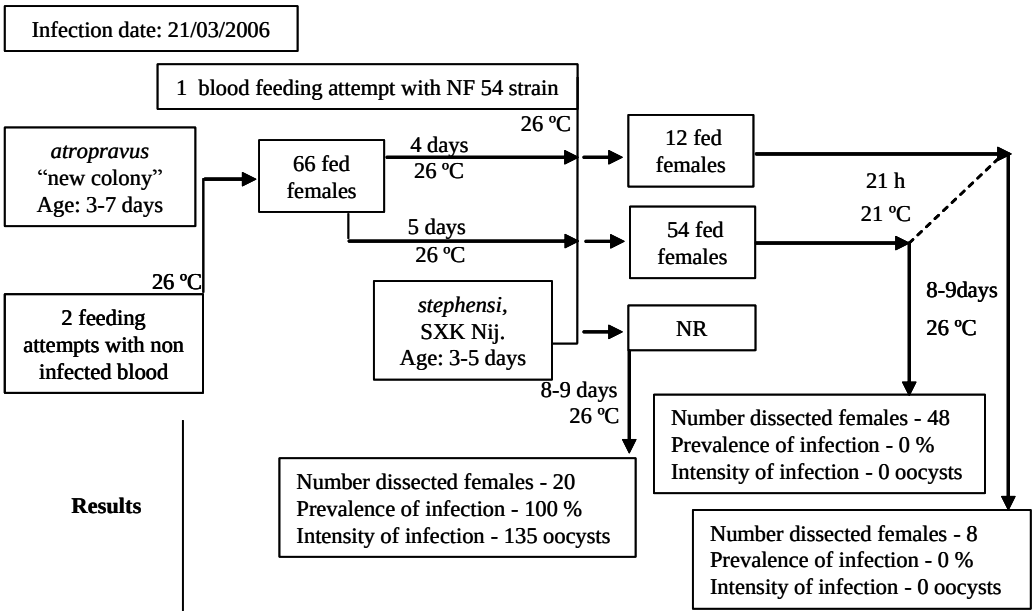


iv. Infection of specimens with *P. falciparum* NF 54 strain – Two blood meals, the first non-infectious; variation of time between feeds.

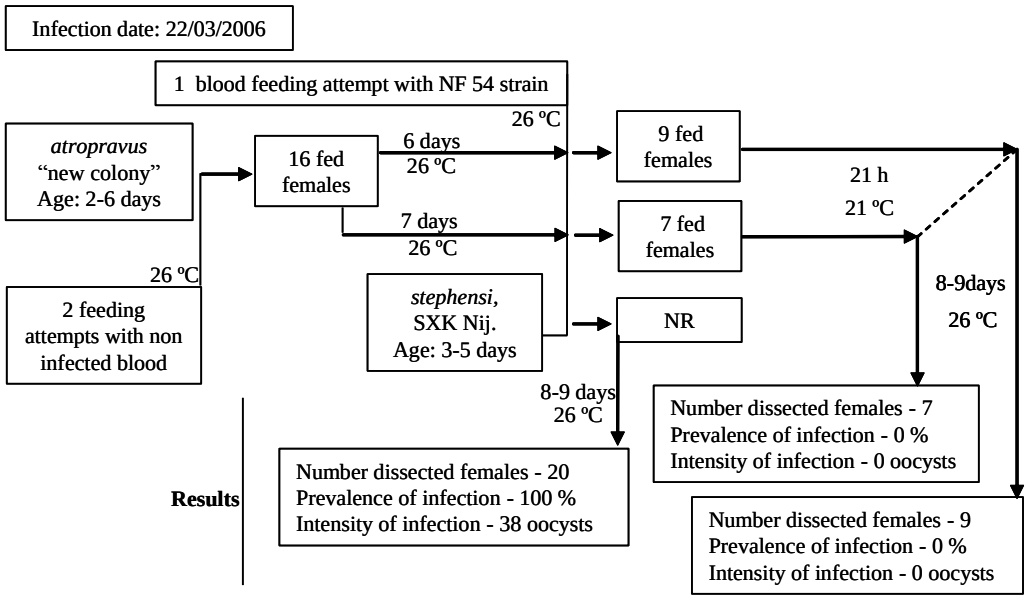
a.



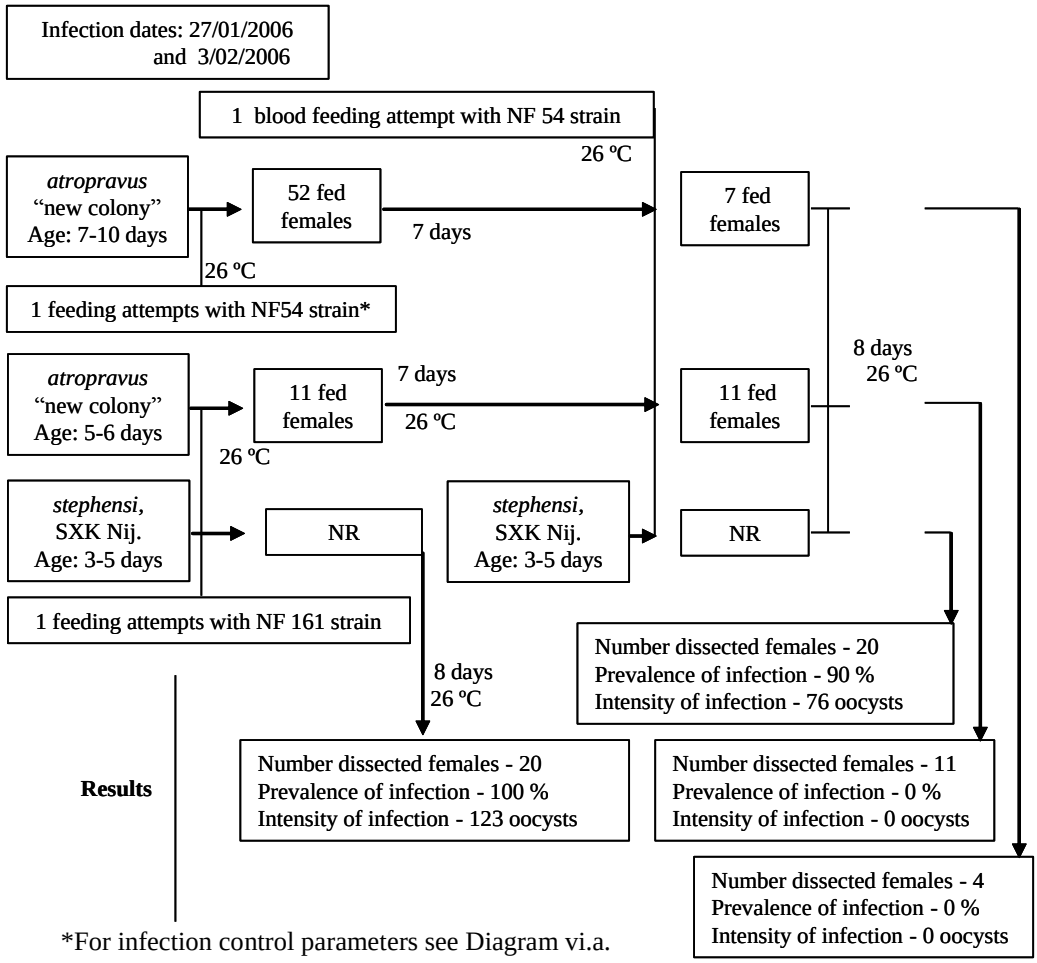
b.



c.



v. Infection of specimens with *P. falciparum* NF 54 strain and NF 161– Two infectious blood meals with variation of *Plasmodium* strains.

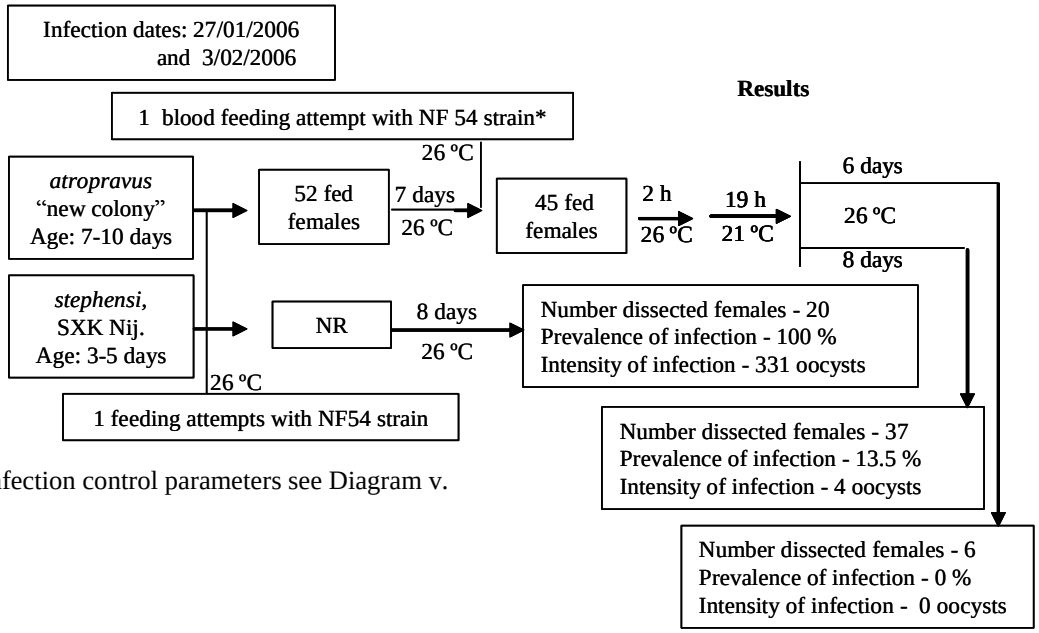


*For infection control parameters see Diagram vi.a.

vi. Infection of specimens with *P. falciparum* NF 54 strain– Two infectious blood meals, variation of room temperature and time between infective feeds.

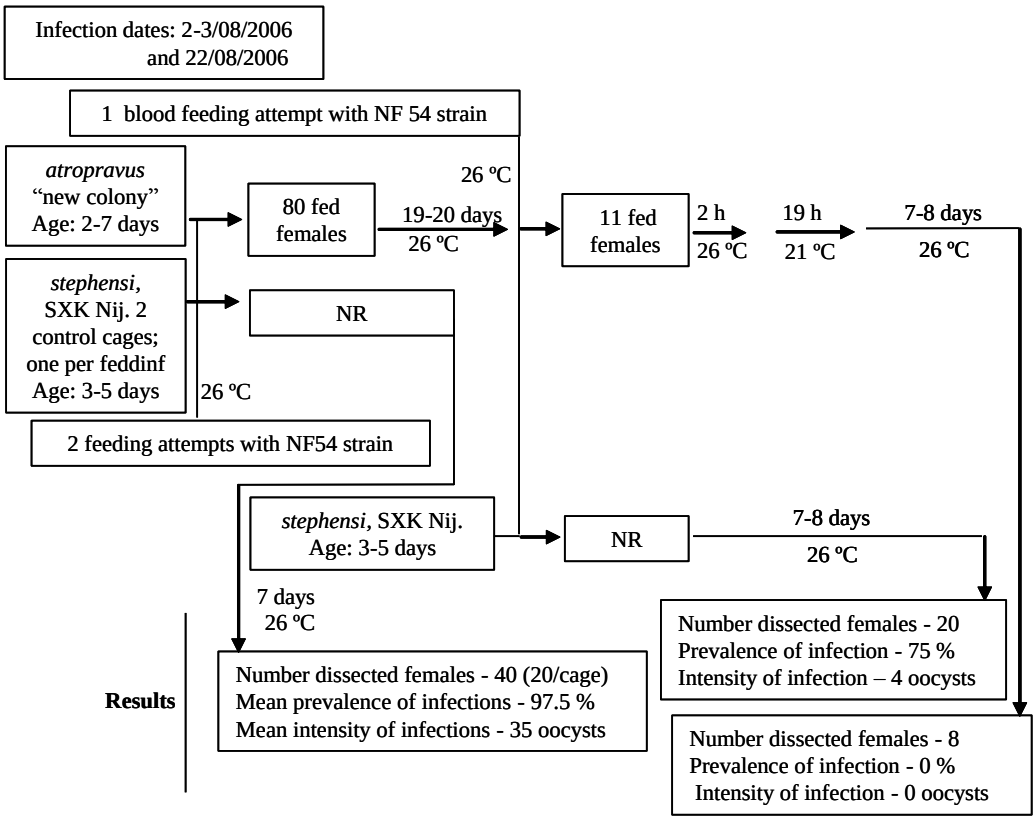
a.

.

