UNIVERSIDADE da MADEIRA

Nº de ordem 11/D/07 **Ano de 2007**

TESE DE DOUTORAMENTO

apresentada na
UNIVERSIDADE DA MADEIRA

Para obtenção do grau de Doutor

Marta Isabel Marreiros Santa Ana Viegas Gouveia

Susceptibility of mosquito vectors to *Dirofilaria immitis* on Madeira Island, Portugal

Júri: Prof. Doutor Pedro Telhado Pereira (Universidade da Madeira)
Prof. Doutor António Santos Grácio (Instituto de Higiene e Medicina Tropical)
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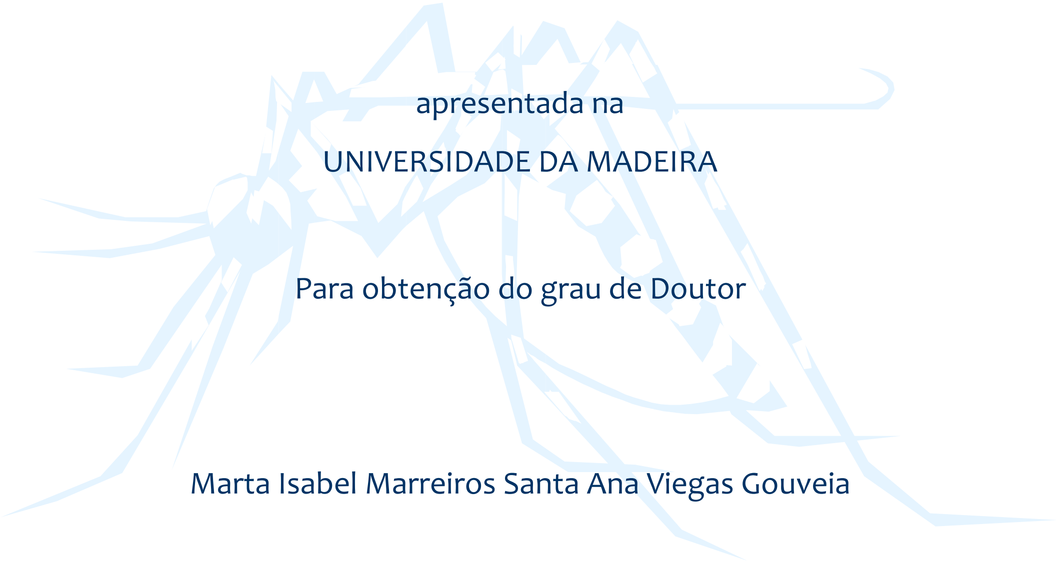

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CITMA
CENTRO DE CIÊNCIA E TECNOLOGIA DA MADEIRA

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Estamos sempre limitados aos nossos olhos, aos nossos modos de representação. Só a Natureza sabe o que de facto quer ou o que quis.

Goethe *In* Nachlaß

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RESUMO GERAL

Dirofilaria immitis (Leidy, 1856), agente da dirofilariase canina, é um importante parasita, quer do ponto de vista veterinário, quer como modelo de estudo da filaríase humana. O parasita, no seu estado adulto, ocupa o ventrículo direito e artérias pulmonares dos canídeos. Os culicideos são os seus vectores naturais.

D. immitis é um importante agente da dirofilariase na Ilha da Madeira, onde cerca de 30% dos cães apresentam esta doença. Contudo, nunca tinham sido feitos estudos sobre os vectores da dirofilaríase canina nesta região, a interacção entre parasita e vector, ou sobre as variáveis ambientais que possam ter influência na transmisão da doença.

A susceptibilidade inata à infecção é apenas um dos componentes da competência vectorial e o isolamento de mosquitos naturalmente infectados demonstra uma grande capacidade de *D. immitis* em explorar uma grande diversidade de espécies vectoras em condições naturais.

O propósito deste trabalho foi determinar quais os mosquitos vectores da dirofilaríase, a relação entre a densidade populacional destes vectores e o ambiente, e a associação entre a resposta imune do vector e o parasita.

A abundância sazonal de *Culex theileri* e *Cx. pipiens molestus* é aqui apresentada. Testes de correlação e análise de correspondência canónica foram efectuados, usando os dados sobre a dinâmica populacional destas espécies, relacionando-os com variáveis ambientais seleccionadas, incluindo temperatura, humidade relativa e precipitação mensal acumulada. O factor limitativo mais importante para determinar a abundância de *Cx. theileri* demontrou ser a precipitação acumulada, enquanto que a variação

populacional de *Cx. p. molestus* não se deveu a qualquer relação com as variáveis estudadas.

Estudos de campo foram realizados para verificar se *Cx. theileri* operava como vector de *D. immitis* na Ilha da Madeira. Demonstrou-se, pela primeira vez, que *Cx. theileri* apresentava competência vectorial para este parasita.

Os mesmos estudos foram efectuados para *Cx. p. molestus*. Uma fêmea capturada naturalmente por armadilhas EVS apresentava duas larvas de segundo estado nos túbulos de Malpighi; no entanto, estas apresentavam-se deformadas. Foram infectadas duas estirpes de *Cx. p. molestus* em laboratório, para melhor analisar a sua susceptibilidade a *D. immitis*. Nenhuma fêmea apresentou larvas infectantes do terceiro estado.

Finalmente, este estudo explorou o facto de *Cx. p. molestus* ser um mosquito autogénico para avaliar os custos reprodutivos quando esteja infectado por *D. immitis,* sem a utilização de refeições sanguíneas. Este mosquito demonstrou uma resposta de encapsulação melanótica quando inoculado intratoracicamente com microfilárias. Os ovários de *Cx. p. molestus,* que apresentavam as filárias melanóticamente encapsuladas, desenvolveram mais ovos do que aquelas que não melanizavam o parasita. Este facto contradiz estudos prévios relativos a custos reprodutivos em *Armigeres subalbatus* e *Aedes trivittatus*. Foi, no entanto, a primeira vez que se utilizou um mosquito autogénico em estudos nesta matéria.

GENERAL ABSTRACT

Dirofilaria immitis (Leidy, 1856), an agent of heartworm disease, is an important parasite from both the veterinary standpoint and as a model to study human filariasis. It is a mosquito-borne filarial nematode which inhabits the right ventricle and pulmonary arteries of dogs.

D. immitis is an important disease agent on Madeira Island with about 30% of dogs testing positive for this worm. Nevertheless, the vectors of this parasite in Madeira have never been studied, nor has the interaction between pathogen and vector, or the environmental variables that might influence heartworm transmission.

Innate susceptibility to infection is only one component of vector competence, and field isolation of naturally infected mosquitoes has shown the capability of *D. immitis* to exploit a great diversity of vector species under natural conditions.

The purpose of this work was to determine which mosquitoes are vectors of heartworm disease, the relation between population density and environment, and the association between immune response of the vector to the filarial parasite.

Seasonal abundance of *Culex theileri* and *Culex pipiens molestus* was studied. Correlation and canonical correspondence analysis were performed using abundance data of these two species with selected weather variables, including mean temperature, relative humidity and accumulated precipitation. The most important factor determining *Cx. theileri* abundance was accumulated

precipitation, while *Cx. pipiens molestus* abundance did not have any relationship with weather variables.

Field studies were performed to verify whether *Cx. theileri* Theobald functions as a natural vector of *D. immitis* on Madeira Island, Portugal. *Cx*. *theileri* tested positive for *D*. *immitis* for the first time.

The same study was made regarding *Cx. p. molestus*. Two abnormal L2 stage filarial worms were found in Malpighian tubules in field caught *Cx. p. molestus*. In the laboratory, two strains of *Cx. p. molestus* were studied for their susceptibility to *D. immitis*. None presented infective-stage larvae.

Finally, because *Cx. p. molestus* is an autogenous mosquito, we evaluated the reproductive costs when this mosquito mounts an immune response against *D. immitis* in the absence of a blood meal. This mosquito showed an active immune response when inoculated intrathoracically with microfilariae (mf) of the heartworm. The ovaries from mosquitoes undergoing melanotic encapsulation developed more eggs than those which could not melanize the mf. This fact is contradictory with some previous studies of reproductive costs in *Armigeres subalbatus* and *Ochlerotatus trivittatus,* and it was the first time that an autogenous mosquito was used to study this subject.

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Chapter 1

Introduction

STATE OF ART

Dirofilaria immitis (Leidy, 1856), an agent of heartworm disease, is an important parasite from both the veterinary standpoint and as a model to study human filariasis (Grieve et al. 1983; Beerntsen 2000; Lok 2000). It is a mosquito-borne filarial nematode which inhabits the right ventricle and pulmonary arteries of dogs. Because of its close biological relationship to the filarial parasites of man, this nematode serves as an important experimental and epidemiological model which facilitates the development of vaccines, diagnostic aids, and prophylactic and curative drugs (e. g. Ivermectin).

Dirofilaria immitis shows a cosmopolitan distribution in warmer climates. It is believed to be the most prevalent in southern Europe, India, China, Japan, Australia, and North and South America (Grieve et al. 1983). In Africa, the distribution of countries reporting canine filariasis to the 1983 WHO/FAO/OIE survey were Morocco, Algeria, Tunisia, Ghana, Burkina Faso and Nigeria (Lok 2000). The distribution of *Dirofilaria* worms is not homogeneous and the highest prevalence occurs in the valleys of rivers and in humid zones, where the environmental conditions are more favourable for the breeding of vectors (WHO 2004). At present, there is clear evidence that *Dirofilaria* infections are spreading in animal populations (Rossi et al. 1996).