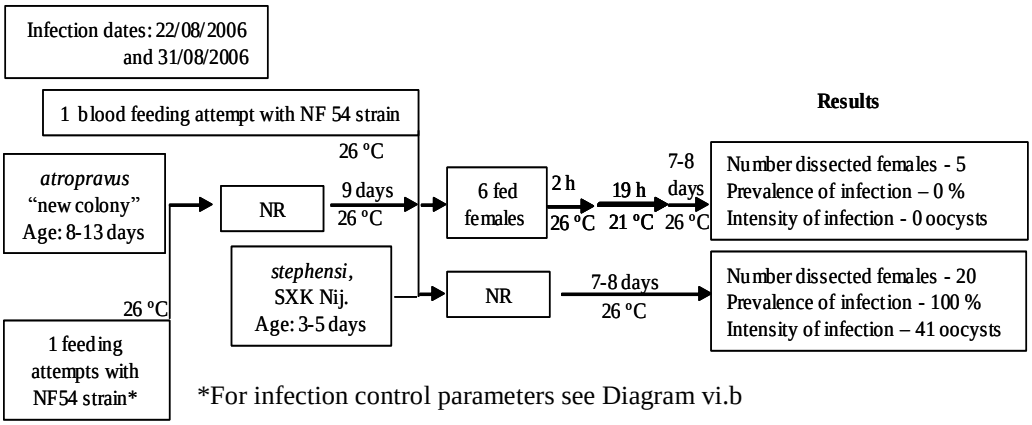


* For infection control parameters see Diagram v.

b .

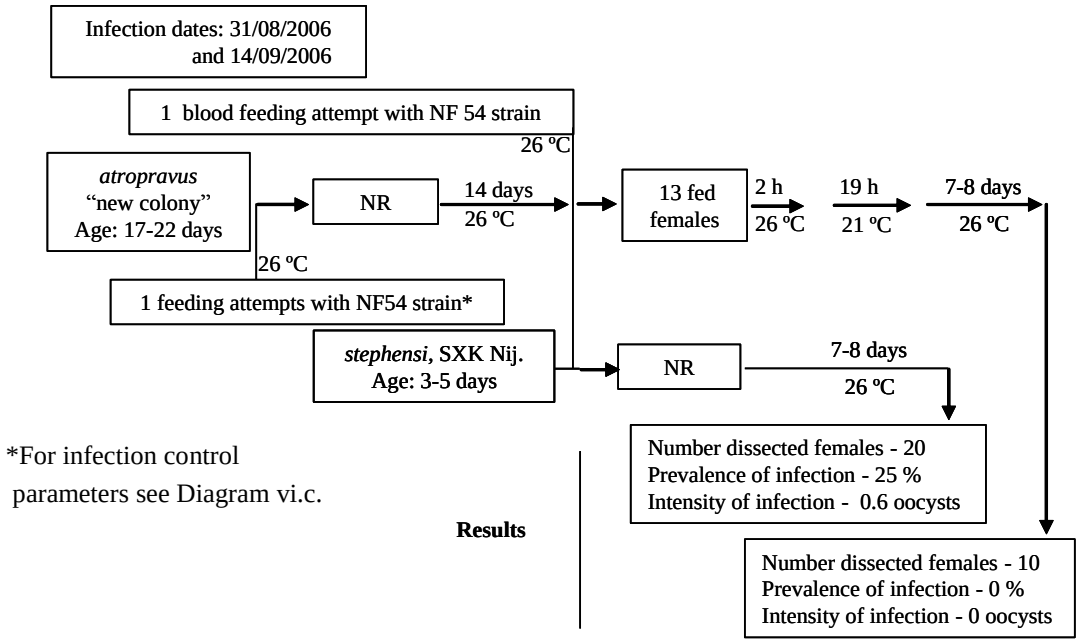


c.



*For infection control parameters see Diagram vi.b

d.



*For infection control parameters see Diagram vi.c.

Prevalence of infection in control specimens were always equal or above 90%, with the exception of experiments of days 22/8/2006 and 14/09/2006 (Diagrams vi.b. and vi.d., respectively) that presented percentages of infection of 75% and 25%, respectively. Mean intensity of infection ranged from 0.6 up to 331 oocysts/midgut. The infection of *An. stephensi* and *An. gambiae* specimens of IHMT colonies was achieved by applying the standard procedures. *Anopheles gambiae* prevalence and intensity of infection were similar to *An. stephensi* NXK Nij. colony specimens. However, the results of the latter compared with those of *An. stephensi* IHMT colony were found to be significantly different (Table VI.5.).

Table VI.5. Comparison of prevalence and intensity of infection with *Plasmodium falciparum* NF 54 between *Anopheles gambiae* and *An. stephensi* IHMT colony specimens and *An. stephensi* NXK Nij. females.

	Colony		N	Statistics
Prevalence of infection	***An. gambiae* IHMT***	100 %	52	ND
	An. stephensi* NXK Nij.	100 %	55	
	An. stephensi* IHMT*	69 %	36	X^2_y= 10,73; d.f.=1 *P*= **0,0011**
	An. stephensi* NXK Nij.*	100 %	35	
Intensity of infection	***An. gambiae* IHMT***	65[a]	52	*U*=898.50 *P*= 0.921
	An. stephensi* NXK Nij.	59 [a]	55	
	An. stephensi* IHMT*	5 [a]	36	*U*=20.00 ***P*<0.001**
	An. stephensi* NXK Nij.*	39 [a]	35	

N: number of dissected females. * Data referring to infection's days 25-26/01/2006. ** Data referring to infection's day 25/01/2006. [a] : mean number of oocysts *per* dissected female. X^2_y : Chi-square test of 2X2 contingency tables with Yates correction for continuity. *U*: Mann-Whitney statistic, 2-tailed test. Bold: significant at level < 0.01.

The infection of *An. atroparvus* females was achieved only once (Diagram vi.a.). Specimens took two infective feeds with a seven days interval. Mosquitoes were kept always at 26ºC with the exception of a 19 h period that occurred two hours after the second meal and during which females were placed at 21ºC. Infection prevalence was 13.5% and the mean number of oocysts *per* infected female was 14, ranging between 2 to 75 oocysts *per* infected midgut.

VI.5. DISCUSSION AND CONCLUSIONS

Anopheles stephensi NXK Nij. colony was selected for its high susceptibility to *P. falciparum* and protocols were optimised to achieve 100% of prevalence in all infections. Therefore, it is not surprising that in all infection experiments control specimens were always successfully infected. In 11 of the 25 infective feeds carried out during this study, *An. stephensi* NXK Nij. exhibited 100% of infection prevalence. The possibility of *P. falciparum* strain NF 54 being less effective in infecting mosquitoes from other colonies/species was discarded, as using the same procedures, *An. gambiae* and *An. stephensi* from IHMT colonies were also successfully infected. The reduced prevalence of infection and oocyst load of *An. stephensi* IHMT specimens compared with *An. stephensi* NXK Nij. may be due to the known intraspecific variation in mosquito susceptibility (*e.g.* Boyd, 1949b; Warren *et al.*, 1977; Kitthawee *et al.*, 1990), in this case resulting from genetic and environmentally-induced differences between colonies.

Ookinete intensity of infection was smaller in *An. atroparvus* when compared with control mosquitoes. Twenty one hours after feeding *An. atroparvus* females showed smaller blood meals or nearly empty stomachs and less intact red cells than *An. stephensi* NXK Nij. These differences may result from a reduced intake of blood during the infective meal or a faster digestion of the blood meal performed by *An. atroparvus* females, two factors that can influence infection success (Vanderberg, 1988; Ponnudurai *et al.*, 1989; Feldmann *et al.*, 1990; Vaughan, 2007). Although none of the above-mentioned hypothesis can actually be proven, it was observed that *An. atroparvus* tended to take smaller meals when compared with control females. This may happen for two reasons: female's natural feeding behaviour, which leads them to take more than one meal during their first gonotrophic cycle (see Chapter V), being the first feed, as in other Anopheline species, a partial meal (Hogg *et al.*, 1996; Charlwood *et al.*, 2003), or; the lesser adaptation of *An. atroparvus* to membrane feeding, since specimens came from a recently established colony where females were fed on live hosts. *Anopheles atropa*rvus females also showed a higher ratio of old retort cells/ookinetes which can result from a slower or retarded parasite development.

Of all the attempts carried out during this study to artificially infect *An. atroparvus* specimens, oocysts were observed in female midguts only once. This was accomplished after changing protocol procedures regarding the number of infective blood meals and the room temperature during mosquito infection. The decision for submitting females to an extra feed derived from above-mentioned observations and from the fact that mosquito's nutritional resources may play a relevant role in vector competence. It has been observed that mosquito cohorts previously fed on blood show higher percentage of infected specimens (Okech *et al.*, 2004a). Moreover, temperature-related differences in *Plasmodium* infection prevalence and intensity have been known for long (Young & Burgess, 1961). Although temperatures, between 21ºC and 27ºC, seem to have no influence in *P. falciparum* ookinete and oocyst infection rates or densities (Noden *et al.*, 1995), infection success is reduced at 30ºC and 32ºC (Noden *et al.*, 1995; Okech *et al.*, 2004b). In our study area, a former malaria region where *P. falciparum* was responsible for 70% of infections (Cambournac, 1942), averages of mean daily temperature of the hottest months are usually bellow 21ºC (see Figure III.1, Chapter III). In order to mimic natural ambient conditions and also to delay blood meal digestion, females fed for the second time were submitted to the following temperature cycle: 2 h at 26ºC, which mimics sunset period, 19 h at 21ºC, corresponding to an extended night and morning periods and then back to 26ºC to allow early sporogony to be completed. Lowering ambient temperature to 21ºC during a period of 19-21 h should not affect parasite development (Noden *et al.*, 1995) and may decrease the detrimental effect of accelerated digestion on the transition of ookinetes to oocysts (Vaughan *et al.*, 1994). Based on these results, both factors (temperature and extra blood meal) seem to equally contribute, either to the successful

outcome of the oocyst formation or co-interacting with a key factor of parasite development, since no infection was produced when only one of the parameters was changed (Diagrams iii.a, b and v). The exact procedure which led to oocyst formation was never entirely repeated. In replicas (Diagrams vi.b,c and d) both time between blood meals and mosquito age varied and the number of dissected females was always small. Furthermore, in these assays, the control mosquitoes showed the lowest values of infection prevalence and intensity ever recorded in this study. This reduced parasite infectivity may also have concurred to *An. atroparvus* infection's failure.

Anopheles atroparvus was always considered a less efficient malaria vector in comparison to its sibling species from North Africa, *An. labranchie*, and from Asia, *An. sacharovi* (Zulueta *et al.*, 1975; Bruce-Chwatt & Zulueta, 1980a). Regarding its competence, it is believed that only after a process of selection this species was able to transmit tropical strains of *P. falciparum* originally transported to Europe by traders, slaves and soldiers (Bruce-Chwatt & Zulueta, 1980a). This evolution path was interrupted by the disease eradication in Europe and the disappearance of European *P. falciparum* strains. Present *An. atroparvus* populations are considered to be refractory to tropical strains of this parasite. The results of this study partially support this assumption, since oocyst formation was observed in only five mosquitoes out of 736 dissected females in all experiments. Unfortunately, experiments were not carried out beyond the oocyst phase; in fact, in the single occasion when *An. atroparvus* infection was successful all mosquitoes were dissected for oocyst observation. Quantifying oocysts provides only limited information regarding sporogony dynamics and no conclusion can be drawn regarding sporozoite formation and invasion of salivary glands. However, infection intensity was the highest recorded (Table VI.1.), prevalence rate was within the limits observed for other highly competent, artificially infected malaria vectors (Okech *et al.*, 2004c; 2007), and oocysts appeared to be completely developed and viable under microscopic observation. Considering that direct feeding may produce significantly higher infection rates than membrane feeding (Bonnet *et al.*, 2000), and mortality during the transition ookinete-oocysts is much greater for NF 54 *falciparum* strain than for African and Asian wild populations (Vaughan, 2007), early and mid sporogony may be more easily achieved by *P. falciparum* when parasitizing *An. atroparvus* in nature, rather than in artificial conditions. Furthermore, failure to become infected is not an absolute guide to refractoriness and when assessing transmission data sample sizes should not be less than 50, ideally being 100 mosquitoes (Medley *et al.*, 1993). In this study only 75 mosquitoes received two infective blood meals and went through the cycle of temperature changes mentioned above. Of these, only 19 specimens were in comparable conditions concerning mosquito age and parasite infectivity of the cohort which presented the infected specimens.

These sample sizes are therefore too small to sustain any definitive conclusion regarding *An. atroparvus* susceptibility status to tropical strains of *P. falciparum.*.

This study confirms that *An. atroparvus* is, at the most, a low competent vector regarding tropical strains of *P. falciparum*. It stresses the role of temperature and nutritional factors on infection success. This supports the hypothesis of Lambrechts *et al.* (2006) that environmental variation can greatly reduce the importance of genes in modulating mosquito resistance to *Plasmodium* infection. This study emphasises the importance of adapting laboratory-controlled experiments to conditions as similar as possible to those existing in nature. Although these results should be approached with care due to the above-mentioned study limitations, at this stage, *An. atroparvus* complete refractoriness to tropical *P. falciparum* strains seems less certain than at the beginning of this study.

Chapter VII

CONCLUDING REMARKS

VII.1. MAIN FINDINGS

This study has produced an update on some aspects of *Anopheles atroparvus* bionomics in Portugal and, for the first time, a comprehensive assessment of its vectorial capacity and competence for the transmission of malaria parasites. It has also been attempted to determine if this species' biology and behaviour has suffered any major switches since the time that malaria was endemic in Portugal.

As in the past, *An. atroparvus* remains the most frequent Anopheline and one of the most abundant mosquito species in Comporta Region. Although it is difficult to compare present data with that of previous studies, due to methodological differences, evidences suggest that the present abundance estimates should not be far different from those during the malaria period.