Since mosquitoes were first reported as intermediate hosts of *D. immitis* by Grassi and Noè (1900), several species have been described as vectors of heartworm disease. Appendix 1 summarizes the mosquito species known to support natural development of *D. immitis*.

Although the association of the nematode with a mosquito vector limits its transmission both seasonally and geographically, *D. immitis* seems unique among the filarial worms in its ability to exploit a wide range of mosquito species in a variety of habitat types. Innate susceptibility to infection is only one component of vector competence, and field isolation of natural infected mosquitoes showed the capability of *D. immitis* to exploit a great diversity of vector species under natural conditions (Lok 2000).

Aside from the inherently different biology and ecology of the mosquito vectors, other factors, such as global warming, the increasing abundance of mosquitoes, the movement of domestic hosts throughout the continents, and the abundance of wild reservoirs (Abraham 2000) act as favourable forces for the distribution of filarial infections (Genchi et al. 2005).

The vector competence (factors that determine the compatibility between mosquitoes and pathogens), as a component of vector capability, is regulated by intrinsic factors (genetic) which affect the capability of vectors to transmit pathogens (Hardy et al. 1983). Any trait, such as feeding preferences of a host, that has a genetic factor, will affect the vector competence of the mosquito (Beerntsen et al. 2000).

It was demonstrated in several genetic studies of a variety of vector species that a single gene can profoundly affect vector competence (James and Fallon 1996). The genetic map of *Aedes aegypti* based on isozymes and morphological mutant markers, enabled a number of investigators to determine chromosomal regions of genes with major influence on the susceptibility to several pathogens, e. g., *Plasmodium gallinaceum* (Kilama and Craig 1969), *Brugia* spp. (MacDonald 1962a, 1962b) and *D. immitis* (McGreevy et al. 1974). A recessive gene(s) (designated as f^t), located on chromosome 1 in *Ae. aegyti,* was shown to control the susceptibility to the filarial worm *D. immitis*. It was clear in this study that other genes should be involved in determining parasite susceptibility in this mosquito (Beerntsen et al.2000).

Although many molecular and genetic tools have been developed to assess molecular determinants of vector competence, these studies have focused on two main genera, *Anopheles* (Holt et al. 2002) and *Aedes* (Knudson et al. 2002). The genome projects of these species are an outstanding achievement that will enable further characterization of candidate genes useful for disease control.

Genetic markers provide a powerful tool to locate genes or genome segments and to evaluate their influence on a particular phenotype. Severson and collaborators (1994a) provided the first demonstration of the feasibility and power of using DNA-based markers in linkage studies to identify genetic loci implicated on the vector competence to transmit *Brugia* parasites. This study mainly followed the experiments of MacDonald (1962a, 1962b) with *Ae. aegypti* and *B. malayi* using restriction fragment length polymorphism markers (RFLP), allowing them to conduct whole-genome scans for loci involved in susceptibility. An important feature of *Ae. aegypti* RFLP map is that most of the markers are random cDNA clones of *Ae. aegypti* genes (Severson et al. 1993). Therefore, these sequences probably represent single loci that will be conserved across species. Severson and collaborators (1994b) demonstrated that *Ae. aegypt*i RFLP markers derived from cDNA clone could be extremely useful for comparative gene mapping. This study showed that homology of these loci could be examined through hybridization with genomic DNA from several culicidae mosquitoes, including *Ae. albopictus*, *Ae. togoi*, *Armigeres subalbatus*, *Cx. pipiens* and *Anopheles gambiae*, as was confirmed by later studies (Ferdig et al. 1998; Mori et al. 1999; Severson et al. 2004).

Another robust genetic linkage map based on random amplified polymorphic DNA from PCR was constructed for *Ae. aegypti*. However, these markers have limited use to families or strains from which the map was derived (Beerntsen et al. 2000).

Recently, Hoti and Sillanpää (2006) presented a new method based on a Bayesian gene mapping that analyzed quantitative traits using both genotypic and microarray data. This study looked for possible interactions between marker genotypes and gene expression levels. This is a standard design appropriate for a traditional quantitative trait loci analysis (QTL). They additionally propose, for all the individuals in a cross, that measurements are taken of gene expression, using a microarray assay to monitors several genes. This work opens huge possibilities in new approaches to access molecular determinants of vector competence.

The result is an increasing amount of information on the genetic basis for susceptibility of mosquitoes to various pathogens. In fact, the technologies to manipulate mosquito genomes to express genes of interest are now available. This could be pottencially used to create refractory populations in genetic-based control strategies. Transgenic technologies, knock-out and gene silencing approaches are now being used to identify parasite “resistance genes” (Allen et al. 2001; Tamang et al. 2004; Huang et al. 2005); however, extensive experiments must be conducted before of any of these genes could be used in wild mosquitoes. For instance, *Cx. pipiens* strains that are susceptible to *Wuchereria bancrofti* are refractory to *B. malayi* (Bartholomay and Christensen 2002). Another problem is the variation in population size of vector species because of seasonal fluctuations such as temperature, desiccation, or the reduced efforts to control populations. At these times, random chance and genetic drift can have significant consequences on gene frequencies. (Tabachnick and Black 1996).

In the following paragraphs the biology of vectors will be discussed, followed by a description of the relationship between pathogens and mosquitoes. Emphasis is placed in the biology of vectors of *D. immitis* existing on Madeira Archipelago (Chapter 2), and the filarial worm interaction with these vectors (Chapters 3-5).

RESEARCH TOOLS

Mosquito species

Natural vectors of *D. immitis* include mosquitoes in the genera *Aedes*, *Anopheles*, *Culex*, *Ochlerotatus*, *Psorophora*, and *Wyeomyia*; however, the majority of research investigating susceptibility of mosquitoes to this parasite utilized selected strains of *Ae. aegypti*. This latter, although not a common natural vector of *D. immitis*, can be easily reared in the laboratory, both classical and molecular marker linkage maps of the genome exist, and efforts to sequence the genome is nearly completed (Christensen, personal communication).

On Madeira archipelago there are 6 known species of mosquitoes: *Anopheles hispaniola* (Theobald), *Culex hortensis maderensis* Mattingly, *Culex pipiens* L., *Culex theileri* Theobald, *Culiseta longiareolata* (Macquart), and *Ochlerotatus eatoni* (Edwards) (Capela, 1982). *An. hispaniola* was signalized only in Porto Santo Island and *Oc. eatoni* was recorded only for Madeira Island (Capela, 1982). *An. hispaniola*, *Cx. theileri* and *Cx. pipiens* (Capela 1981, 1982) all have a clear preference for mammals as hosts for blood feeding; however, in the last 4 years, surveys of Porto Santo recovered no *An. hispaniola*, which could indicate that this species is no longer present on this island. The presence of the *Cx. pipiens molestus* and *Cx. theileri* in the Archipelago implicates them as potential vectors of *D. immitis*; therefore, the present study only adresses to this two species.

Like all mosquitoes these two species are in the family Culicidae, which is subdivided into three subfamilies: Anophelinae, Toxorhynchitinae and Culicinae. The genus *Culex* is a member of the subfamily Culicinae (Knight and Stone 1977).

Numerous researchers have examined the biology and population genetics of *Cx. pipiens* (Knight 1951; Harbach et al. 1984; Vinagradova 2000; Fonseca et al. 2004; Keyghobadi et al. 2004), but we know very little about its vector competence. Recently, several investigators started to use molecular/genetic tools needed to identify those factors that contribute to the susceptibility *vs*. resistance of *Culex* mosquitoes with the nematodes they transmit (Da Silva et al. 2000; Allen et al. 2001; Bartholomay et al. 2003; Allen and Christensen 2004). *Cx. pipiens* represents the species complex of major medical and veterinary importance, serving as vector for St. Louis encephalitis virus (Tsai and Mitchell 1989), West Nile virus (Hubalek and Halouzka 1999; Lanciotti et al. 2000), Rift Valley fever virus (Meegan 1979), and other arboviruses. It also transmits the causative agents of lymphatic filariasis, *W. bancrofti* (Farid et al. 2001; Bartholomay et al. 2003) and dog heartworm disease, *D. immitis* (Hu 1931; Villavaso and Steelman 1970; Lowrie 1991; Rossi et al. 1999; Lai et al. 2001), as well as several avian *Plasmodium* species (Atkinson et al. 1995).

Members of this complex have a known reputation to develop resistance to insecticides, including organophosphates, carbamates and pyrethroids (Georghiou 1965; Bisset et al. 1997; Raymond et al. 2001). The evolution and diffusion, between continents, of resistant genes in members of the *pipiens* complex has become a topic of scientific interest (Labbe et al. 2005, Xu et al. 2005).

The referred complex has interpretational difficulties and controversy associated with a number of bewildering morphological, behavioural/physiological and genetic issues (Harbach et al. 1985). Discussions and debates regarding the taxonomy of the *pipien*s complex still continue involving primarily the *pipiens*/*molestus* issue. Many authors believe that the *pipiens* and *molestus* forms are only one species, because their differences are only due to physiological variations (Harbach et al. 1984). Others considered them as two distinct species (Miles and Peterson 1979;

Capela 1981; Fonseca et al. 2004). Between those extremes, some authors considered them to be two subspecies or semi-species (Bullini 1982). In fact, the differentiation between the two forms seems to vary from location to location. In addition, intermediate forms with characteristics of both species have been detected, suggesting that hybridization can occur between *Cx. pipiens* and *Cx. molestus* (Byrne and Nichols 1997).

Morphologic and behaviour characters (Christophers 1951; Senevet and Anderelli 1959; Harbach et al., 1984, 1985; Vinagradova 2000; Cornel et al. 2003), enzyme electrophoresis profiles ((Byrne and Nichols 1997; Cornel et al. 2003) and microssatelite analysis (Fonseca et al. 2004) have been developed to separate the 3 most commons members of pipiens complex (*Cx. pipiens*, *Cx. molestus* and *Cx. quinquefasciatus*).

Behaviour characters (autogeny vs. anautogeny) and microssatelites (Fonseca et al. 2004) have been used to identify the member of this complex present in Madeira Archipelago as *Cx. molestus.* However, because the discussion on the taxonomy of this complex member is still open (species, semi-species, sub-species), herein this form will be denoted as *Culex pipiens molestus*.

Culex theileri is not as well known as *Cx. pipiens*, in part due to the fact that this species is very difficult to rear in the laboratory. Nevertheless, the medical and veterinary importance of *Culex theileri* has been demonstrated in several studies. *Cx. theileri* is a naturally infected vector of West Nile virus, Rift Valley fever virus and Sindbis virus (Jupp et al. 1966; Oelofsen et al. 1990; Burt et al. 2002; Acha and Szyfres 2003) and the filarial worm *D. immitis* (Santa-Ana et al.

2006, chapter 3). *Cx. theileri* females are zoo-anthropophilic, feeding mainly on mammals. The distribution of this species includes the Afro-tropical region and they widely invade the Palearctic and the Oriental regions. It is present in southern Europe (Schaffner et al. 2001).

Mosquito surveillance

Carbon dioxide is included among several factors, such as odour, visual stimuli and heat, generally considered to be involved in host attraction (Clements 1999). Because carbon dioxide attracts at least some mosquito species, it has been commonly used in various traps (Service 1976). Several field studies were made to evaluate the best attractants for mosquitoes (for example Becker et al. 1995; Rueda et al. 2001; Russell 2004; Drummond et al. 2006). Russell (2004) caught more *Cx. quinquefasciatus* with EVS-trap (encephalitis vector surveillance trap) baited with CO_2 than with CDC-trap baited with the same attractant. When octenol was added, the number of mosquitoes collected was reduced drastically. Cooperband and Cardé (2006) tested several traps in a large field wind tunnel and the number of mosquitoes approaching the different traps was compared the number of mosquitoes captured. Although *Cx. quinquefasciatus*, *Cx. restuans* and *Cx. tarsalis* spend more time oriented to EVS trap, only 13-16% of them were captured by this trap. However, Webb and Russell (2005) demonstrated in a field survey with four commercially available adult mosquito traps that EVS-trap collected the most mosquitoes (137% more than the mosquito magnet pro trap, the second most efficient). These studies show great variablility with respect to relative numbers and particular species. For the present study, EVS-trap was selected based on the mosquito species present on Madeira Island.

Infection of mosquitoes with *D. immitis*

Several ways to infect mosquitoes with parasites for experimental purposes in the laboratory are possible: 1) mosquitoes can feed directly on infected experimental hosts, 2) parasites can be presented to mosquitoes through an artificial membrane on a glass feeding apparatus, and 3) parasites can be inoculated directly using pulled glass capillary needles (allowing an assessment of parasite development without a blood meal) (Bartholomay 2004). This method of inoculating *D. immitis* into the hemocoel has become an established method to trigger and evaluate the melanization immune response. A previous investigation showed that all microfilaria (mf) that accidentally penetrated through the Malpighian tubules into the hemocoel of *Ochlerotatus trivittatus* (=*Aedes trivittatus*) were completely melanized and encapsulated (Christensen 1981a). Moreover, this technique is ideal to test the relationship between melanization and autogeny in the absence of a blood meal.

THE BIOLOGY OF MOSQUITO-PARASITE INTERACTIONS

D. immitis development in the mosquito host.

Following ingestion via blood meal, all the pathogens enter the midgut. When filarial worms responsible for dog heartworm are ingested, they travel through the midgut lumen, migrate up the lumen of Malpighian tubules, and enter the distal cells of the tubules, where they develop intracellularly.

Following the period of development, and after going through two moults, infective third-stage larvae break out of the Malpighian tubules and enter the hemocoel where they migrate thorough the open circulatory system to the head region. Then, the infective-stage filarial worms actively emerge from the tip of the proboscis and are deposited on the surface of the vertebrate skin, while the mosquito feeds. They then enter through the wound made by the mosquito bite.

Filarial worm development in the mosquito is not a benign process. In general, the physiology of the host is affected due to structural damages or deregulation of physiological balance (Clements 1999). Several studies with *D. immitis* showed an increasing mortality rate in infected mosquitoes when compared with non-infected ones (Kartman 1953; Intermill 1973). Additional studies also revealed a strong negative correlation between parasites intensity and mosquito survival (Christensen 1978; Nayar and Knight 1999; Lai et al. 2001). The mortality rates of mosquitoes infected with *D. immitis* could be explained by the fact that this filarial worms cause serious damage in the Malpighian tubules. Palmer and collaborators (1986) suggested that *D. immitis* mf completely damaged the Malpighian tubule cells, and a heavy infection could be responsible for the complete destruction of the excretory system, leading to the host death.

The influence of parasitism in the fecundity of mosquitoes has been frequently studied. Both melanotic encapsulation of parasites and egg tanning require several common substrates, for example, tyrosine and phenylalanine (Li and Christensen 1993, Uchida 1993, Christensen et al. 2005) and a competition for limited resources might result in a lower fecundity of the mosquito

(Beerntsen et al. 2000). Christensen (1981b) shows that in *Oc. trivittatus,* infected with *D. immitis* had a negative impact on fertility, and as the intensity of infection increased, the mean number of eggs produced by *Oc. trivittatus* decreased.

Barriers to parasite development

All pathogens transmitted by mosquitoes are acquired with a blood meal and, although the life cycle of each pathogen is distinct from each other, all of them share the same events of being ingested, exposed to the midgut environment, and traversing the hemolymph-filled hemocoel to reach their tissue site of development and then suitable sites for transmission back to the vertebrate host (Beernsten et al. 2000).

The anti-hemostatic factors present in mosquito saliva facilitate and contribute to the success of blood-feeding. Nevertheless, the consistency of the ingested blood can vary between different species. Blood coagulation inside the midgut can inhibit the migration of pathogens inside the vector, influencing the prevalence and intensity of infection (Kartman 1953).

The digestive tract of mosquitoes is often interrupted by a variety of sclerotinized spines that are projected from the gut wall into the lumen. In the fore-gut, these structures can be concentrated in rows or in groups (called the pharyngeal or cibarial armature). These armatures are the first line of defence against microfilariae, and in certain mosquito species can cause lethal lacerations of the worms at the time they are ingested with the blood meal (Coluzzi and Trabucchi 1968). These observations suggested that the dynamic variation of filariae transmission in different vector species could depend partially on the degree of development of its armature. McGreevey and collaborators (1978) analysed the fore-gut of 25 mosquito species, of which, 14 had well developed cibarial armature. Unlike several *Anopheles*, where the cibarial armatures has big sharp theeth and strong pointed spines (for example *An. gambiae* and *An. farauti*), *Culex* species show small and delicate teeth (figures 1.1 a and b). The pharyngeal armature in every *Culex* species analysed was composed of small spines.

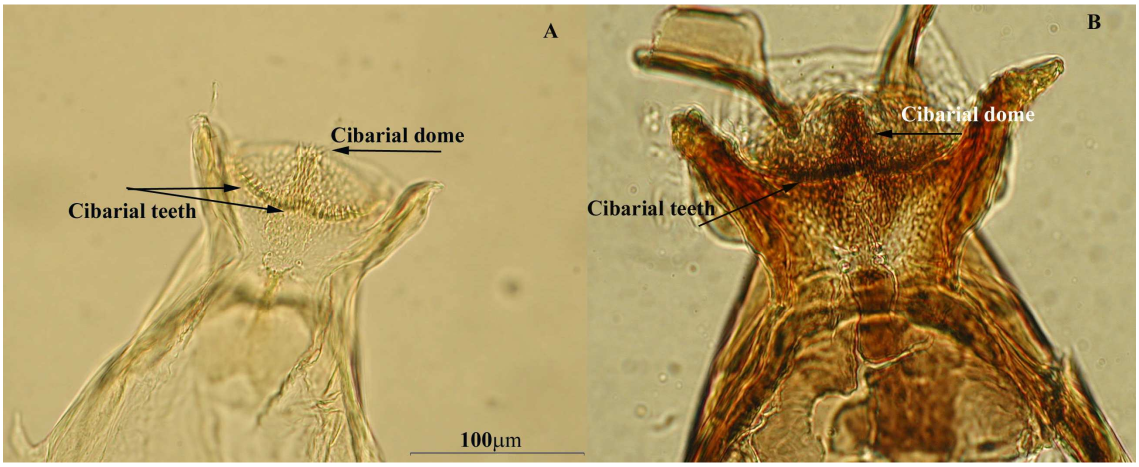

Fig. 1.1- Cibarial armature of *Cx. pipiens molestus* (A) and *Cx. theileri* (B)

Another environment potentially hostile to the parasites is the midgut. Inside of the midgut, the temperature and pH change drastically, proteolytic enzymes start the digestion of the blood meal, the blood loses its natural fluidity and the peritrophic matrix is produced, isolating the blood from the midgut epithelium (Beernten et al. 2000). The digestive enzymes can have a negative or positive impact on parasites, thereby influencing vector competence (Beerntsen et al. 2000; Okuda et al. 2002).