Anopheles atroparvus showed to be a species with marked endophilic behaviour and a crepuscular biting activity. Females were found to be mainly zoophilic as previously described but the *HBI* computed was smaller than that recorded during the time of endemic malaria. However, when data from the 1940's was re-analysed using only the same type of mosquito sampling also used in this study (*i.e.* resting collections in animal dwellings) differences were less striking or even absent. Therefore, these differences are more likely to reflect sampling effects rather than any changes in mosquito behaviour.

To establish the receptivity of Comporta region for malaria re-introduction the vectorial capacity (C) of *An. atroparvus* was assessed under several hypothetical conditions. Results showed that in the worst case scenario, nine potential new cases per day could be generated in the human population, if a gametocyte carrier came into the area. This value was estimated for *Plasmodium vivax* considering that females rarely or never ingest sugar meals and that, independently of their abundance, they inflicted 38 bites *per* day in each human that lives in the Comporta region (*ma*=38). For *P. falciparum* the highest C was eight, obtained with the same conditions as for *P. vivax*. With the exception of August 2001, the C=1 threshold (*i.e.* the possible occurrence of one autochthonous case) was only surpassed during winter/spring months due to an extremely high parity rate (and thus also a high daily survival rate) of the female population. However, these are the same months when mosquito abundance was lowest and thus the real *ma* is likely to be much smaller than the one used for estimating C. Therefore, one can foresee that the receptivity of the area to the re-emergence of malaria is very limited.

Only one batch of 37 *A. atroparvus* out of more than 2,200 that were sent to Nijmegen Medical Centre was infected with *P. falciparum*. In this experiment, infection prevalence was 13.5% and oocysts seemed viable. It would be desirable to repeat this procedure in order to

determine if produced sporozoites would be able to generate an infection in a naive host. It would also be of major importance to determine the infectivity of *An. atroparvus* for *P. vivax*. Given that the estimated vectorial capacity of *An. atroparvus* was higher for this parasite, such in the same way should be the risk of re-introduction of *vivax* malaria. Furthermore, the claimed refractoriness of *An. atroparvus* to tropical strains of *P. falciparum* was never observed for *P. vivax* strains (Table VI.1, Chapter VI).

VII.2. MALARIA RISK ASSESSMENT

The results obtained in Chapters V and VI provide the means to assess the risk of malaria re-emergence using the basic reproduction rate (R_0) model (Alten *et al.*, 2007; Smith *et al.*, 2007). This model describes the rate of potential secondary cases originating from a single case throughout its duration (see Chapter I). In malaria-free regions R_0 is given by the product of vectorial capacity and competence (*i.e.* R_0=C*c) estimated for the local mosquito population. Thus, in Comporta Region, R_0 for *P. falciparum* should be equal to 1.08 (*i.e.* 8*0.135). A R_0 above one indicates that the number of people infected by the parasite increases. Therefore, theoretically, an outbreak of malaria in Comporta region is possible if all the required conditions meet at a proper time. However, the risk is still very low as the R_0 value barely surpasses the threshold limit, in spite of all parameters having been overestimated.

A low R_0 value agrees with the post-eradication history of malaria in Portugal. A single autochthonous case has been detected in Portugal after malaria eradication (Bruce-Chwatt & Zulueta, 1980a). Even during the great influx of repatriates from the former Portuguese territories of Africa (1975-76) malaria transmission did not re-emerge in Portugal. The main reason advocated for explaining this phenomenon was the refractoriness of *An. atroparvus* to transmit tropical strains of *P. falciparum*. Although this definitely has contributed to the final outcome, it does not explain the absence of autochthonous cases by *P. vivax.* As to *An. atroparvus* biology and behaviour, these traits do not seem to have dramatically changed over the past five decades. Therefore any explanation based on a behavioural switch or dramatic decrease of abundances is not sustained by the current evidences.

What has altered from malaria endemic days was in fact the rice culture. Nowadays the number of workers and the type of labour developed in the rice fields is completely different. Rice is seeded by aeroplane instead of being planted by hand. Harvesting is made by machinery. The displacement of thousands of labours into the rice fields each year, living in badly constructed huts next to the paddies, no longer occurs. The close contact between

Man and mosquitoes was broken. In spite of *An. atroparvus* tendency to feed on animals, the high availability of human hosts inside resting facilities and next to the breeding sites would have greatly promoted malaria transmission in the past. These conditions have disappeared.

Altogether, the results obtained in this study support the idea that the establishment of malaria in Portugal is a possible but unlikely event in the present ecological conditions.

VII.3. PREDICTING THE FUTURE

In what conditions may malaria re-emerge in Portugal? Three scenarios can be hypothesised:

(i) A first scenario would imply little change on ecological conditions other than climate with an increase in the number of days *per* year with favourable temperature for both parasite and mosquito development. In this case the increased risk should not be significant if the population of *An. atroparvus* favoured hosts (pigs, sheep, goats and rabbits) was able to accommodate the extra number of blood feeds induced by the rise of temperature.

(ii) A second scenario would involve a drastic reduction of the population of non-human *An. atroparvus* hosts. If by human decision or due to an exceptional event (*e.g.* veterinary disease) most livestock disappeared from the area but mosquito breeding conditions were kept unchanged, malaria risk would increase due to enhanced human-vector contact. This could generate the most similar situation to the one that existed in malaria endemic time.

(iii) The third scenario concerns the possibility of the introduction of a new malaria vector species. The establishment of tropical species, in particular from the African continent, may be regarded as difficult in a short-term, due to two main reasons: the effect of the Sahara desert as a barrier to mosquito dispersal, and; the ecological and climatic differences between tropical and temperate ecosystems, that only long-term changes would ameliorate.

There is also the risk of other more efficient malaria vector species of the *An. maculipennis* complex to extend their distribution areas further northwest. However, species such as *An. labranchiae* and *An. sacharovi* would have first to overcome the apparent superior competitiveness of *An. atroparvus* in order to successfully establish themselves into Northwestern-European regions. Even if this would occur, the impact in malaria re-emergence is not straightforward. Currently facing much higher numbers of malaria imported cases than Portugal (Jelinek *et al.*, 2002), Italy remains a malaria-free Country in spite of having these three vector species (*An. atroparvus*, *An. labranchiae* and *An. sacharovi*) in its territory (Jetten & Takken, 1994).

A shift in medical importance of a secondary vector should also be taken into account. *Anopheles plumbeus* has been incriminated more than once in malaria transmission but its scanty distribution and sylvatic breeding habits (tree-hole breeder) do not render it a relevant role as a vector. However, certain populations have started breeding in cess-pools (Alten *et al.*, 2007). Such a behavioural shift may result in a significant increase of abundance of highly anthropophagic *An. plumbeus*, with potential to establish malaria transmission in the presence of a gametocyte carrier.

Concurrent with these three scenarios there is always the possibility of a large-scale introduction of a parasite species or strain for which local *An. atroparvus* populations are highly competent. From this study, based on the vectorial capacity estimates of local *An. atroparvus*, it can be concluded that the risk occurrence of a malaria focus in Portugal due to *P. vivax* is higher when compared with *P. falciparum*. This is derived from the differences between the two parasite species in the length of their sporogonic cycle. It is generic conclusion valid for all Europe and shared by others (Jetten & Takken, 1994). However, the vectorial competence of *An. atroparvus* for strains of *P. falciparum* from places like Turkey or Countries of the East Europe region from where malaria never entirly disappear was never investigated. Addressing this issue may help to understand what is the real risk of malaria re-emergence in Europe.

VII.4. FUTURE PROSPECTS

Some aspects of the biology of *An. atroparvus* deserve a more detailed characterisation. One of those aspects is the species feeding behaviour and biology during the first gonotrophic cycle (i_0). Do the females of this species in nature feed mainly on blood, rarely or never ingesting sugar meals? Are the values for i_0 length estimated in laboratorial conditions, a true measure of what takes place in the field? How can one better assess host preferences in order to estimate a more accurate human blood index? These are all subjects that clearly deserve to be further investigated due to their importance in malaria epidemiology.

The studies on the susceptibility of *An. atroparvus* to tropical strains of human malaria parasites should continue. The exact procedure which led to oocyst formation must be repeated with larger number of mosquitoes in order to follow up infection until the sporozoite invasion of salivary glands. Results of these experiments are of obvious importance for the fully comprehension of the risk of malaria re-emergence in Portugal and in Europe. Independently of the outcome of these studies regarding *An. atroparvus* vectorial competence, this type of experimental approach could also help to understand the influence of non-genetic factors in modulation of mosquito resistance to *Plasmodium* infection. It can also give

insights regarding mosquito refractoriness mechanisms that could be useful for transgenic mosquito technology. All this type of information can eventually be incorporated in the design of novel control strategies to be applied in malaria endemic areas.

When facing the possibility of a malaria outbreak it seems to be important to have information regarding the insecticide susceptibility status of local *An. atroparvus* populations. Insecticides remain a crucial component of vector control. No information is available on current levels and mechanisms of insecticide resistance of *An. atroparvus* and this will be an essential requirement if any vector control strategy needs to be implemented. It is noteworthy that, besides malaria, *An. atroparvus* has also been implicated in the transmission of other pathogens, such as West Nile virus (Filipe, 1972).

Information on the genetic structure of *An. atroparvus* is also desirable. Knowledge on levels of genetic variation and the degree of genetic differentiation among populations could be of major importance to infer the potential for vector-mediated dissemination of malaria infections under a scenario of local introduction of parasites. It is also important for the implementation of vector control programs since it can help to predict the spread of genes of interest (*e.g.* genes that confer insecticide resistance) and to monitor the impact of control strategies.

What has been learnt for *An. atroparvus* population of Comporta region can be extrapolated to the rest of the Country by means of mathematical modelling. Based on mosquito abundance rates estimated over a period of four years together with data regarding climate and environment one can elaborate models to predict mosquito abundance variation in time and/or space. This practical tool could help in assessing at that given moment, what are the risk areas for malaria transmission. This type of tools can be brought quickly into action and contribute to real-time decision making.

Regardless of what the future may bring for malaria introduction in Portugal, the negative outcome of this disease will most likely be hampered by the socio-economic standards of the Country. Even in the advent of a large epidemic, Portugal has an organised health system. Efficient anti-malarial drugs are available as well as powerful synthetic insecticides and operational knowledge. Its impact would only be remarkable if heath care facilities collapsed. Still the political contextualisation in a European Union environment would most probably promote unprecedented integrated efforts towards the containment of the disease. Above all, malaria was a poverty disease in European countries. It still is a poverty disease in developing nations.

BIBLIOGRAPHY

BIBLIOGRAPHY

Afrane Y.A., Lawson B.W., Githeko A.K. & Yan G. 2005. Effects of microclimatic changes caused by land use and land cover on duration of gonotrophic cycles of *Anopheles gambiae* (Diptera: Culicidae) in western Kenya highlands. *Journal of Medical Entomology* 42: 974-980.

Afrane Y.A., Zhou G., Lawson B.W., Githeko A.K. & Yan G. 2006. Effects of microclimatic changes caused by deforestation on the survivorship and reproductive fitness of *Anopheles gambiae* in western Kenya highlands. *The American Journal of Tropical Medicine and Hygiene* 74: 772-778.

Akbari M., Hansen M.D., Halgunset J., Skorpen F. & Krokan H.E. 2005. Low copy number DNA template can render polymerase chain reaction error prone in a sequence-dependent manner. *The Journal of Molecular Diagnostics* 7: 36-39.

Alberts B., Bray D., Lewis J., Raff M., Roberts K. & Watson J.D. 1994. *Molecular biology of the cell.* Garland Publishing, Inc., New York. 1294 pp.

Almeida A.P.G., Galão R.P., Sousa C.A., Novo M.T., Pinto J., Piedade J. & Esteves A. 2008 (in press). Potential mosquito vectors of arboviruses in Portugal: species, distribution, abundance and West Nile infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene* TRSTMH-D-07-00446.

Alsop Z. 2007. Malaria returns to Kenya's highlands as temperatures rise. *Lancet* 370: 925-926.

Alten B., Kampen H. & Fontennile D. 2007. Malaria in southern Europe: resurgence from the past?, pp. 35-58, *Emerging pest and vector-borne diseases in Europe.* Wageningen Academic Publishers, Wageningen.

Antunes F., Forte M., Tavares L., Botas J., Carvalho C., Carmona H. & Araujo F.C. 1987. Malaria in Portugal 1977-1986. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 81: 561-562.

Arav-Boger R. & Shapiro T.A. 2005. Molecular mechanisms of resistance in antimalarial chemotherapy: the unmet challenge. *Annual Review of Pharmacology and Toxicology* 45: 565-585.

Ayala F.J., Escalante A.A., Lal A.A. & Rich S.M. 1998. Evolutionary relationships of human malaria parasites, pp. 285-300, *Malaria: parasite biology, pathogenesis and protetion*. American Society for Microbiology Press, Washington.

Baldari M., Tamburro A., Sabatinelli G., Romi R., Severini C., Cuccagna G., Fiorilli G., Allegri M.P., Buriani C. & Toti M. 1998. Malaria in Maremma, Italy. *Lancet* 351: 1246-1247.

Bali B. 1936. Étude sur les nervures transversales dans les ailes d'*Anopheles maculipennis* de l'Asie mineure. *Bulletin de la Société de Pathologie Exotique* 29: 518-521.

Ballinger-Crabtree M.E., Black IV W.C. & Miller B.R. 1992. Use of genetic polymorphisms detected by the random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for differentiation and identification of *Aedes aegypti*

subspecies and populations. *The American Journal of Tropical Medicine and Hygiene* 47: 893-901.