The molecules of the immune system provide mosquitoes with an innate defence system against foreign organisms. Although this system does not acquire a response memory, as is typical with antibodies of vertebrates, it does however possess internal defence mechanisms that are surprisingly specific and effective in destroying or limiting the development of pathogens and parasites (Paskewitz and Christensen 1996). The immune response of mosquitoes caused by foreign organisms involves humoral and cellular components. The humoral components include the phenoloxidase cascade system of parasite melanization and wound healing (Hernandez-Martinez et al. 2002; Lai et al. 2002; Christensen et al. 2005; Nappi and Christensen 2005), inducible antimicrobial peptides (Beerntsen and Christensen 1990; Lowenberg 2001; Vizioli et al. 2001; Bartholomay et al. 2003), and reactive oxygen and nitrogen intermediates (Luckhart et al. 1998; Kumar et al. 2003). The cellular components include phagocytosis (Da Silva et al. 2000; Hillyer et al. 2003; Hillyer et al. 2005) and encapsulation by hemocytes (Forton et al. 1985; Christensen et al. 2005).

One of the essential components of the immune response in mosquitoes is melanization. This unique defence mechanism is a fascinating process which involves an elaborated genetic and biochemical regulation. This response is usually mediated by hemocytes (Christensen and Forton 1986; Paskewitz and Christensen 1996; Bartholomay and Christensen 2002; Christensen et al. 2005) and culminates with the deposition of melanotic material that surrounds the parasite. This response has been observed in all of the mosquito species studied, including mosquitoes susceptible to parasites (Paskewitz and Riehle

1994). The mechanisms of filarial parasite recognition in mosquito are just beginning to be understood, but the biological process leading to melanotic encapsulation have largely been elucidated and characterized (Bartholomay 2004). The process begins when melanotic materials are deposited on a filarial worm, which becomes a dark and hardened capsule. Figure 1.2 shows the biochemical pathway of the melanin synthesis in mosquitoes.

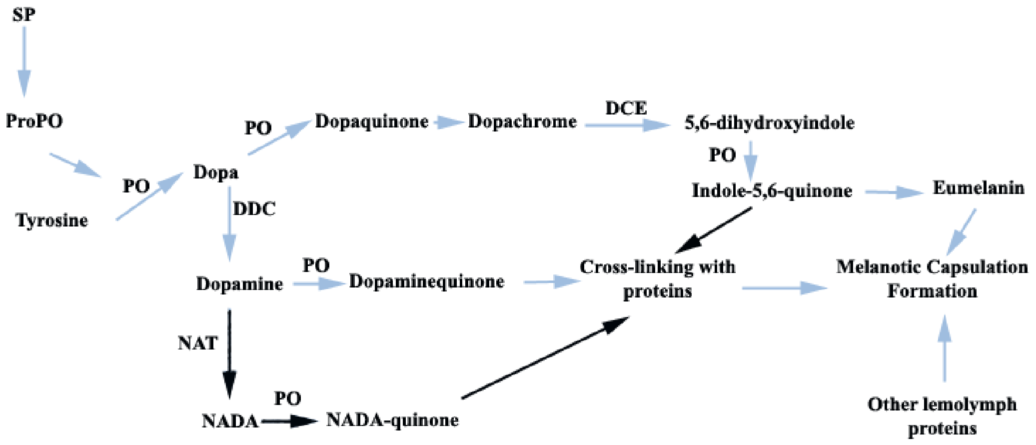

Fig 1.2- Melanotic encapsulation pathway in mosquitoes (SP- serine protease; ProPO- prophenol oxidase; PO- phenol oxidase; DDC- dopa decarboxylase; DCE- dopachrome conversion enzyme; NAT- *N*-acetyltransferase; NADA- *N*-acetyldopamine; → major pathways; → minor pathways). Adapted from Beerntsen et al. 2000.

The pathway of melanin biosynthesis involves a complex cascade of reactions with enzymatic and non-enzymatic reactions starting with tyrosine hydroxylation by phenoloxidase to form 3,4 di-hydroxyphenylalanine (DOPA) and ends with the oxidative polymerization of indolequinones. This polymerization is non-enzymatic, producing eumelanin, a dark brown polymer (Christensen et al. 2005).

In addition to immunity, melanin production is crucial for other physiological processes, including egg chorion tanning, wound healing and cuticle tanning. Ferdig and collaborators (1993) suggested that the process of egg development and melanotic encapsulation must compete for the same resources. By melanizing parasites, mosquitoes avoid the damage inflicted by developing worms, but by doing so they may reduce their reproductive output.

THE PRESENT STUDY

Dirofilaria immitis is an important disease agent on Madeira Island with about 30% of dogs testing positive for this worm (Clemente 1996). Nevertheless, the vectors of this disease in Madeira were never studied, nor the interaction between pathogen and vector, or the environmental variables that might influence heartworm transmission. To predict the course of a disease, the dynamics of its transmission must be thoroughly understood. The aim of this study presented herein was to understand the biology of the possible vectors present in the Madeira Archipelago (*Cx. p. molestus* and *Cx. theileri*) and to determine their susceptibility to *D. immitis*. The experiments conducted were performed to understand the natural system that exists between the vectors and the worm.

The vector-borne disease cycle comprises a dynamic interaction between pathogen, the vector (s), the vertebrate host(s) and the environment. One of the most important factors that intervene in vector competence is the environment. In fact, interactions among various elements of the transmission cycle are closely related to environmental conditions (Ba et al. 2005). Several studies have investigated the effect of weather on mosquito populations (Scott et al. 2000; Bolling et al. 2005; Shone et al. 2006). Temperature and humidity affect behaviour periodicities as permissive factors, and the physical effects of precipitation on surface conditions are multiple and the response of different mosquitoes to these effects is varied (Clements 1999, Shaman and Day 2005). In Madeira the temperature and relative humidity is very stable throughout the year. Precipitation, however, varies across the year, with the rainiest months being October to April. Herein, the abundance of *Cx. p. molestus* and *Cx. theileri* was correlated with these three environmental variables (Chapter 2).

Very few studies have been conducted focusing on the biology and vector competence of *Cx. theileri.* The first report concerning *Cx. theileri* and *Dirofilaria* sp. was in Portugal and involved field-collected mosquitoes from which only first and second instars were found in the Malpighian tubules (Ribeiro et al. 1983). However, it was impossible, at that time, to identify with certainty whether the parasite was *D. immitis.* Chapter 3 focus on *Cx. theileri,* because this species is now considered a new vector of *D. immitis.*

The biology of *Cx. pipiens* is very well studied (for example Vinagradova 2000) and, although the transmission of *D. immitis* by *Cx. pipiens* s.l. is known (Kartman 1953; Rossi et al. 1999; Cancrini et al. 2006), the vector competence of *Cx. pipiens molestus* was never been verified in nature. The ecology of Madeira Archipelago is unique, so the behaviour and ecology of this species could be different from other locations. Moreover, the literature presents evidence that the same species of mosquito have shown varying susceptibility to *D. immitis* in different laboratories (Kartman 1953). In chapter 4 further details are provided concerning the susceptibility of *Cx. p. molestus* to *D. immitis* on Madeira Island.

The capacity of *Cx. pipiens molestus* to melanize *D. immitis* was also assessed herein. As we described earlier, both melanotic encapsulation of parasites and egg tanning required several common substrates, e.g. tyrosine and phenylalanine (Li and Christensen, Uchida 1993, Christensen et al. 2005) and a competition for limited resources might result in a lower fecundity of the mosquito (Beerntsen et al. 2000). According to Ferdig and collaborators (1993), melanization of the parasites could have reproductive costs and mosquitoes best equipped genetically to respond may not be as reproductively competent in the event of parasite exposure. In that study *Ar. subalbatus* were fed with unifected and infected gerbils with *B. malayi* and the ovaries from the mosquitoes undergoing melanotic encapsulation reactions did not attain levels of tyrosine equal to ovaries from control mosquitoes. However, the biochemical makeup of ingested blood can have significant influence on vector competence (Beerntsen et al. 2000). Because *Cx. p. molestus* is an autogenous form of *pipiens* complex, it seemed important to assess the relation between melanized mf and egg production without the potentially confounding variable of a blood meal (chapter 5).

REFERENCES CITED

Abraham, D. 2000. Biology of *Dirofilaria immitis*. In: P. F. Boreham and R. B. Atwell (Eds.) Dirofilariasis. Boca Raton, FL, USA: CRC Press, Inc.. p 29-46.

Acha P. N., and B. Szyfres. 2003. Zoonoses and Communicable Diseases Common to Man and Animals: Chlamydioses, Rickettsioses, and Viroses. Vol. 2, 3rd ed. WA, USA; PAHO (Pan American Health Organization); 416 p.

Allen, M.L., and B.M. Christensen. 2004. Flight muscle-specific expression of act88F: GFI in trangenic *Culex quinquefasciatus* Say (Diptera: Culicidae). Parasitology International 53: 307-314.

Allen, M. L., D. A. O'Brochta, P. W. Atkinson, and C. S. Levesque. 2001. Stable, germ-Line transformation of *Culex quinquefasciatus* (Diptera: Culicidae). Journal of Medical Entomology, 38: 701-710.

Atkinson, C. T., K. L. Woods, R. J. Dusek, L. S. Silseo, and W. M. Iko. 1995. Wildlife disease and conservation in Hawai: pathogenicity of avian malaria (Plasmodium relictum) in experimentally infected Iiwi (Vestiaria coccinea). Parasitology 111 (supl.): 58-69.

Ba, Y., D. Diallo, C. M. F. Kebe, I. Dia, and M. Diallo. 2005. Aspects of bioecology of two Rift Valley fever virus vectors in Senegal (West Africa): *Aedes vexans* and *Culex poicilipes* (Diptera: Culicidae). Journal of Medical Entomology 42: 739-750.

Bartholomay, L. C. 2004. Mechanisms of innate immunity in culicine mosquitoes. [DPhil. Thesis], Madison, WI, USA: University of Madison-Wisconsin. 153 p. Available from: Memorial Library UW Madison Theses Basement North, Madison, WI; AWB B286 L875.

Bartholomay, L. C., and B. M. Christensen. 2002. Vector-Parasite interactions in mosquito-borne filariasis, In: T.R. Klei and T.V. Rajan (Ed.) World Class Parasites: volume 5 - The filaria. Massachusetts: USA Kluwer Academic Publishers, p. 9-20.

Bartholomay, L. C., H. A. Farid, R. M. Ramzy, and B. M. Christensen. 2003. *Culex pipiens pipiens*: characterization of immune peptides and the influence of immune activation on development of *Wuchereria bancrofti*. Molecular and Bichemical Parasitology 130: 43-50.

Becker N., M. Zgomba, D. Petric, and M. Ludwig. 1995. Comparison of carbon dioxide, octenol and a host-odour as mosquito attractants in Upper Rhine Valley, Germany. Medical and Veterinary Entomology 9: 377-380.

Beerntsen, B. T., and B. M. Christensen. 1990. *Dirofilaria immitis*: effect on hemolymph polypeptide synthesis in *Aedes aegypti* during melanotic encapsulation reactions against microfilariae. Experimental Parasitology 71: 406-414.

Beerntsen, B. T., A. A. James, and B. M. Christensen. 2000. Genetics of mosquito vector competence. Microbiology and Molecular Biology Reviews 64: 115-137.

Bisset, J., M. Rodriguez, A. Soca, N. Pasteur, and M. Raymond. 1997. Cross-resistance to pyrethroid and organophosphorus insecticides in the southern house mosquito (Diptera: Culicidae) from Cuba. Journal of Medical Entomology 34: 244-246.

Bolling, B. G., J. H. Kernnedy, and E. G. Zimmerman. 2005. Seasonal dynamics of four potential West Nile vector species in north-central Texas. Journal of Vector Ecology 30: 186-194.

Bullini, L. 1982. The electrophoretic approach to the study of parasites and vectors. Parassitologia, 27: 1-11.

Burt, F. J., A. A. Grobbelaar, P. A. Leman, F. S. Anthony, G. V. F. Gibson, and R. Swanepoel. 2002. Phylogenetic relationships of southern African West Nile Virus isolates. Emerging Infectious Diseases 8: 820-826.

Byrne, K., and R. A. Nichols. 1997. *Culex pipiens* in London underground tunnels: differentiation between surface and subterranean populations. Heredity 82: 7-15.

Cancrini, G., M. Maggi, S. Gabrielli, M. Arispici, F. Tolari, M. Dell'Omodarme, and M. C. Prati. 2006. Natural vectors of Dirofilariasis in rural and urban areas of the Tuscan region, Central Italy. Journal of Medical Entomology 43: 574-579.

Capela, R. A. 1981. Contribution to the study of mosquitoes (Diptera: Culicidae) from the Archipelago of Madeira and the Salvages I - Madeira. Arquivos do Museu Bocage Série A 1: 45-66.

Capela, R. A. 1982. Contribuicão para o conhecimento dos mosquitos (Diptera, Culicidae) dos Arquipélagos da Madeira e das Selvagens II - Madeira, Deserta Grande, Porto santo e Selvagem Grande. Boletim do Museu Municipal do Funchal 34: 105-123.

Christensen, B. M. 1978. *Dirofilaria immitis*: Effect on the longevity of *Aedes trivittatus*. Experimental parasitology 44: 116-123.

Christensen, B. M. 1981a. Observation on the immune response of *Aedes trivittatus* against *Dirofilaria immitis*. Transactions of the Royal Society of Tropical Medicine and Hygiene 75: 439-443.

Christensen, B. M. 1981b. Effect of *Dirofilaria immitis* on the fecundity of *Aedes trivittatus*. Mosquito News 41: 78-81.

Christensen, B. M., and K. F. Forton. 1986. Hemocyte-mediated melanization of microfilariae in *Aedes aegypti*. Journal of Parasitology 72: 220-225.

Christensen, B. M., J. Li, C. Chen, and A. J. Nappi. 2005. Melanization immune response in mosquito vectors. Trends in Parasitology 21: 192-199.

Christophers, S. R. 1951. Note on morphological characters differentiating *Culex pipiens* L. from *Culex molestus* Forskal and the status of this forms. Transactions of the Royal Entomological Society of London 102: 372-379.

Clemente, M. L. 1996. Prevalence of *Dirofilaria* in dogs in Madeira Island. Examination and identification of microfilaria. Veterinária Técnica, Agosto: 34-37.

Clements, A. N. 1999. The biology of mosquitoes: sensory reception and behaviour. Vol. 2. Cambridge, UK: CABI Publishing, University Press. 740 p.

Coluzzi, M., and R. Trabucchi. 1968. Importanza dell'armatura bucco-faringea in *Anopheles* e *Culex* in relazione alle infezioni con *Dirofilaria*. Parassitologia 10: 47-59.

Cooperband, M. F., and R. T. Cardé. 2006. Orientation of *Culex* mosquitoes to carbon dioxide-baited traps: flight manoeuvres and trapping efficiency. Medical and Veterinary Entomology 20: 11-26.

Cornel, A. J., R. D. McAbee, J. Rasgon, M. A. Stanich, T. W. Scott, and M. Coetzee. 2003. Differences in extent of genetic introgression between sympatric *Culex pipiens* and *Culex quinquefasciatus* (Diptera: Culicidae) in California and South Africa. Journal of Medical Entomology 40: 36-51.

Da Silva, J. B., C. M. R. Albuquerque, E. C. Araujo, C. A. Peixoto, and H. Hurd 2000. Immune defense mechanisms of *Culex quinquefasciatus* (Diptera:Culicidae) against *Candida albicans* infection. Journal of Invertebrate Pathology 76: 257-262.

Drummond, C. L., J. Drobnack, P. B. Backenson, G. D. Ebel, and L. D. Kramer. 2006. Impact of trap elevation on estimates of abundance, parity rates and body size of *Culex pipiens* and *Culex restuans* (Diptera: Culicidae). Journal of Medical Entomology 43: 177-184.

Farid, H. A., R. E. Hammad, M. M. Hassan, Z. S. Morsy, I. H. Kamal, G. J. Weil, and R. M. R. Ramzy. 2001. Detection of *Wuchereria bancrofti* in mosquitoes by the polymerase chain reaction: a potentially useful tool for large-scale control programmes. Transactions of the Royal Society of Tropical Medicine and Hygiene 95: 29-32.

Ferdig, M. T., B.T. Beerntsen, F. J. Spray, J. Li, and B.M. Christensen. 1993. Reproductive costs associated with resistance in a mosquito-filarial worm system. American Journal of Tropical Medicine and Hygiene 49: 756-762.

Ferdig, M. T., A. S. Taft, D. W. Severson, and B. M. Christensen. 1998. Development of a comparative linkage map for *Armigeres subalbatus* using *Aedes aegypti* RFLP markers. Genome Reserch 8: 41-47.

Fonseca, D., N. Keyghobadi, C. A. Malcolm, C. Mehmet, F. Schaffner, M. Mogi, R.C. Fleischer, and R.C. Wilkerson. 2004. Emerging vectors in the *Culex pipiens* Complex. Science 303: 1535-1538.

Forton, K. F., B. M. Christensen, and D. R. Sutherland. 1985. Ultrastucture of the melanization response of *Aedes trivittatus* against inoculated *Dirofilaria immitis* microfilaria. Journal of Parsitology 71: 331-341.

Genchi, C., L. Rinaldi, C. Cascone, M. Mortarino, and G. Cringoli. 2005. Is heartworm disease really spreading in Europe? Veterinary Parasitology 133: 137-148.