Bates M. 1939a. Variation in the antepalmate hairs of larvae of the *Anopheles maculipennis* complex. *Rivista di Malariologia* 18: 299-312.

Bates M. 1939b. Hydridization experiments with *Anopheles maculipennis* complex. *American Journal of Hygiene, sec. C.* 29: 1-6.

Bates M. 1940. The nomenclature and taxonomic status of the mosquitoes of the *Anopheles maculipennis* complex. *Annals of the Entomological Society of America* 33: 343-356.

Bates M. & Hackett L.W. 1939. The distinguishing characteristics of the populations of *Anopheles maculipenni*s found in southern Europe. *Verhandlungen des VII internationalen Kongresses fur Entomologie 1938* 3: 1555-1569.

Bates M., Beklemishevi W.N. & La Face L. 1949. Anophelines of the Paleartic region, pp. 419-442, *Malariology.* W.S. Saunders Company, Philadelphia and London.

Bayoh M.N. & Lindsay S.W. 2004. Temperature-related duration of aquatic stages of the Afrotropical malaria vector mosquito *Anopheles gambiae* in the laboratory. *Medical and Veterinary Entomology* 18: 174-179.

Beier J.C. 1996. Frequent blood-feeding and restrictive sugar-feeding behavior enhance the malaria vector potential of *Anopheles gambiae s.l.* and *An. funestus* (Diptera:Culicidae) in western Kenya. *Journal of Medical Entomology* 33: 613-618.

Bibikova V.A., Dashkova N.G., Sulaimanov G.D., Rasnitsyn S.P. & Tiburskaia N.A. 1977. Transmission of the Brazilian strain of tertian malaria by *Anopheles maculipennis atroparvus* mosquitoes. *Meditsinskaia Parazitologiia i Parazitarnye Bolezni* 46: 63-67.

Bonnet S., Gouagna C., Safeukui I., Meunier J.Y. & Boudin C. 2000. Comparison of artificial membrane feeding with direct skin feeding to estimate infectiousness of *Plasmodium falciparum* gametocyte carriers to mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94: 103-106.

Borges F. 2001. A malária no vale do rio Sado, pp. 59-64, *Memórias do Instituto de Malariologia de Águas de Moura. Da luta anti-palúdica ao museu.* Câmara Municipal de Palmela/Centro de estudos de vectores e doenças infecciosas, Palmela.

Boyd M.F. 1949a. Historical review, pp. 3-25, *Malariology*. W.S. Saunders Company, Philadelphia and London.

Boyd M.F. 1949b. Epidemiology: Factors related to the defenitive host, pp. 608-697, *Malariology*. W.S. Saunders Company, Philadelphia and London.

Braks M.A., Juliano S.A. & Lounibos L.P. 2006. Superior reproductive success on human blood without sugar is not limited to highly anthropophilic mosquito species. *Medical and Veterinary Entomology* 20: 53-59.

Briegel H. 1990. Fecundity, metabolism, and body size in *Anopheles* (Diptera: Culicidae), vectors of malaria. *Journal of Medical Entomology* 27: 839-850.

Brower V. 2001. Vector-borne diseases and global warming: are both on an upward swing? Scientists are still debating whether global warming will lead to a further spread of mosquitoes and the diseases they transmit. *EMBO Reports* 2: 755-757.

Bruce-Chwatt L.J. 1988. History of malaria from prehistory to eradication, pp. 1-60, *Malaria. Principles of Malariology*. Churchill Livinstone Inc., London.

Bruce-Chwatt L.J. & Zulueta J. 1977. Malaria eradication in Portugal. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 71: 232-240.

Bruce-Chwatt L.J. & Zulueta J. 1980a. *The rise and fall of malaria in Europe. A historico-epidemiological study.* Oxford University Press, Oxford. 240 pp.

Bruce-Chwatt L.J. & Zulueta J. 1980b. *Sezonismo*. Ministério dos Assuntos Sociais, Lisboa. 96 pp.

Bullini A.P.B., Cianchi R., Sabatini A., Coluzzi M. & Bullini L. 1980a. Ricercha elettroforetiche su specie paleartiche del complesso *Anopheles maculipennis* (Diptera, Culicidae), pp. 255-259, *Atti XII Congresso Nazionale Italiano di Entomologia, Roma.*

Bullini L., Bullini A.P.B., Cianchi R., Sabatini A. & Coluzzi M. 1980b. Tassonomia biochimica del complesso *Anopheles maculipennis*. *Parassitologia* 22: 290-292.

Buonomini G. & Mariani M. 1946. Qual'è il valore sistematico del nome *maculipennis* Meig.? *Rivista di Malariologia* 25: 101-107.

Buonomini G. & Mariani M. 1953. World Anophelines belonging to the subgenus *Maculipennia* Buonomini e Mariani, 1946. *Rivista di Malariologia* 32: 173-188.

Burkot T.R., Goodman W.G. & DeFoliart G.R. 1981. Identification of mosquito blood meals by enzyme-linked immunosorbent assay. *The American Journal of Tropical Medicine and Hygiene* 30: 1336-1341.

Butler B. 2004. Power to the people. *Nature* 430: 928-929.

Calheiros J.M. & Casimiro E. 2002. Human health, pp. 241-300, *Climate change in Portugal. Scenarios, impacts and adaptation measures. SIAM Project.* Gradiva-Publicações, Lda, Lisboa.

Cambournac F.J. 1942. *Sobre a epidemiologia do sezonismo em Portugal*. Sociedade Industrial de Tipografia, Lda, Lisboa. 235 pp.

Cambournac F.J. 1944. Culicidae (Diptera, Nematocera) da região de Águas de Moura. *Anais do Instituto Tropical* 1: 247-268.

Cambournac F.J. 1994. Contribution to the history of malaria epidemiology and control in Portugal and some other places. *Parassitologia* 36: 215-222.

Cambournac F.J. & Hill R.B. 1938. The biology of *Anopheles atroparvus* in Portugal. *Transactions of the Third International Congress of Tropical Medicine and Malaria* 2: 178-184.

Cambournac F.J. & Hill R.B. 1940. Observation on the swarming of *Anopheles maculipennis*, var. *atroparvus*. *The American Journal of Tropical Medicine* 20: 133-140.

Canalis A., De Negri A., Frizzi G. & Sepulcri P. 1954. Le specie del grupo "Maculipennis" identificate col metodo citogenetico dai focolai larvali in zona trattata con D.D.T. nelle province di Rodrigo e Venezia, pp. 7-28, *XVII Congresso Nazionale d'Igiene, Venezia.*

Canalis A., De Negri A. & Sepulcri P. 1956a. Nuovi orientamenti per lo studio delle specie anofeliche del grupo "Maculipennis" in zona malarica trattata con DDT. *Rivista di Malariologia* 35: 21-37.

Canalis A., De Negri A., Frizzi G. & Sepulcri P. 1956b. L'anofelismo nel Veneto dopo dieci anni di trattamento con disinfestanti ad azione residua. *Rivista di Malariologia* 35: 39-57.

Cano J., Descalzo M.A., Moreno M., Chen Z., Nzambo S., Bobuakasi L., Buatiche J.N., Ondo M., Micha F. & Benito A. 2006. Spatial variability in the density, distribution and vectorial capacity of Anopheline species in a high transmission village (Equatorial Guinea). *Malaria Journal* 5: 21.

Casimiro E., Calheiros J.M., Santos F.D. & Kovats S. 2006. National assessment of Human Health effects of climate change in Portugal: Approach and key findings. *Environmental Health Perspectives* 114: 1950-1956.

Castro L., Cardoso A.I., Queiros L. & Goncalves G. 2004. Malária na região norte de Portugal (1993-2002). Caracterização epidemiológica. *Acta Médica Portuguesa* 17: 291-298.

Cha R.S. & Thilly W.G. 1993. Specificity, efficiency, and fidelity of PCR. *PCR Methods and Applications* 3: 18-29.

Charlwood J.D., Pinto J., Sousa C.A., Ferreira C., Petrarca V. & Rosario Vdo E. 2003. 'A mate or a meal'-pre-gravid behaviour of female *Anopheles gambiae* from the islands of Sao Tome and Principe, West Africa. *Malaria Journal* 2: 9.

Christophers R., Hackett L.W., James S.P., Missiroli A., Pittaluga G., Sergent E. & Swellengrebel N.H. 1935. Indirizzo alla determinazione delle varietá di *Anopheles maculipennis*. *Rivista di Malariologia* 14: 110-115.

Cianchi R., Sabatini A., Boccolini D., Bullini L. & Coluzzi M. 1980. Due entitá riproduttivamente isolate sotto il nome di *Aedes caspius* (Diptera, Culicidae). *Atti XII Congreso Nazionale Italiano di Entomologia, Roma.* 2: 269-272.

Cianchi R., Sabatini A., Bullini L. & Coluzzi M. 1981. Differenziazione morfologica e genetica nei complessi *Anopheles maculipenni*s e *Anopheles claviger*. *Parassitologia* 23: 158-163.

Clements A.N. 1999. *The biology of mosquitoes. Volume 2: Sensory reception and behavior*. CABI Publishing, New York. 740 pp.

Collado J.G. 1938. La distribución geográfica de las variedades de *Anopheles maculipennis* en Espana, con breves consideraciones acerca de su biología. *Rivista di Malariologia* 17: 276-279.

Collins F.H., Mendez M.A., Rasmussen M.O., Mehaffey P.C., Besansky N.J. & Finnerty V. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *American Journal of Tropical Medicine and Hygiene* 37: 37-41.

Coluzzi M. & Petrarca V. 1973. Aspirator with paper cup for collecting mosquitoes and other insects. *Mosquito News* 33: 249-250.

Constantinescu P. & Negulici E. 1967. The experimental transmission of *Plasmodium malariae* to *Anopheles labranchiae atroparvus*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 61: 182-188.

Cornejo O.E. & Escalante A.A. 2006. The origin and age of *Plasmodium vivax*. *Trends in Parasitology* 22: 558-563.

Corradetti A. 1934. Richerche sugli incroci tra le varietà *Anopheles maculipennis*. *Rivista di Malariologia* 13: 707-720.

Corradetti A. 1937. Sui caratteri morfologici degli ibridi derivati dall'incrocio tra *Anopheles maculipennis* var. *elutus* e *Anopheles maculipennis* var. *atroparvus*. *Rivista di Malariologia* 16: 42-45.

Costero A., Edman J.D., Clark G.G. & Scott T.W. 1998. Life table study of *Aedes aegypti* (Diptera: Culicidae) in Puerto Rico fed only human blood *versus* blood plus sugar. *Journal of Medical Entomology* 35: 809-813.

Cowman A.F. & Foote S.J. 1990. Chemotherapy and drug resistance in malaria. *International Journal for Parasitology* 20: 503-513.

Cox J. & Abeku T.A. 2007. Early warning systems for malaria in Africa: from blueprint to practice. *Trends in Parasitology* 23: 243-246.

Crabb C. 2002. Blaming malaria rise on climate change is simplistic. *Bulletin of the World Health Organization* 80: 334-335.

Cuadros J., Calvente M.J., Benito A., Arevalo J., Calero M.A., Segura J. & Rubio J.M. 2002. *Plasmodium ovale* malaria acquired in central Spain. *Emerging Infectious Diseases* 8: 1506-1508.

D'Alessandro G., Smiraglia C.B. & Lavagnino A. 1971. Futher studies on the biology of *Anopheles labranchiae labranchiae* Falleroni in Sicily. *World Health Organization, WHO/MAL/71.754*: 1-13.

Daskova N.G. & Rasnicyn S.P. 1982. Review of data on susceptibility of mosquitoes in the USSR to imported strains of malaria parasites. *Bulletin of the World Health Organization* 60: 893-897.

Davidson G. 1954. Estimation of the survival-rate of Anopheline mosquitoes in nature. *Nature* 174: 792-793.

De Buck A. & Swellengrebel N.H. 1929. Behaviour of dutch *Anopheles atroparvus* and *messeae* in winter under artificial conditions. *Annales de l' Institut Pasteur* 42: 1370-1389.

De Buck A., Shoute E. & Swellengrebel N.H. 1930. Racial differentiation of *Anopheles maculipennis* in Netherlands and its relation to malaria. *Rivista di Malariologia* 9: 97-109.

De Buck A., Schoute E. & Swellengrebel N.H. 1932. Further investigations on the racial differentiation of *Anopheles maculipennis* in Netherlands and its bearing on malaria. *Rivista di Malariologia* 11: 137-156.

De Buck A., Torren G.V.D. & Swellengrebel N.H. 1933. Report for the year 1932 on investigations into the racial composition of *Anopheles maculipennis* in Holland. *Rivista di Malariologia* 12: 265-280.

De Buck A., Schoute E. & Swellengrebel N.H. 1934. Crossbreeding experiments with dutch and foreign races of *Anopheles maculipennis*. *Rivista di Malariologia* 13: 237-263.

Degaetano A.T. 2005. Meteorological effects on adult mosquito (*Culex*) populations in metropolitan New Jersey. *International Journal of Biometeorology* 49: 345-353.

Detinova T.S. 1963. *Méthodes a appliquer pour classer par groupes d'âge les diptéres présentant une importance médicale*. World Health Organization, Geneve. 220 pp.