Georghiou, G. P. 1965. Development and characterization of resistance to o-Isopropoxyphenyl methyl-carbamate in the mosquito *Culex pipiens quinquefasciatus* Say. Nature 207: 883-884.

Grassi, B., and G. Noè. 1900. The propagation of the filariae of the blood exclusively by means of the puncture of peculiar mosquitoes. British Medical Journal, 2: 1306.

Grieve, R. B., J. B. Lok, and L. T. Glickman. 1983. Epidemiology of canine heartworm infection. Epidemiologic Reviews 5: 220-246.

Harbach, R. E., C. Dahl, and C. B. White. 1985. *Culex* (*Culex*) *pipiens* L. (Diptera: Culicidae): concepts, type designation and description. Proceedings of the Entomological Society of Washington 87: 1-24.

Harbach, R. E., B. A. Harrison, and A. M. Gad. 1984. *Culex* (*Culex*) *molestus* Forskal (Diptera:Culicidae) neotype designation, description, variation and taxonomic states. Proceedings of the Entomological Society of Washington 86: 521-542.

Hardy, J. L., E. J. Houk, L. D. Kramer, and W. C. Reeves. 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annual Review of Entomology 28: 229-262.

Hernández-Martínez, S., H. Lanz, M. H. Rodríguez, L. González-Ceron, and V. Tsutsumi. 2002. Cellular-mediated reactions to foreign organisms inoculated into hemocoel of *Anopheles albimanus* (Diptera: Culicidae). Journal of Medical Entomology 39: 61-69.

Hillyer, J. F., S. L. Schmidt, J. F. Fuchs, J. P. Boyle, and B. M. Christensen. 2005. Age-associated mortality in immune challenged mosquitoes (*Aedes aegypti*) correlates with decrease in haemocyte numbers. Cellular Microbiology 7: 39-51.

Hillyer, J. F., S. L. Schmidt, and B. M. Christensen. 2003. Rapid phagocythosis and melanization of bacteria and *Plasmodium* sporozoites by hemocytes of the mosquito *Aedes aegypti*. Journal of Parasitology 89: 62-69.

Holt, R. A. et.al. 2002. The genome sequence of the Malaria mosquito *Anopheles gambiae*. Science 298: 129-165.

Hoti F. and M. J. Sillanpää. 2006. Bayesian mapping of genotypes x expression interactions in quantitative and qualitative traits. Heredity 97: 4-18.

Hu, S. M. K. 1931. Studies on host-parasite relationships of *Dirofilaria immitis* Leidy and its culicine intermediate hosts. American Journal of Hygiene 14: 614-631.

Huang, C.-Y., S.-Y. Chou, L. C. Bartholomay, B. M. Christensen, and C.-C. Chen. 2005. The use of gene silencing to study the role of dopa decarboxylase in mosquito melanization reactions. Insect Molecular Biology 14: 237-244.

Hubalek, Z., and J. Halouzka. 1999. West Nile fever: a re-emerging mosquito borne viral disease in Europe. Emerging Infectious Diseases 5: 643-650.

Intermill, R. W. 1973. Development of *Dirofilaria immitis* in *Aedes triseriatus* Say. Mosquito News 33: 176-180.

James A. A., and A. M. Fallon. 1996. Gene expression in vectors, In: B. J. Beaty and W. C. Marquardt (Eds.) The Biology of Disease Vectors. Niwot, Colo. USA: University Press of Colorado. p. 229-245.

Jupp, P. G. B. M. McIntosh, and R. G. Brown. 1966. Laboratory transmission experiments with West Nile and Sindbis viruses and *Culex* (*Culex*) *theileri* Theobald. South African Journal of Medical Science 31: 95-97.

Kartman, L. 1953. Factors influencing infection of the mosquito with *Dirofilaria immitis* (Leidy, 1856). Experimental Parasitology 2: 27-78.

Keyghobadi, N., M. A. Matrone, G. D. Ebel, L. D. Kramer, and D. M. Fonseca 2004. Microsatellite loci from the northern house mosquito (*Culex pipiens*), a principal vector of West Nile virus in North America. Molecular Ecology Notes 4: 20-22.

Kilama, W. L., and G. B. Craig, Jr. 1969. Monofactorial inheritance of susceptibility to *Plasmodium gallinaceum* in *Aedes aegypti*. Annals of Tropical Medicine and Parasitology 63: 419-432.

Knight, K. L. 1951. A review of the *Culex pipiens* Complex in the Mediterranean subregion (Diptera, Culicidae). Transactions of the Royal Entomological Society of London 102: 354-364.

Knight, L. K., and A. Stone. 1977. A catalog of the mosquitoes of the world (Diptera: Culicidae) - The Thomas Say Foundation volume VI. 2nd ed. College Park: Entomological Society of America. 611 p.

Knudsen D. L., S. E. Brown, and D. W. Severson 2002. Culicine genomics. Insect Biochemistry Molecular Biology 32: 1193-1197.

Kumar, S., G. K. Christophides, R. Cantera, B. Charles, Y. S. Han, S. Meister, G. Dimopoulos, F. C. Kafatos, and C. Barillas-Mury. 2003. The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America 100: 14139-14144.

Labbe, P., T. Lenormand, and M. Raymond. 2005. On the worldwide spread of an insecticide resistance gene: a role for local selection. Evolutionary Biology 18: 1471-1484.

Lai, S.-C., C.-C. Chen, and R.F. Hou. 2002. Immunolocalization of prophenoloxidase in the process of wound healing in the mosquito *Armigeres subalbatus* (Diptera: Culicidae). Journal of Medical Entomology 39: 266-274.

Lai, C.-H., K.-C. Tung, H.-K. Ooi, and J.-S. Wang. 2001. Susceptibility of mosquitoes in Central Taiwan to natural infections of *Dirofilaria immitis*. Medical and Veterinary Entomology 15: 64-67.

Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, and J. T. Roehrig. 2000. Rapid detection of

West Nile virus from human clinical specimens, field-collected mosquitoes and avian samples by TaqMan reverse transcriptase-PCR assay. Journal of Clinical Microbiology 38: 4066-4071.

Leidy, J. 1856. Worms in the heart of the dog. Procedings of the Academy of Natural Sciences of Philadelphia 8: 2.

Li J., and B. M. Christensen. 1993. Involvement of L-tyrosine and phenol oxidase in the tanning of *Aedes aegypti* eggs. Insect Biochemistry and Molecular Biology 23: 739-748.

Lowenberg, C. 2001. Innate immune response of *Aedes aegypti*. Insect Biochemistry and Molecular Biology 31: 219-229.

Lowrie Jr., R. C. 1991. Poor vector of *Culex quinquefasciatus* following infection with *Dirofilaria immitis*. Journal of the American Mosquito Control Association 7: 30-36.

Lok, J. B. 2000. *Dirofilaria* sp.: taxonomy and distribution. In: P.F.L. Boreham and R.B. Atwell (Eds.) Dirofilariasis. Boca Raton, Florida, USA: CRC Press, Inc. p. 2-28.

Luckhart, S., Y. Vodovotz, L. Cui, and R. Rosenberg. 1998. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. Proceedings of the National Academy of Sciences of the United States of America 95: 5700-5705.

MacDonald, W. W. 1962a. The selection of a strain of *Aedes aegypti* susceptible to infection with semi-periodic *Brugia malayi*. Annals of Tropical Medicine Parasitology 56: 368-372.

MacDonald, W. W. 1962b. The genetic basis of susceptibility to infection with semi-periodic *Brugia malayi* in *Aedes aegypti*. Annals of Tropical Medicine and Parasitology 56: 373-382.

McGreevy, P. B., J. H. Bryan, P. Oothuman, and N. Kolstrup. 1978. The lethal effects of the cibarial and pharyngeal armatures of mosquitoes on microfilariae. Transactions of the Royal Society of Tropical Medicine and Hygiene 72: 361-368.

McGreevy, P. B., G. A. H. McClelland, and M. M. J. Lavoipierre. 1974. Inheritance of susceptibility to *Dirofilaria immitis* infection in *Aedes aegypti*. Annals of Tropical Medicine and Parasitology 68: 97-109.

Meegan, J. M. 1979. The Rift Valley fever epizootic in Egypt 1977-1978. Transactions of the Royal Society of Tropical Medicine and Hygiene 73: 618-623.

Miles, S. J., and H. E. Peterson. 1979. Protein variation and systematics in the *Culex pipiens* group of species. Mosquito Systematics 11: 187-202.

Mori, A., D. W. Severson, and B. M. Christensen. 1999. Comparative linkage maps for the mosquitoes (*Culex pipiens* and *Aedes aegypti*) based on common RFLP loci. The Journal of Heredity 90: 160-164.

Nappi, A. J., and B. M. Christensen. 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochemistry and Molecular Biology 35: 443-459.

Nayar, J. K., and J. W. Knight 1999. *Aedes albopictus* (Diptera: Culicidae): an experimental and natural host of *Dirofilaria immitis* (Filarioidea: Onchocercidae) in Florida, U.S.A. Journal of Medical Entomology 36: 441-448.

Oelofsen M. J., A. Gericke, M. S. Smith, and T. C. van der Linde. 1990. Establishment and characterization of a cell line from the mosquito *Culex* (*Culex*) *theileri* (Diptera: Culicidae) and its susceptibility to infection with arboviruses. Journal of Medical Entomology 27: 939-944.