Devi P.N. & Jauhari R.K. 2006. Relationship between *Anopheles fluviatilis* & *A. stephensi* (Diptera: Culicidae) catches & the prevalence of malaria cases at Kalsi area in Dehradun district (Uttaranchal). *The Indian Journal of Medical Research* 123: 151-158.

Dias A.A. 2001. Estuário do Sado - Valores económicos, ecológicos e culturais, pp. 85-104, *Memórias do Instituto de Malariologia de Águas de Moura. Da luta anti-palúdica ao museu.* Câmara Municipal de Palmela/Centro de estudos de vectores e doenças infecciosas, Palmela.

Djadid N.D., Gholizadeh S., Tafsiri E., Romi R., Gordeev M. & Zakeri S. 2007. Molecular identification of Palearctic members of *Anopheles maculipennis* in northern Iran. *Malaria Journal* 6: 6.

Dobson M.J. 1994. Malaria in England: a geographical and historical perspective. *Parassitologia* 36: 35-60.

Donnelly M.J., Cuamba N., Charlwood J.D., Collins F.H. & Townson H. 1999. Population structure in the malaria vector, *Anopheles arabiensis* Patton, in East Africa. *Heredity* 83: 408-417.

Doudier B., Bogreau H., DeVries A., Poncon N., Stauffer W.M., 3rd, Fontenille D., Rogier C. & Parola P. 2007. Possible autochthonous malaria from Marseille to Minneapolis. *Emerging Infectious Diseases* 13: 1236-1238.

Downes J.A. 1958. The feeding habits of biting flies and their significance in classification. *Annual Review of Entomology* 3: 249-266.

Dunning A.M., Talmud P. & Humphries S.E. 1988. Errors in the polymerase chain reaction. *Nucleic Acids Research* 16: 10393.

Dye C. 1990. Epidemiological significance of vector-parasite interactions. *Parasitology* 101: 409-415.

Dzhavadov R.B., Trofimov G.K. & Kurochenko G.N. 1977. Susceptibility of *Anopheles maculipennis atroparvus* V. Thiel to infection with the Azerbaijan strains of *Plasmodium* of tertian malaria (*Plasmodium vivax*). *Meditsinskaia Parazitologiia i Parazitarnye Bolezni* 46: 190-191.

Edman J.D. & Webber L.A. 1975. Effect of vertebrate size and density on host-selection by caged *Culex nigripalpus*. *Mosquito News* 35: 508-512.

Edman J.D., Strickman D., Kittayapong P. & Scott T.W. 1992. Female *Aedes aegypti* (Diptera: Culicidae) in Thailand rarely feed on sugar. *Journal of Medical Entomology* 29: 1035-1038.

Encinas Grandes A. 1982. *Taxonomia y biologia de los mosquitos del area Salmantina (Diptera, Culicidae)*. Europa, Artes Gráficas, S.A., Salamanca. 437 pp.

Escalante A.A. & Ayala F.J. 1994. Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequences. *Proceedings of the National Academy of Sciences of the United States of America* 91: 11373-11377.

Escalante A.A., Freeland D.E., Collins W.E. & Lal A.A. 1998. The evolution of primate malaria parasites based on the gene encoding cytochrome b from the linear mitochondrial genome. *Proceedings of the National Academy of Sciences of the United States of America* 95: 8124-8129.

Escalante A.A., Cornejo O.E., Freeland D.E., Poe A.C., Durrego E., Collins W.E. & Lal A.A. 2005. A monkey's tale: the origin of *Plasmodium vivax* as a human malaria parasite. *Proceedings of the National Academy of Sciences of the United States of America* 102: 1980-1985.

Fantini B. 1994. Anophelism without malaria: an ecological and epidemiological puzzle. *Parassitologia* 36: 83-106.

Faustino V.M. 2006. Mosquitos, arroz e sezões. A erradicação da malária no vale do Sado, pp. 133, Instituto de Ciências Sociais. Universidade de Lisboa, Lisboa.

Feldmann A.M. & Ponnudurai T. 1989. Selection of *Anopheles stephensi* for refractoriness and susceptibility to *Plasmodium falciparum*. *Medical and Veterinary Entomology* 3: 41-52.

Feldmann A.M., Billingsley P.F. & Savelkoul E. 1990. Bloodmeal digestion by strains of A*nopheles stephensi* Liston (Diptera: Culicidae) of differing susceptibility to *Plasmodium falciparum*. *Parasitology* 101 Pt 2: 193-200.

Fernandes L. & Briegel H. 2005. Reproductive physiology of *Anopheles gambiae* and *Anopheles atroparvus*. *Journal of Vector Ecology* 30: 11-26.

Filipe A.R. 1972. Isolation in Portugal of West Nile virus from *Anopheles maculipennis* mosquitoes. *Acta Virológica* 16: 361.

Filipe A.R. 2001. Em memória do Instituto de Malariologia e seus investigadores, pp. 17-18, *Memórias do Instituto de Malariologia de Águas de Moura. Da luta anti-palúdica ao museu.* Câmara Municipal de Palmela/Centro de estudos de vectores e doenças infecciosas, Palmela.

Fischer S. & Schweigmann N. 2004. *Culex* mosquitoes in temporary urban rain pools: seasonal dynamics and relation to environmental variables. *Journal of Vector Ecology* 29: 365-373.

Foster W.A. 1995. Mosquito sugar feeding and reproductive energetics. *Annual Review of Entomology* 40: 443-474.

Foster W.A. & Eischen F.A. 1987. Frequency of blood-feeding in relation to sugar availability in *Aedes aegypti* and *Anopheles quadrimaculatus*. *Annals of the Entomological Society of America* 80: 103-108.

Foster W.A. & Takken W. 2004. Nectar-related vs. human-related volatiles: behavioural response and choice by female and male *Anopheles gambiae* (Diptera: Culicidae) between emergence and first feeding. *Bulletin of Entomological Research* 94: 145-157.

Frizzi G. 1947a. Cromosomi salivari in *Anopheles maculipennis*. *Scientia Genetica* 3: 67-79.

Frizzi G. 1947b. Salivary gland chromosomes of *Anopheles*. *Nature* 160: 226-227.

Frizzi G. 1953. Étude cytogénetique d'*Anopheles maculipennis* en Italie. *Bulletin of the World Health Organization* 9: 335-344.

Frizzi G. 1956. Studio della fauna anofelica nella piana di Valencia (Spagna). *Bollettino di Zoologia* 23: 406-410.

Gaffigan T.V. & Ward R.A. 1985. Index to the second supplement of the "A catalog of the mosquitoes of the World" with corrections and additions (Diptera, Culicidae). *Mosquito Systematics* 17: 52-63.

Garnham P.C.C. 1988. Malaria parasites of Man: life cycles and morphology (excluding ultrastructure), pp. 61-96, *Malaria. Principles of Malariology*. Churchill Livinstone Inc., London.

Garrett-Jones C. 1964a. Prognosis for interruption of malaria transmission through assessment of the mosquito's vectorial capacity. *Nature* 204: 1173-1175.

Garrett-Jones C. 1964b. The human blood index of malaria vectors in relation to epidemiological assessment. *Bulletin of the World Health Organization* 30: 241-261.

Garrett-Jones C. & Grab B. 1964. The assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the proportion of parous females. *Bulletin of the World Health Organization* 31: 71-86.

Garrett-Jones C. & Shidrawi G.R. 1969. Malaria vectorial capacity of a population of *Anopheles gambiae*: an exercise in epidemiological entomology. *Bulletin of the World Health Organization* 40: 531-545.

Garrett-Jones C., Boreham P.F.L. & Pant C.P. 1980. Feeding habits of Anophelines (Diptera:Culicidae) in 1971-78, with reference to the human blood index: a review. *Bulletin of Entomological Research* 70: 165-185.

Gary Jr. R.E. & Foster W.A. 2001. Effects of available sugar on the reproductive fitness and vectorial capacity of the malaria vector *Anopheles gambiae* (Diptera: Culicidae). *Journal of Medical Entomology* 38: 22-28.

Gary Jr. R.E. & Foster W.A. 2006. Diel timing and frequency of sugar feeding in the mosquito *Anopheles gambiae*, depending on sex, gonotrophic state and resource availability. *Medical and Veterinary Entomology* 20: 308-316.

Ghavani M.B. 2005. Estimation and comparison of *Anopheles maculipennis s.l.* (Diptera:Culicidae) survival rates with light-trap and indoor resting data. *Iranian Journal of Public Health* 34: 48-57.

Gilles H.M. 1993. The malaria parasites, pp. 124-163, *Bruce-Chwatt's Essential Malariology*. Edward Arnold, Kent.

Gilles H.M. 2002. Historical outline, pp. 1-8, *Essential Malariology*. Oxford University Press Inc., Oxford.

Gillies M.T. & De Meillon B. 1968. *The Anopheline of Africa south of the Sahara*. The South African Institute for medical research, Johannesburg. 343 pp.

Gordeev M.I., Zvantsov A.B., Goriacheva, I.I., Shaikevich E.V. & Ezhov M.N. 2005. Description of the new species *Anopheles artemievi sp.n*. (Diptera, Culicidae). *Meditsinskaia Parazitologiia i Parazitarnye Bolezni* 2: 4-5.

Grmek M.D. 1994. Malaria in the eastern Mediterranean in prehistory and antiquity. *Parassitologia* 36: 1-6.

Guimarães A.E., Gentile C., Lopes C.M. & Sant'Anna A. 2001. Ecologia de mosquitos em áreas do Parque Nacional da Serra da Bocaina. II - Frequência mensal e factores climáticos. *Revista de Saúde Publica* 35: 392-399.

Guy Y., Salières A. & Boeliger E. 1976. Contribution a l'étude du "complexe Maculipennis" (Diptera-Culicidae-Anophelinae). Mise au point en 1975. *Année Biologique*: 227-282.

Hackett L.W. 1934. The present status of our knowledge of the sub-species of *Anopheles maculipennis. Transactions of the Royal Society of Tropical Medicine and Hygiene* 28: 109-140.

Hackett L.W. & Missiroli A. 1931. The natural disappearance of malaria in certain regions of Europe. *American Journal of Hygiene* 13: 57-78.

Hackett L.W. & Missiroli A. 1935. The varieties of *Anopheles maculipennis* and their relation to the distribution of malaria in Europe. *Rivista di Malariologia* 14: 45-109.

Hackett L.W. & Lewis D.J. 1935. A new variety of *Anopheles maculipennis* in southern Europe. *Rivista di Malariologia* 14: 377-383.

Hackett L.W., Martini E. & Missiroli A. 1932. The races of *A. maculipennis*. *American Journal of Hygiene* 16: 137-162.

Harant H., Rioux J.-A., Attisso M. & Ruffié J. 1957. Variabilité génétique chez *Anopheles atroparvus*. *Comptes rendus des séances de la Société de Biologie* 151: 2158-2162.

Harbach R.E. 2004. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. *Bulletin of Entomological Research* 94: 537-553.

Harinasuta T. & Bunnag D. 1988. The clinical features of malaria, pp. 709-734, *Malaria. Principles of Malariology*. Churchill Livinstone Inc., London.

Hay S.I., Cox J., Rogers D.J., Randolph S.E., Stern D.I., Shanks G.D., Myers M.F. & Snow R.W. 2002a. Climate change and the resurgence of malaria in the East African highlands. *Nature* 415: 905-909.

Hay S.I., Rogers D.J., Randolph S.E., Stern D.I., Cox J., Shanks G.D. & Snow R.W. 2002b. Hot topic or hot air? Climate change and malaria resurgence in East African highlands. *Trends in Parasitology* 18: 530-534.

Hay S.I., Were E.C., Renshaw M., Noor A.M., Ochola S.A., Olusanmi I., Alipui N. & Snow R.W. 2003. Forecasting, warning, and detection of malaria epidemics: a case study. *Lancet* 361: 1705-1706.

Hay S.I., Shanks G.D., Stern D.I., Snow R.W., Randolph S.E. & Rogers D.J. 2005. Climate variability and malaria epidemics in the highlands of East Africa. *Trends in Parasitology* 21: 52-53

Hedenqvist P. & Hellebrekers L.J. 2003. Laboratory animal analgesia, anesthesia, and euthanasia, pp. 413-456, *Handbook of laboratory animal science*. CRC Press LLC, Boca Raton.

Hess A.D., Hayes R.O. & Tempelis C.H. 1968. The use of the forage ratio technique in mosquito host preference studies. *Mosquito News* 28: 386-389.

Hocking B. 1953. The intrinsic range and speed of flight of insects. *Transactions of the Royal Entomology Society of London* 104: 223-345.

Hogg J.C., Thomson M.C. & Hurd H. 1996. Comparative fecundity and associated factors for two sibling species of the *Anopheles gambiae* complex occurring sympatrically in The Gambia. *Medical and Veterinary Entomology* 10: 385-391.

Holliday-Hanson M.L., Yuval B. & Washino R.K. 1997. Energetics and sugar-feeding of field-collected Anopheline females. *Journal of Vector Ecology* 22: 83-89.

Huldén L. & Heliovaara K. 2005. Endemic malaria: an 'indoor' disease in northern Europe. Historical data analysed. *Malaria Journal* 4: 19.

Jafari S., Durand R., Lusina D. & Le Bras J. 2002. Molecular characterisation of airport malaria: four cases in France during summer 1999. *Parasite* 9: 187-191.

James P. 1930-1931. Some general results of a study of induced malaria in England. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 24: 477-538.

Jelinek T., Schulte C., Behrens R. & *et al.* 2002. Imported *falciparum* malaria in Europe: sentinel surveillance data from the European Network on surveillance of imported infectious diseases. *Clinical Infectious Disease.*

*(*http://www.tropnet.net/publications/pdf_publications/mal_clininfdis_2002.pdf): (http://www.tropnet.net/publications/pdf_publications/mal_clininfdis_2002.pdf).

Jetten T.H. & Takken W. 1994. *Anophelism without malaria in Europe. A review of the ecology and distribution of the genus Anopheles in Europe*. Veenman Drukkers, Wageningen. 69 pp.

Joy D.A., Feng X., Mu J., Furuya T., Chotivanich K., Krettli A.U., Ho M., Wang A., White N.J., Suh E., Beerli P. & Su X.Z. 2003. Early origin and recent expansion of *Plasmodium falciparum*. *Science* 300: 318-321.

Kaufmann C. & Briegel H. 2004. Flight performance of the malaria vectors *Anopheles gambiae* and *Anopheles atroparvus*. *Journal of Vector Ecology* 29: 140-153.

Ke X., Collins A. & Ye S. 2001. PIRA PCR designer for restriction analysis of single nucleotide polymorphisms. *Bioinformatics* 17: 838-839.

Kirkwood B.R. 1988. *Essentials of medical statistics*. Blackwell Scientific Publications, Oxford. 234 pp.

Kitthawee S., Edman J.D. & Sattabongkot J. 1990. Evaluation of survival potential and malaria susceptibility among different size classes of laboratory-reared *Anopheles dirus*. *The American Journal of Tropical Medicine and Hygiene* 43: 328-332.

Kitzmiller J.B. 1967. Mosquito cytogenetics, pp. 133-150, *Genetics of insect vectors of disease*. Elsevier Publishing Company, Amsterdam.

Knell A.J. 1991. *Malaria*. Oxford University Press, Oxford. 89 pp.

Knight K.L. 1978. *Supplement to "A catalog of the mosquitoes of the World -VII"*. Entomological Society of America, Maryland. 107 pp.

Knight K.L. & Stone A. 1977. *A catalog of the mosquitoes of the World-VII*. The Geo. W. King Company, Maryland and Baltimore. 611 pp.

Koella J.C., Sorensen F.L. & Anderson R.A. 1998. The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proceedings of the Royal Society of London* 265: 763-768.

Kraytsberg Y. & Khrapko K. 2005. Single-molecule PCR: an artifact-free PCR approach for the analysis of somatic mutations. *Expert Review of Molecular Diagnostics* 5: 809-815.

Kruger A., Rech A., Su X.Z. & Tannich E. 2001. Two cases of autochthonous *Plasmodium falciparum* malaria in Germany with evidence for local transmission by indigenous *Anopheles plumbeus*. *Tropical Medicine & International Health* 6: 983-985.

Krzywinski J. & Besansky N.J. 2003. Molecular systematics of *Anopheles*: from subgenera to subpopulations. *Annual Review of Entomology* 48: 111-139.

Krzywinski J., Wilkerson R.C. & Besansky N.J. 2001. Toward understanding Anophelinae (Diptera, Culicidae) phylogeny: insights from nuclear single-copy genes and the weight of evidence. *Systematic Biology* 50: 540-556.

Kuhn K.G., Campbell-Lendrum D.H. & Davies C.R. 2002. A continental risk map for malaria mosquito (Diptera: Culicidae) vectors in Europe. *Journal of Medical Entomology* 39: 621-630.

Laarman J.J. 1968. The intake of sugars by females of wild *Anopheles gambiae* and *Anopheles funestus*. *Acta Leiden* 36: 137-144.

Laganier R., Randimby F.M., Rajaonarivelo V. & Robert V. 2003. Is the Mbita trap a reliable tool for evaluating the density of Anopheline vectors in the highlands of Madagascar? *Malaria Journal* 2: 42.

Laird M. 1988. *The natural history of larval mosquito habitats*. Academic Press Limited, London. 555 pp.

Lambrechts L., Chavatte J.M., Snounou G. & Koella J.C. 2006. Environmental influence on the genetic basis of mosquito resistance to malaria parasites. *Proceedings. Biological Sciences / The Royal Society* 273: 1501-1506.

Landeiro F. & Cambournac F.J. 1933. *O sezonismo em Portugal*. Divisão de Publicações e Biblioteca, Agência Geral das Colónias, Lisboa. 142 pp.

Legros F. & Danis M. 1998. Survailance of malaria in European Union countries. *Eurosurveillance-European Communicable Disease Bulletin* 3: 45-47.

Linton Y.-M., Harbach R.E., Smith L., Koliopoulus G., Nicolescu G., Sedeghat M.M., Samanidou-Voyadjoglou A. & Zounos A.K. 2002a. The paleartic *Anopheles maculipennis* complex: new approaches to old taxonomic problems. *Acta Tropica.* 83, Suppl.1: S82.

Linton Y.-M., Smith L. & Harbach R.E. 2002b. Observations on the taxonomic status of *Anopheles subalpinus* Hackett & Lewis and *An. melanoon* Hackett. *European Mosquito Bulletin* 13: 1-7.

Linton Y.-M., Smith L. & Harbach R.E. 2002c. Molecular confirmation of sympatric populations of *Anopheles messeae* and *Anopheles atroparvus* overwintering in Kent, southeast England. *European Mosquito Bulletin* 13: 8-16.

Lupascu G. 1941. Su alcune variazioni delle ouva di *Anopheles maculipennis* var. *labranchiae*. *Estratto dai rendiconti dell'Istituto di Sanità Pubblica* 4: 704-707.

Lupascu G., Constantinescu P., Negulici E., Shute P.G. & Maryon M.E. 1968. Parasitological and clinical investigations on infections with the VS Romanian strain of *Plasmodium malariae* transmitted by *Anopheles labranchiae atroparvus*. *Bulletin of the World Health Organization* 38: 61-67.

Mabaso M.L., Craig M., Ross A. & Smith T. 2007. Environmental predictors of the seasonality of malaria transmission in Africa: the challenge. *The American Journal of Tropical Medicine and Hygiene* 76: 33-38.

Macdonald G. 1956. Epidemiological basis of malaria control. *Bulletin of the World Health Organization* 15: 613-626.

Macdonald G. 1957. *Epidemiology and control of malaria*. Oxford University Press, London. 210 pp.

Marchant P., Eling W., van Gemert G.J., Leake C.J. & Curtis C.F. 1998. Could British mosquitoes transmit *falciparum* malaria? *Parasitology Today* 14: 344-345.

Maroco J. 2003. *Análise estatística com utilização do SPSS*. Sílabo, Lisboa. 508 pp.

Martens P., Kovats R.S., Nijhol S., De Vries P., Livermore M.T.J., Bradley D.J., Cox J. & McMichael A.J. 1999. Climate change and future populations at risk of malaria. *Global Environmental Change* 9: S89-S107.

Mayr E. 1969. *Principles of systematic zoology*. McGraw-Hill Book Company, New York, Toronto, London and Sydney. 428 pp.

McCrae A.W.R. 1989. Differences in sugar feeding activity between tropical and temperate mosquitoes: field observations and their implications. *Vector Ecology Newsletter* 20: 16.

McCrae A.W.R., Boreham P.F.L. & Ssenkubuge Y. 1976. The behavioural ecology of host selection in *Anopheles implexus* (Theobald) (Diptera, Culicidae). *Bulletin of Entomological Research* 66: 587-631.

McHugh C.P. 1989. Ecology of a semi-isolated population of adult *Anopheles freeborni*: abundance, trophic status, parity, survivorship, gonotrophic cycle length, and host selection. *The American Journal of Tropical Medicine* 41: 169-176.

Medley G.F., Sinden R.E., Fleck S., Billingsley P.F., Tirawanchai N. & Rodriguez M.H. 1993. Heterogeneity in patterns of malarial oocyst infections in the mosquito vector. *Parasitology* 106: 441-449.

Minakawa N., Sonye G., Mogi M., Githeko A. & Yan G. 2002. The effects of climatic factors on the distribution and abundance of malaria vectors in Kenya. *Journal of Medical Entomology* 39: 833-841.

Miranda P.M.A. & Moita R. 2006. Estudo de caso da região do Sado, pp. 385-480, *Alterações climáticas em Portugal. Cenários, impactos e medidas de adaptação. Projecto SIAM II*. Gradiva-Publicações, Lda, Lisboa.

Missiroli A. 1935. Nuova varietà di *Anopheles maculipennis*. *Annali d'Igiene* 45: 333.

Missiroli A. & Hackett L.W. 1927. La regressione spontanea della malaria in alcune regioni d'Italia. *Rivista di Malariologia* 6: 193-243.

Missiroli A., Hackett L.W. & Martini E. 1933. Le razze di *Anopheles maculipennis* e loro importanza nella distribuzione della malara in alcune regioni d'Europa. *Rivista di Malariologia* 12: 1-56.

Molineaux L. 1988. The epidemiology of human malaria as an explanation of its distribution, including some implications for its control, pp. 913-998, *Malaria. Principles and practice of malariology*. Churchill Livingstone Inc., London.

Molineaux L., Muir D.A., Spencer H.C. & Wernsdorfer W.H. 1988. The epidemiology of malaria and its mesurement, pp. 999-1090, *Malaria. Principles and practice of malariology*. Churchill Livingstone Inc., London.

Morales A.L. 1946. Contribución al estudio de la biologia del *A. maculipennis* var. *atroparvus* en función del ambiente. *Revista de Sanidad y Higiene Pública* 20: 239-250.

Mostowy W.M. & Foster W.A. 2004. Antagonistic effects of energy status on meal size and egg-batch size of *Aedes aegypti* (Diptera:Culicidae). *Journal of Vector Ecology* 29: 84-93.

Mu J., Joy D.A., Duan J., Huang Y., Carlton J., Walker J., Barnwell J., Beerli P., Charleston M.A., Pybus O.G. & Su X.Z. 2005. Host switch leads to emergence of *Plasmodium vivax* malaria in humans. *Molecular Biology and Evolution* 22: 1686-1693.

Murphy R.W., Sites; J.W., Buth D.G. & C.H. H. 1990. Proteins I: isozyme electrophoresis, pp. 45-126, *Molecular systematics*. Sinauer Associates, Inc., Massachusetts.

Naksathit A.T. & Scott T.W. 1998. Effect of female size on fecundity and survivorship of *Aedes aegypti* fed only human blood *versus* human blood plus sugar. *Journal of the American Mosquito Control Association* 14: 148-152.

Nanostat ™. 1987. Computer program, version Imperial College of Science, Techonology and Medicine. By Nanostat ™.

Nayar J.K. & Sauerman Jr. D.M. 1975. The effects of nutrition on survival and fecundity in Florida mosquitoes. Part 3. Utilization of blood and sugar for fecundity. *Journal of Medical Entomology* 12: 220-225.

Nicolescu G., Linton Y.M., Vladimirescu A., Howard T.M. & Harbach R.E. 2004. Mosquitoes of the *Anopheles maculipennis* group (Diptera: Culicidae) in Romania, with the discovery and formal recognition of a new species based on molecular and morphological evidence. *Bulletin of Entomological Research* 94: 525-535.

Noden B.H., Kent M.D. & Beier J.C. 1995. The impact of variations in temperature on early *Plasmodium falciparum* development in *Anopheles stephensi*. *Parasitology* 111: 539-545.

Novikov L.M. & Alekseev A.N. 1989. *Anopheles maculipennis* northeastern and *Anopheles beklemishevi* southwestern distribution areas. *Meditsinskaia Parazitologiia i Parazitarnye Bolezni* 1: 16-19.

Okech B.A., Gouagna L.C., Kabiru E.W., Beier J.C., Yan G. & Githure J.I. 2004a. Influence of age and previous diet of *Anopheles gambiae* on the infectivity of natural *Plasmodium falciparum* gametocytes from human volunteers. *Journal of Insect Science* 4: 33.

Okech B.A., Gouagna L.C., Kabiru E.W., Walczak E., Beier J.C., Yan G. & Githure J.I. 2004b. Resistance of early midgut stages of natural *Plasmodium falciparum* parasites to high temperatures in experimentally infected *Anopheles gambiae* (Diptera: Culicidae). *The Journal of Parasitology* 90: 764-768.

Okech B.A., Gouagna L.C., Walczak E., Kabiru E.W., Beier J.C., Yan G. & Githure J.I. 2004c. The development of *Plasmodium falciparum* in experimentally infected

Anopheles gambiae (Diptera: Culicidae) under ambient microhabitat temperature in western Kenya. *Acta Tropica* 92: 99-108.

Okech B.A., Gouagna L.C., Yan G., Githure J.I. & Beier J.C. 2007. Larval habitats of *Anopheles gambiae s.s.* (Diptera: Culicidae) influences vector competence to *Plasmodium falciparum* parasites. *Malaria Journal* 6: 50.

Pääbo S. 1989. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. *Proceedings of the National Academy of Sciences of the United States of America* 86: 1939-1943.

Pääbo S. & Wilson A.C. 1988. Polymerase chain reaction reveals cloning artefacts. *Nature* 334: 387-388.

Pascual M., Ahumada J.A., Chaves L.F., Rodo X. & Bouma M. 2006. Malaria resurgence in the East African highlands: temperature trends revisited. *Proceedings of the National Academy of Sciences of the United States of America* 103: 5829-5834.