Okuda, K., A. S. Caroci, P. E. M. Ribolla, A. G. de Bianchi, and A. T. Bijovsky. 2002. Functional morphology of adult female *Culex quinquefasciatus* midgut during blood digestion. Tissue and Cell 34: 210-219.

Palmer, C. A., D. D. Wittrock, and B. M. Christensen 1986. Ultrastucture of Malpighian tubules of *Aedes aegypti* infected with *Dirofilaria immitis*. Journal of Invertebrate Pathology 48: 310-317.

Paskewitz, S. M., and B. M. Christensen. 1996. Immune response of vectors. In: B.J. Beaty and W.C. Marquardt (Ed.) The biology of Disease Vectors. Niwot, Colo. USA: University Press of Colorado. p. 371-392.

Paskewitz, S., and M. A. Riehle 1994. Response of *Plasmodium* refractory and susceptible strains of *Anopheles gambiae* to inoculated sephadex beads. Developmental and Comparative Immunology 18: 369-375.

Raymond, M., C. Berticat, M. Weill, N. Pasteur, and C. Chevillon. 2001. Insecticide resistance in the mosquito *Culex pipiens*: what have we learned about adaptation? Genetica 112-113: 287-296.

Ribeiro, H., H. Ramos, and C. A. Pires. 1983. Contribuição para o estudo dos vectores das filaríases animais em Portugal. Jornal da Sociedade de Ciências Médicas 147: 143-146.

Rossi, L., F. Pollono, P. G. Meneguz, and G. Cancrini. 1999. Quattro specie di culicidi come possibili vettori di *Dirofilaria immitis* nella risaia piemontese. Parassitologia 41: 537-542.

Rossi L., F. Pollono, P. G. Meneguz, L. Gribaudo, and T. Balbo. 1996. An epidemiological study of canine filarioses in north-west Italy: what has changed in 25 years? Veterinary Research Community 20: 308–315.

Rueda, L. M., B. A. Harrison, J. S. Brown, P. B. Whitt, R. L. Harrison, and R. C. Gardner. 2001. Evaluation of 1-octen-3-ol, carbon dioxide and light as attractants for mosquitoes associated with two distinct habitats in North Carolina.Journal of the American Mosquito Control Association 17: 61-66.

Russell, R. C. 2004. The relative attractiveness of carbon dioxide and octenol in CDC- and EVS-type light traps for sampling the mosquitoes *Aedes aegypti* (L.), *Aedes polynesiensis* Marks, and *Culex quinquefasciatus* Say in Moorea, French Polynesia. Journal of Vector Ecology 29: 309-314.

Santa-Ana, M., M. Khadem, and R. Capela. 2006. Natural Infection of Cu*l*ex *theileri* (Diptera: Culicidae) with *Dirofilaria immitis* (Nematoda: Filarioidea) on Madeira Island, Portugal. Journal of Medical Entomology 43: 104-106.

Schaffner E. G. A., B. Geoffroy, J. J. P. Henry, A. Rhaiem, and J. Brunhes. 2001. TheMosquitoes of Europe [CD-Rom]. IRD, Montpellier, France: IRD Éditions.

Scott, T. W., A. C. Morrison, L. H. Lorenz, G. G. Clark, D. Strickman, P. Kittayapong, H. Zhou, and J. D. Edman. 2000. Longitudinal studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: Population dynamics. Journal of Medical Entomology 37: 77-88.

Senevet, G., and L. Anderelli. 1959. Les moustiques de l'Afrique du Nord et du basin méditerraéen. Les genres *Culex*, *Uranotaenia*, *Theobaldia*, *Orthopodomyia* et *Mansonia*. Encyclopédie Entomologique (A) 37 Paris, France: Éditions Paul Lechevalier. 383 p.

Service, M. W. 1976. Mosquito ecology: field sampling methods. London, UK: Applied Science Publishers LTD. 583 p.

Severson, D. W., B. DeBruyn, D. D. Lovin, S. E. Brown, D. L. Knudson, and I. Moralis. 2004. Comparative genome analysis of the yellow fever mosquito *Aedes aegypti* with *Drosophila melanogaster* and the malaria vector mosquito *Anopheles gambiae*. The Journal of Heredity 95: 103-113.

Severson, D. W., A. Mori, Y. Zhang, and B. M. Christensen. 1993. Linkage map for Aedes aegypti using restriction fragment length polymorphism. The Journal of Heredity 84: 241-247.

Severson, D. W., A. Mori, Y. Zhang, and B. M. Christensen. 1994a. Chromosomal mapping of two loci affecting filarial worm susceptibility in *Aedes aegypti*. Insect Molecular Biology 3: 67-72.

Severson D. W., A. Mori, Y. Zhang, and B. M. Christensen. 1994b. The suitability of restriction fragment length polymorphism markers for evaluating genetic diversity among and synteny between mosquito species. American Journal of Tropical Medicine and Hygiene 50: 425-432.

Shaman J., and J. F. Day. 2005. Achieving operational hydrologic monitoring of mosquito-borne disease. Emerging Infectious Diseases 11: 1343-1350.

Shone, S. M., F. C. Curriero, C. R. Lesser, and G. E. Glass. 2006. Characterizing population dynamics of *Aedes sollicitans* (Diptera: Culicidae) using metereological data. Journal of Medical Entomology 43: 393-402.

Tabachnick, W. J., and W. C. Black. 1996. Population genetics in vector biology. In: B.J. Beaty and W.C. Marquardt (Ed.) The biology of Disease Vectors. Niwot, Colo. USA: University Press of Colorado, p. 417-437.

Tamang, D., S. M. Tseng, C. Y. Huang, I. Y. Tsao, S. Z. Chou, S. Higgs, B. M. Christensen, and C. C. Chen. 2004. The use of a double subgenomic Sidbis virus expression system to a study mosquito gene function: effects of antisense nucleotide number and duration of viral infection on gene silencing efficiency. Insect Molecular Biology 13: 595-602.

Tsai, T. F., and C. J. Mitchell. 1989. St. Louis Encephalitis. In: T.P. Monath (ed.) The arboviroses: epidemiology and ecology IV. FL., USA: CRC Boca Raton. p.113-143.

Uchida, K. 1993. Balanced amino acid composition essential for infusion-induced egg development in the mosquito (*Culex pipiens pallens*). Journal of Insect Physiology 39: 615-621.

Villavaso, E.J., and C.D. Steelman. 1970. Laboratory and field study of the southern house mosquito, *Culex pipiens quinquefasciatus* Say, infected with the dog heartworm, *Dirofilaria immitis* (Leidy), in Louisiana. Journal of Medical Entomology 7: 471-476.

Vinogradova, E. 2000. *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Sofia, Bulgaria: Pensoft Publishers. 250 p.

Vizioli, J., P. Bulet, J. A. Hoffmann, F. C. Kafatos, H.-M. Müller, and G. Dimopoulos. 2001. Gambicin: a novel immune responsive antimicrobial peptide from malaria vector *Anopheles gambiae*. Proceedings of the National Academy of Sciences of the United States of America 98: 12630-12635.

Xu, Q., H. Liu, L. Zhang, N. Liu. 2005. Resistence in the mosquito *Culex quinquefasciatus* and possible mechanisms for resistance. Pest Management Science 61: 1096-1102.

Webb, C., and R. C. Russel (Mosquito Control Systems Australia). 2005. A comparison of four commercially available adult mosquito traps. Institute of Clinical Pathology and Medical Research, Western Sydney Area Health Services, 6 p. Availability from:

http://www.mozziemagnet.com.au/research/mosquitomagnetstudy2005.pdf

WHO (World Health Organization). 2004. The vector-borne human infections of Europe: Their distribution and burden on public health. Copenhagen, Denmark: WHO Regional Office for Europe. 144 p. Available from:

http://www.euro.who.int/document/E82481.pdf

Chapter 2

Seasonal abundance of two potential *Dirofilaria immitis* (Nematoda: Filarioidea) vectors*, Culex theileri* and *Culex pipiens molestus* (Diptera: Culicidae) in Funchal, Portugal

ABSTRACT

Monthly average adult mosquito catches from one dry-ice baited light trap was carried out between March 2002 and May 2004 in Quebradas, Funchal. Seasonal abundance of *Culex theileri* and *Culex pipiens molestus* was studied. Correlation and canonical correspondence analysis were performed using abundance data of these two species with selected weather variables, including mean temperature, relative humidity and accumulated precipitation. The most important factor determining *Cx. theileri* abundance was accumulated precipitation, while *Cx. pipiens molestus* abundance did not have any relationship with weather variables. Both mosquito species tested positive for *Dirofilaria immitis* during the study period.

The medical and veterinary importance of *Culex theileri* Theobald has been demonstrated in several studies. *Cx. theileri* is a natural vector of West Nile virus, Rift Valley fever virus, Sindbis virus (Jupp et al. 1966; Burt et al. 2002; Acha and Szyfres 2003) and the filarial worm, *Dirofilaria immitis* (Leidy) (Santa-Ana et al. 2006). *Cx. theileri* females are zoo-anthropophilic, feeding mainly on mammals. The distribution of this species includes the Afro-tropical region, the Palearctic and the Oriental regions, as well as being present in southern Europe (Schaffner et al. 2001).