Patz J.A. & Olson S.H. 2006. Malaria risk and temperature: influences from global climate change and local land use practices. *Proceedings of the National Academy of Sciences of the United States of America* 103: 5635-5636.

Patz J.A., Hulme M., Rosenzweig C., Mitchell T.D., Goldberg R.A., Githeko A.K., Lele S., McMichael A.J. & Le Sueur D. 2002. Climate change: Regional warming and malaria resurgence. *Nature* 420: 627-628; discussion 628.

Pereira A. 2003. *SPSS. Guia prático de utilização. Análise de dados para ciências sociais e psicologia*. Edições Sílabo, Lisboa. 221 pp.

Pires C.A., Ribeiro H., Capela R.A. & Ramos H.C. 1982. Research on the mosquitoes of Portugal (Diptera, Culicidae). VI-The mosquitoes of Alentejo. *Anais do Instituto de Higiene e Medicina Tropical* 8: 79-102.

Ponçon N., Toty C., L'Ambert G., Le Goff G., Brengues C., Schaffner F. & Fontenille D. 2007. Biology and dynamics of potential malaria vectors in southern France. *Malaria Journal* 6: 18.

Ponnudurai T., Lensen A.H., Leeuwenberg A.D. & Meuwissen J.H. 1982a. Cultivation of fertile *Plasmodium falciparum* gametocytes in semi-automated systems. 1. Static cultures. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 76: 812-818.

Ponnudurai T., Meuwissen J.H., Leeuwenberg A.D., Verhave J.P. & Lensen A.H. 1982b. The production of mature gametocytes of *Plasmodium falciparum* in continuous cultures of different isolates infective to mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 76: 242-250.

Ponnudurai T., Lensen A.H., Meis J.F. & Meuwissen J.H. 1986. Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system. *Parasitology* 93: 263-274.

Ponnudurai T., Lensen A.H., Van Gemert G.J., Bensink M.P., Bolmer M. & Meuwissen J.H. 1989. Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes. *Parasitology* 98: 165-173.

Poolsuwan S. 1995. Malaria in prehistoric southeastern Asia. *The Southeast Asian Journal of Tropical Medicine and Public Health* 26: 3-22.

Post R.J. & Millest A.L. 1991. Sample size in parasitological and vector surveys. *Parasitology Today* 7: 141.

Postiglione M., Bruno-Smiraglia C., Lavagnino A., Gokberk C. & Ramsdale C. 1970. A preliminary note on the occurrence in Turkey of the *subalpinus* form of the *A. maculipennis* complex. *Rivista di Parassitologia* 31: 155-158.

Postiglione M., Tabanli B. & Ramsdale C.D. 1973. The *Anopheles* of Turkey. *Rivista di Parassitologia* 34: 127-159.

Prakash A., Bhattacharyya D.R., Mohapatra P.K. & Mahanta J. 2001. Estimation of vectorial capacity of *Anopheles dirus* (Diptera: Culicidae) in a forest-fringed village of Assam (India). *Vector Borne and Zoonotic Diseases* 1: 231-237.

Price R.N. & Nosten F. 2001. Drug resistant *falciparum* malaria: clinical consequences and strategies for prevention. *Drug Resistance Update* 4: 187-196.

Proft J., Maier W.A. & Kampen H. 1999. Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. *Parasitology Research* 85: 837-843.

Pusch C.M. & Bachmann L. 2004. Spiking of contemporary human template DNA with ancient DNA extracts induces mutations under PCR and generates nonauthentic mitochondrial sequences. *Molecular Biology Evolution* 21: 957-964.

Pusch C.M., Broghammer M., Nicholson G.J., Nerlich A.G., Zink A., Kennerknecht I., Bachmann L. & Blin N. 2004. PCR-induced sequence alterations hamper the typing of prehistoric bone samples for diagnostic achondroplasia mutations. *Molecular Biology and Evolution* 21: 2005-2011.

Ramos H.C. 1983/84. Contribuição para o estudo dos mosquitos limnodendrófilos de Portugal (Diptera, Culicidae). *Garcia de orta, Sér. Zool.* 11: 133-154.

Ramos H.C., Ribeiro H., Pires C.A. & Capela R.A. 1977/78. Research on the mosquitoes of Portugal (Diptera, Culicidae). II-The mosquitoes of Algarve. *Anais do Instituto de Higiene e Medicina Tropical* 5: 237-256.

Ramos H.C., Ribeiro H., Pires C.A. & Capela R.A. 1982. Research on the mosquitoes of Portugal (Diptera, Culicidae). VII-Two new Anopheline records. *Anais do Instituto de Higiene e Medicina Tropical* 8: 103-109.

Ramos H.C., Ribeiro H. & Novo M.T. 1992. Mosquito ecology in southeastern Portugal, an area receptive to African Horsesickness. *Bulletin of the Society for Vector Ecology* 17: 85-93.

Ramos H.C., Novo M.T., Sousa C.A., Almeida A.P.G., Parreira R., Diniz M.C., Galão R., Carvalho L. & Lopes D.K. 2001. Avaliação da situação culicideológica na área do complexo turístico da Comporta/Atlantic Company, pp. 86, *Unpublished*.

Ramsdale C. & Coluzzi M. 1975. Studies on the infectivity of tropical African strains of *Plasmodium falciparum* to some southern European vectors of malaria. *Parassitologia* 17: 39-48.

Ramsdale C. & Snow K. 2000. Distribution of the genus *Anopheles* in Europe. *European Mosquito Bulletin* 7: 1-26.

Ramsdale C. & Wilkes T.J. 1985. Some aspects of overwintering in southern England of the mosquitoes *Anopheles atroparvus* and *Culiseta annulata* (Diptera:Culucidae). *Ecological Entomology* 10: 449-454.

Rathore D., Wahl A.M., Sullivan M. & McCutchan T.F. 2001. A phylogenetic comparison of gene trees constructed from plastid, mitochondrial and genomic DNA of *Plasmodium* species. *Molecular and Biochemical Parasitology* 114: 89-94.

Reiter P. 1996. Oviposition et dispersion d'*Aedes aegypti* dans l'environnent urbain. *Bulletin de la Société de Pathologie Exotique* 89: 120-122.

Reiter P. 2001. Climate change and mosquito-borne disease. *Environmental Health Perspectives* 109 Suppl 1: 141-161.

Ribeiro H. 1967. A solidifiable formic acid-PVA solution for transporting, preserving and mounting mosquito larvae and pupae. *Stain Technology* 42: 159-160.

Ribeiro H. & Ramos H.C. 1999. Identification keys of the mosquitoes (Diptera:Culicidae) of Continental Portugal, Açores and Madeira. *European Mosquito Bulletin* 3: 1-11.

Ribeiro H., Ramos H.C., Capela R.A. & Pires C.A. 1977. Research on the mosquitoes of Portugal (Diptera, Culicidae). III-Further five new mosquito records. *Garcia de orta, Sér. Zool.* 6: 51-60.

Ribeiro H., Ramos H.C., Pires C.A. & Capela R.A. 1977/78. Research on the mosquitoes of Portugal (Diptera, Culicidae). I-Four new culicine records. *Anais do Instituto de Higiene e Medicina Tropical* 5: 203-214.

Ribeiro H., Ramos H.C., Pires C.A. & Capela R.A. 1980a. Research on the mosquitoes of Portugal (Diptera, Culicidae). IV-Two new Anopheline records. *Garcia de orta, Sér. Zool.* 9: 129-138.

Ribeiro H., Ramos H.C., Capela R.A & Pires C.A. 1980b. *Os mosquitos de Cabo Verde (Diptera: Culicidae). Sistemática, distribuição, bioecologia e importância médica.* Junta de investigações científicas do Ultramar, Lisboa. 139 pp.

Ribeiro H., Ramos H.C. & Pires C.A. 1983. Contribuição para o estudo dos vectores das filaríases animais em Portugal. *Jornal da Sociedade de Ciências Médicas* 147: 143-146.

Ribeiro H., Ramos H.C., Pires C.A. & Capela R.A. 1985. Research on the mosquitoes of Portugal (Diptera, Culicidae). IX-A new Anopheline record. *Garcia de orta, Sér. Zool.* 12: 105-112.

Ribeiro H., Ramos H.C., Pires C.A. & Capela R.A. 1988. An annotated checklist of the mosquitoes of continental Portugal (Diptera, Culicidae). *Actas do III Congresso Ibérico de Entomologia*: 233-254.

Ribeiro H., Batista J.L., Ramos H.C., Pires C.A., Champalimaud J.L., Costa J.M., Araújo C., Mansinho K. & Pina M.C. 1989. An attempt to infect *Anopheles atroparvus* from Portugal with African *Plasmodium falciparum*. *Revista Portuguesa de Doenças Infecciosas* 12: 81-82.

Ribeiro H., Ramos H.C., Pires C.A. & Capela R.A. 1992. Research on the mosquitoes of Portugal (Diptera, Culicidae). XI-The mosquitoes of the Beiras. *Garcia de orta, Sér. Zool.* 16: 137-161.

Ribeiro H., Pires C.A. & Ramos H.C. 1996. Os mosquitos do Parque Natural da Arrábida (Insecta, Diptera, Culicidae). *Garcia de orta, Sér. Zool.* 21: 81-110.

Ribeiro H., Ramos H.C. & Pires C.A. 1999a. Os mosquitos do Parque Natural de Montesinho (Insecta, Diptera, Culicidae). *Garcia de orta, Sér. Zool.* 23: 23-67.

Ribeiro H., Ramos H.C. & Pires C.A. 1999b. Os mosquitos do Parque Natural da Serra da Estrela (Insecta, Diptera, Culicidae). *Garcia de orta, Sér. Zool.* 23: 119-168.

Ribeiro H., Pires C.A., Ramos H.C. & Capela R.A. 2002. Research on the mosquitoes of Portugal (Diptera, Culicidae). XII-The mosquitoes of Minho and Douro Litoral. *Garcia de orta, Sér. Zool.* 24: 31-50.

Richards O.W. & Davies R.G. 1977. *Imm's general book of entomology II: General classification and biology.* Chapman and Hall, New York. 1354 pp.

Rioux J.-A. 1958. *Les culicides do "Midi" méditerranéen*. Éditions Paul Lechavalier, Paris. 303 pp.

Rioux J.-A. & Ruffié J. 1957. Présence d'*Anopheles maculipennis s.sp. subalpinus* Hackett et Lewis, 1937 en Bas-Languedoc. *Bulletin de la Société de Pathologie Exotique* 50: 831-837.

Rioux J.-A., Ruffié J. & Pech J. 1959. Évolution des idées en entomologie médicale. *Extrait de la Biologie Médicale* 48: 215-237.

Robert V., Le Goff G., Ariey F. & Duchemin J.B. 2002. A possible alternative method for collecting mosquito larvae in rice fields. *Malaria Journal* 1: 4.

Rogers D.J. & Randolph S.E. 2000. The global spread of malaria in a future, warmer world. *Science* 289: 1763-1766.

Romi R., Boccolini D., Di Luca M., La Rosa G. & Marinucci M. 2000. Identification of the sibling species of the *Anopheles maculipennis* complex by heteroduplex analysis. *Insect Molecular Biology* 9: 509-513.

Roubaud E. 1918. Recherches sur la transmission du paludisme par les anophèles français de régions non palustres (Yonne et région parisiense). *Annales de l' Institut Pasteur* 31: 430-462.

Roubaud E. 1920. Les conditions de nutrition des anophèles en Franceen France *A. maculipennis* et le rôle du bétail dans la prophylaxie du paludisme. *Annales de l' Institut Pasteur* 34: 181-228.

Roubaud E. 1921. La différenciation des races zootropiques d'anophèles et la régression spontanée du paludisme. *Bulletin de la Société de Pathologie Exotique* 14: 577-595.

Roubaud E. 1928. Nouvelles recherches sur l'évolution zoophile des faunes d'anophèles en Europe (*A. maculipennis*) d'après les données de l'armement maxillaire. *Annales de l' Institut Pasteur* 42: 553-618.

Roubaud E. 1934. Un type racial nouveau de l' *Anopheles maculipennis*. *Bulletin de la Société de Pathologie Exotique* 27: 737-740.

Roubaud E. 1935. Variété nouvelle de l'*Anopheles maculipennis* au Maroc. *A. maculipennis sicaulti. Bulletin de la Société de Pathologie Exotique* 28: 107-111.

Roubaud E. 1937. Variations de l'oeuf chez *Anopheles maculipennis* (biotype *atroparvus*). *Bulletin de la Société de Pathologie Exotique* 30: 279-283.

Roubaud E. & Gaschen H. 1933. Insuffisance des caractères de l'oeuf pour la distinction des races trophiques et biologiques de l'*Anopheles maculipennis*. *Bulletin de la Société de Pathologie Exotique* 26: 447-451.

Roubaud E. & Treillard M. 1936. Sur une variété portugaise de l'*Anopheles maculipennis* (groupe *atroparvus*). *Bulletin de la Société de Pathologie Exotique* 29: 383-392.

Roubaud E. & Treillard M. 1937. Observations biologiques sur le biotype *cambournaci* de l´*Anopheles maculipennis*. *Bulletin de la Société de Pathologie Exotique* 30: 383-392.

Rúa G.L., Quinones M.L., Vélez I.D., Zuluaga J.S., Rojas W., Poveda G. & Ruiz D. 2005. Laboratory estimation of the effects of increasing temperatures on the duration of gonotrophic cycle of *Anopheles albimanus* (Diptera: Culicidae). *Memórias do Instituto Oswaldo Cruz* 100: 515-520.

Sallares R., Bouwman A. & Anderung C. 2004. The spread of malaria to southern Europe in antiquity: new approaches to old problems. *Medical History* 48: 311-328.

Santos R.L., Forattini O.P. & Burattini M.N. 2002. Laboratory and field observations on duration of gonotrophic cycle of *Anopheles albitarsis s.l.* (Diptera: Culicidae) in southeastern Brazil. *Journal of Medical Entomology* 39: 926-930.

Sarmento M. & França C. 1901. Nota sobre alguns culicidios portuguezes. *Revista Portuguesa de Medicina e Cirurgia Práticas* 11: 38-43.

Sautet J. & D'Ortoli M.F. 1944. Variations biologiques et morphologiques de l'*Anopheles maculipennis*. *Biologie Comptes Rendus.* 138: 680-682.

Scott T.W., Naksathit A., Day J.F., Kittayapong P. & Edman J.D. 1997. A fitness advantage for *Aedes aegypti* and the viruses it transmits when females feed only on human blood. *The American Journal of Tropical Medicine and Hygiene* 57: 235-239.

Scott T.W., Morrison A.C., Lorenz L.H., Clark G.G., Strickman D., Kittayapong P., Zhou H. & Edman J.D. 2000. Longitudinal studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: population dynamics. *Journal of Medical Entomology* 37: 77-88.

Sedaghat M.M., Linton Y.M., Oshaghi M.A., Vatandoost H. & Harbach R.E. 2003. The *Anopheles maculipennis* complex (Diptera: Culicidae) in Iran: molecular characterization and recognition of a new species. *Bulletin of Entomological Research* 93: 527-535.

Senevet G. 1958. *Les Anopheles du Globe.* Éditions Paul Lechevalier, Paris. 214 pp.

Senevet G. & Andarelli G. 1956. *Les anophèles de l'Afrique du Nord et du Bassin Méditerranéen.* Éditions Paul Lechevalier, Paris. 280 pp.

Senevet G. & Andarelli. 1959. *Les genres Culex, Uranotaenia, Theobaldia, Orthopodomyia et Mansonia*. Éditions Paul Lechavalier, Paris. 383 pp.

Sergent E. & Sergent E. 1903. Observations sur les moustiques des environs d'Alger. *Annales de l' Institut Pasteur* 17: 60-65.

Sergent E., Sergent E., Parrot L. & Foley H. 1922. L'armature maxillaire des *Anopheles maculipennis* en pays paludéen. *Bulletin de la Société de Pathologie Exotique* 15: 19-30.

Service M.W. 1976. *Mosquito ecology. Field sampling methods.* Applied Science Publishers, London. 583 pp.

Service M.W. 1993. Mosquitoes (Culicidae), pp. 120-240, *Medical insects and arachnids*. Chapman & Hall, London.

Service M.W. & Townson H. 2002. The *Anopheles* vector, pp. 59-84, *Essential Malariology*. Oxford University Press Inc., Oxford.

Shute P.G. 1935. The value of the external spine of the harpago in identifying varieties of *Anopheles maculipennis. Parasitology* 27: 561-563.

Shute P.G. 1936. A study of laboratory-bred *Anopheles maculipennis* var. *atroparvus*, with special reference to egg-laying. *Annals of Tropical Medicine and Parasitology* 30: 11-16.

Shute P.G. 1940. Failure to infect English specimens of A*nopheles maculipennis* var. *atroparvus* with certain strains of *Plasmodium falciparum* of tropical origin. *The Journal of Tropical Medicine and Hygiene* 43: 175-178.

Shute P.G. & Maryon M.E. 1955. Transmission of *Plasmodium malariae* by laboratory-bred *Anopheles maculipennis* var. *atroparvus* Meigen. *Annals of Tropical Medicine and Parasitology* 49: 451-454.

Shute P.G. & Ungureanu E. 1938. Comparative studies of the eggs of *Anopheles maculipennis* var. *atroparvus* in the field and in the laboratory. *Rivista di Malariologia* 12: 358-361.

Simões M.J., Próspero M.I. & Ribeiro H. 1995. Optimização da técnica ELISA "two sites" utilizada na identificação de refeições sanguíneas de mosquitos. *Revista Portuguesa de Doenças Infecciosas* 18: 225-229.

Smith D.L., McKenzie F.E., Snow R.W. & Hay S.I. 2007. Revisiting the basic reproductive number for malaria and its implications for malaria control. *PLoS Biology* 5: e42.

Snow K. 1999. Malaria and mosquitoes in Britain: the effect of global climate change. *European Mosquito Bulletin* 4: 17-25.

Snow R.W. & Gilles H.M. 2002. The epidemiology of malaria, pp. 85-106, *Essential Malariology*. Oxford University Press Inc., Oxford.

Sokal R.R. & Rohlf F.J. 1981. *Biometry*. W.H. Freeman and Company, San Francisco. 859 pp.

Sousa C.A., Pinto J., Almeida A.P.G., Ferreira C., Rosário V.E. & Charlwood J.D. 2001. Dogs as favored host choice of *Anopheles gambiae* sense stricto (Diptera:Culicidae) of São Tomé, West Africa. *Journal of Medical Entomology* 38: 122-125.

Stegnii V.N. & Kabanova V.M. 1978. Cytoecological study of indigenous populations of the malaria mosquito in the territory of the U.S.S.R.. I. Identification of a new species of *Anopheles* in the *maculipennis* complex by the cytodiagnostic method. *Mosquito Systematics* 10: 1-12.

Stein M., Oria G.I., Almiron W.R. & Willener J.A. 2005. Fluctuación estacional de *Aedes aegypti* en Chaco, Argentina. *Revista de Saúde Pública* 39: 559-564.

Straif S.C. & Beier J.C. 1996. Effects of sugar availability on the blood-feeding behavior of *Anopheles gambiae* (Diptera:Culicidae). *Journal of Medical Entomology* 33: 608-612.

Sudia W.D. & Chamberlain R.W. 1988. Battery-operated light trap, an improved model. By W. D. Sudia and R. W. Chamberlain, 1962. *Journal American Mosquito Control Association* 4: 536-538.

Takken W., Kager P.A. & Verhave J.P. 2007. Will malaria return to North-West Europe?, pp. 23-34, *Emerging pest and vector-borne diseases in Europe.* Wageningen Academic Publishers, Wageningen.

Teodorescu C. 1983. Experimental infection of an indigenous strain of *Anopheles atroparvus* with imported species of *Plasmodium*. *Archives Roumaines de Pathologie Expérimentales et de Microbiologie* 42: 365-370.

Thomson M.C. & Connor S.J. 2001. The development of Malaria Early Warning Systems for Africa. *Trends in Parasitology* 17: 438-445.

Ungureanu E. & Shute P.G. 1947. The value of the wing scales as an aid to the taxonomy of adult *Anopheles maculipennis*. *Proceedings of the Royal Entomological Society of London, ser.B* 16: 79-85.

Van den Hurk A.F., Cooper R.D., Beebe N.W., Williams G.M., Bryan J.H. & Ritchie S.A. 2000. Seasonal abundance of *Anopheles farauti* (Diptera: Culicidae) sibling species in far north Queensland, Australia. *Journal of Medical Entomology* 37: 153-161.

Van Thiel P.H. 1927. Sur l'origine des variations de taille de l'*Anopheles maculipennis* dans les Pays-Bas. *Bulletin de la Société de Pathologie Exotique* 20: 366-390.

Van Thiel P.H. 1933. Investigations on the range and differentiation of *Anopheles maculipennis* races and their bearing on the existence or the absence of malaria in Italy. *Rivista di Malariologia* 12: 281-318.

Van Thiel P.H. 1934. Insuffisance des caractères de l'oeuf pour la distinction des races trophiques et biologiques de l'*Anopheles maculipennis*. *Bulletin de la Société de Pathologie Exotique* 27: 154-158.

Vanderberg J.P. 1988. In vitro cultivation of malaria parasites: sporogonic stages, pp. 331-348, *Malaria. Principles and practice of malariology*. Churchill Livingstone, London.

Vargas L. 1950. Nota sobre la distribución holártica del grupo *maculipennis* y del subgénero *Coelodiazesi*s. *Missirolimyia,* nov. subg. de *Anopheles* (Diptera: Culicidae). *Rivista di Parassitologia* 11: 73-84.

Vaughan J.A. 2007. Population dynamics of *Plasmodium* sporogony. *Trends in Parasitology* 23: 63-70.

Vaughan J.A., Noden B.H. & Beier J.C. 1994. Prior blood feeding effects on susceptibility of *Anopheles gambiae* (Diptera: Culicidae) to infection with cultured *Plasmodium falciparum* (Haemosporida: Plasmodiidae). *Journal of Medical Entomology* 31: 445-449.

Vercruysse J. 1985. Estimation of the survival rate of *Anopheles arabiensis* in an urban area (Pikine-Senegal). *Journal of Animal Ecology* 54: 343-350.

W.H.O. 1994. *Techniques entomologiques pratiques pour la lutte antipaludique. Partie I. Guide du stagiaire.* World Health Organization, Geneve. 55 pp.

W.H.O. 2001a. *Malaria vectors and approaches to their control in malaria affected countries of the WHO European Region*. World Health Organization, Regional Office for Europe, Copenhagen (http://www.euro.who.int/document/E76649.pdf). 58 pp.

W.H.O. 2001b. *Malaria early warning systems. Concepts, indicators and partners.* World Health Organization, Roll Back Malaria, Geneve. (http://www.who.int/malaria/cmc_upload/0/000/014/807/mews2.pdf). 80 pp.

W.H.O. 2002. *Epidemiological survaillance of Malaria in countries of Central and Eastern Europe and selected newly independent states*. World Health Organization, Regional Office for Europe, Copenhagen, (http://www.euro.who.int/document/E77302.pdf). 30 pp.

W.H.O. 2004. *Malaria epidemics: forescasting, prevention, early detection and control. From policy to practice*. World Health Organization, Geneve. (http://www.who.int/malaria/docs/Leysinreport.pdf). 52 pp.

W.H.O. 2006. *Regional strategy: From Malaria control to elimination in the WHO European region, 2006-2015*. World Health Organization, Regional Office for Europe, Copenhage, (http://www.euro.who.int/Document/E88840.pdf). 50 pp.

Ward R.A. 1984. Second supplement to "A catalog of the mosquitoes of the World" (Diptera, Culicidae). *Mosquito Systematics* 16: 227-270.

Ward R.A. 1992. Third supplement to "A catalog of the mosquitoes of the world" (Diptera, Culicidae). *Mosquito Systematics* 24: 177-230.

Warrel C.A. 2002. Clinical features of malaria, pp. 191-205, *Essential Malariology*. Oxford University Press Inc., Oxford.

Warren M., Collins W.E., Richardson B.B. & Skinner J.C. 1977. Morphologic variants of *Anopheles albimanus* and susceptibility to *Plasmodium vivax* and *P. falciparum*. *The American Journal of Tropical Medicine and Hygiene* 26: 607-611.

White G.B. 1978. Systematic reappraisal of the *Anopheles maculipennis* complex. *Mosquito Systematics* 10: 13-43.

White G.B. 1981. Academic and applied aspects of mosquito cytogenetics, pp. 245-274, Insect Cytogenetics. Symposia of the Royal Entomological Society of London, London.

White G.B. 2003. Medical acarology and entomology, pp. 1717-1806, *Manson's tropical diseases*. Elsevier Science Limited, Edinburgh.

Wiersch S.C., Maier W.A. & Kampen H. 2005. *Plasmodium* (Haemamoeba) *cathemerium* gene sequences for phylogenetic analysis of malaria parasites. *Parasitology Research* 96: 90-94.

Young M.D. & Burgess R.W. 1961. The infectivity to mosquitoes of *Plasmodium malariae*. *The American Journal of Hygiene* 73: 182-192.

Yuval B. 1992. The other habit: sugar feeding by mosquitoes. *Bulletin of the Society for Vector Ecology* 17: 150-156.

Zar J.H. 1984. *Biostatistical analysis*. Prentice-Hall, Inc., New Jersey. 718 pp.

Zhou G., Minakawa N., Githeko A.K. & Yan G. 2004. Association between climate variability and malaria epidemics in the East African highlands. *Proceedings of the National Academy of Sciences of the United States of America* 101: 2375-2380.

Zhou G., Minakawa N., Githeko A.K. & Yan G. 2005. Climate variability and malaria epidemics in the highlands of East Africa. *Trends in Parasitology* 21: 54-56.

Zulueta J. 1973. Malaria and Mediterranean history. *Parassitologia* 15: 1-15.

Zulueta J. 1994. Malaria and ecosystems: from prehistory to posteradication. *Parassitologia* 36: 7-15.

Zulueta J., Ramsdale C.D. & Coluzzi M. 1975. Receptivity to malaria in Europe. *Bulletin of the World Health Organization* 52: 109-111.

Zulueta J., Ramsdale C., Cianchi R., Bullini L. & Coluzzi M. 1983. Observations on the taxonomic status of *Anopheles sicaulti*. *Parassitologia* 25: 73-92

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APPENDIX

Map of Comporta study region

Adapted form Google Earth images (©2007 Google ™).