Culex pipiens molestus Forskål is a well studied mosquito that is used for numerous biological queries (Vinogradova 2000). *Culex pipiens* s. l. serves as a vector for St. Louis encephalitis virus (Tsai and Mitchell 1989), West Nile virus, (Hubalek and Halouzka 1999; Lanciotti et al. 2000) Rift Valley virus (Meegan, 1979) and for several filarial worms, e. g., *Wuchereria bancrofti* (Farid et al. 2001) and *Dirofilaria immitis* (Hu 1931; Villavaso and Steelman 1970; Lowrie 1991; Rossi et al. 1999; Lai et al. 2000), and for *Plasmodium spp.* in birds (Atkinson et al. 1995).

Several studies have investigated the effect of weather on mosquito populations with results varying by species. Temperature and humidity affect behaviour periodicities as permissive factors and the physical effects of precipitation on surface conditions are multiple, with the response of different mosquitoes to these effects being varied (Clements 1999; Shaman and Day 2005).

Under natural conditions, it is not easy to establish the nature of relationships between temperature, humidity and abundance. The climate conditions to which mosquitoes are exposed differ substantially at different latitudes and microclimates differ between habitats at any altitude (Clements 1999). For instance, DeGaetano (2005) studied the meteorological effects on *Culex* populations in New Jersey and concluded that total precipitation was the strongest predictor of catch variability, followed by temperature. Nevertheless, individual heavy rainfall diminished catch. The latter could be explained by the possible flush of immature mosquitoes from breeding sites (Shaman and Day 2005). On the other hand, Lee and Rowley (2000) showed that the changes in the abundance of *Cx. pipiens* in Iowa could not be explained by changes in ambient temperature (both minimum and maximum) or relative humidity, either within or among years. Cupp et al. (2004) studied the fluctuations of *Cx. erraticus* in relation to seasonal rainfall in Mississippi and the population abundance of this specie fluctuated inversely with the amount of rainfall occurring during the 6 month mosquito season, suggesting that this variability could reflect the agility of the larval stage and the tropical nature of this species to flourish in alternating wet/dry habitats.

On Madeira Island heartworm disease is a major problem, with an estimated prevalence in dogs of 30% (Clemente 1996). Potential vectors of *D. immitis* on Madeira are *Cx. pipiens* s.l. and *Cx. theileri* (Cancrini et al. 2006; Santa-Ana et al. 2006; M. S-A., R. C. and B. M. C., unpublished data), but climate information is necessary for the development of efficient mosquito control strategies (Alten et al. 2000) and disease transmission management. On Madeira Island there is no information on the relationship between meteorological factors and mosquito abundance. Fifteen locations on Madeira

Island were investigated but, outside Quebradas collection site, the numbers of mosquitoes collected were not in sufficient to draw statistical conclusions about the relationships between mosquito populations and environmental variables. Therefore, the present study was performed to better understand the seasonal population abundance of adult vectors of *D. immitis* in Quebradas, Funchal, on Madeira Island.

MATERIAL AND METHODS

Study Area

Madeira Island is situated in the Atlantic Ocean, and is the largest island of the Madeira Archipelago at 741 km². It has a length of 30 geographical miles (57 km), with an extreme breadth of 13 miles (22 km). The area of study was situated at Quebradas, in Funchal, wich is one of the most important foci of heartworm disease on Madeira (M.S-A., R.C. and B.M.C., unpublished data).

The collection area is surrounded by 5.4 ha of subtropical fruit trees (mango, papaya, avocado-pear, passion-fruit trees) and contains two large open ponds, always with water, and measuring 5.5x11x3 m and the other 11x22x6 m.

Metereological data

Daily temperature, rainfall and relative humidity data from Quebradas (32º 38' 39.834'' -16º 57' 30.590'') were obtained from the Regional Meteorology Institute. The station was located in Lazareto, Funchal (32º 38' 48.928'' -16º 53'

31.367"). Monthly mean temperature, total rainfall and mean relative humidity were calculated and used for this analysis.

Collection, processing and assaying of mosquito specimens

Adult mosquitoes were collected every 15 days (for 1 night) at one sampling station with a dry-ice baited light trap (EVS trap, Bioquip, Gardena, CA, USA). The sampling station was chosen based on prevalence of *D. immiti*s and the presence of two dogs infected with this nematode. Mosquito sampling was carried out between March 2002 and May 2004. A single trap with 0.6 Kg dry ice was placed about 1.5 meters above ground level, activated before sunset and retrieved two hours after sunrise. Mosquitoes were placed in plastic containers and returned to the lab for species identification. Samples were processed and identified to species with the aid of keys (Ribeiro and Ramos 1999; Shaffner et al. 2001).

Statistical analysis

Statistical analysis were performed on abundance of *Cx. p. molestus* and *Cx. theileri* collected during the study to determine which weather data were most influential on species distributions.

Pearson correlation analysis was used to examine the possible relationship between mosquito abundance and climate variables, and linear regression was used to determine the form and strength of the relationship between monthly total precipitation and *Cx. theileri* abundance using SPSS®14.0 (©SPSS Inc., Chicago, IL. 2005). Canonical correspondence analysis (CCA) (ter Braak 1986) was used to further examine the relationships between mosquito abundance with environmental variables. CCA is a multivariate direct gradient analysis technique, where species composition is directly related to a set of environmental variables. This analysis was carried out using CANOCO 4 for Windows (ter Braak and Smillauer 1998). Because in CANOCO the distribution of the test statistic under the null hypothesis of independence is not known, the Monte Carlo Permutation test is used to simulate this distribution. This test is completely distribution-free, meaning that it does not depend on any assumption about distributions of species abundance values (Lepš and Šmilauer 2003). The main advantages of weighted averaging ordinations include the simultaneous ordering of sites and species, rapid computation and very good performance when species have nonlinear and unimodal relationships to environmental gradients (Palmer 1993).

RESULTS

Mosquito species

A total of 1,634 mosquitoes were collected during this study, belonging to three different species: *Cx. p. molestus*, *Cx. theileri* and *Culiseta longiareolata*. Only eight individuals of the latter species were collected in the trap (0.5%), therefore *Cs. longiareolata* was not considered in this study.

Culex theileri was the most abundant species, comprising 84.3% of the total collection, followed by *Culex p. molestus* (15.2%).

Figure 2.1 is a summary for monthly abundance patterns of *Cx. p. molestus* and *Cx. theileri* females caught in EVS trap.

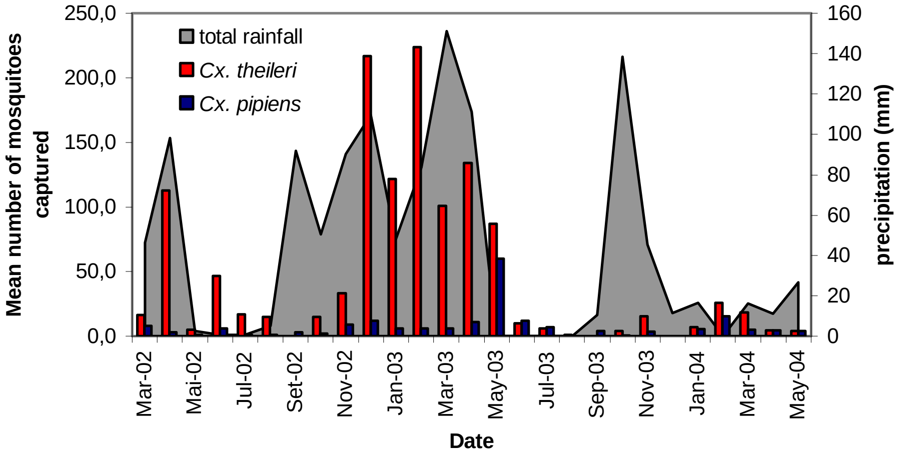

Fig. 2.1- Seasonal distribution of *Cx. pipiens. molestus* and *Cx. theileri* collected in Quebradas, Funchal and monthly accumulated precipitation.

Environmental data

Weather variables used in this study included monthly average temperatures, monthly average of relative humidity and monthly accumulated precipitation. Over the sample period temperatures ranged from 9.9ºC (March 3rd, 2002) and 34.2ºC (March 21st, 2003). The largest rain event occurred on March 28th, 2003, with 64.5mm^3. The total amount of precipitation occurring throughout this study was 1159.1 mm^3. Average relative humidity (R.H.) during the study period was 64% (ranged 8% - 93%).

Dispersal graphics

The influence of environmental variables on abundance of mosquitoes species, was demonstrated using analysis of dispersal graphics.

The number of *Culex pipiens molestus* collected was constant, regardless of variation in temperature, R.H. and precipitation. In contrast, *Culex theileri* abundance was increased when precipitation was over 2.5 mm^3. Changes in temperature and R.H. did not significantly influence abundance of *Cx. theileri* (figure 3).

Correlation and regression analysis

Pearson correlation analysis was performed on abundance data of *Cx. theileri*. There was a significant correlation between *Cx. theileri* and accumulative precipitation (R= 0.322, p<0.01) and a negative correlation between this mosquito and temperature (R=-0.3075, p=0.0118). The number of *Cx. theileri* collected was not affected by R.H..

It was used a linear regression model to explain the variation of the mean number of captured mosquitoes (figure 2.2).

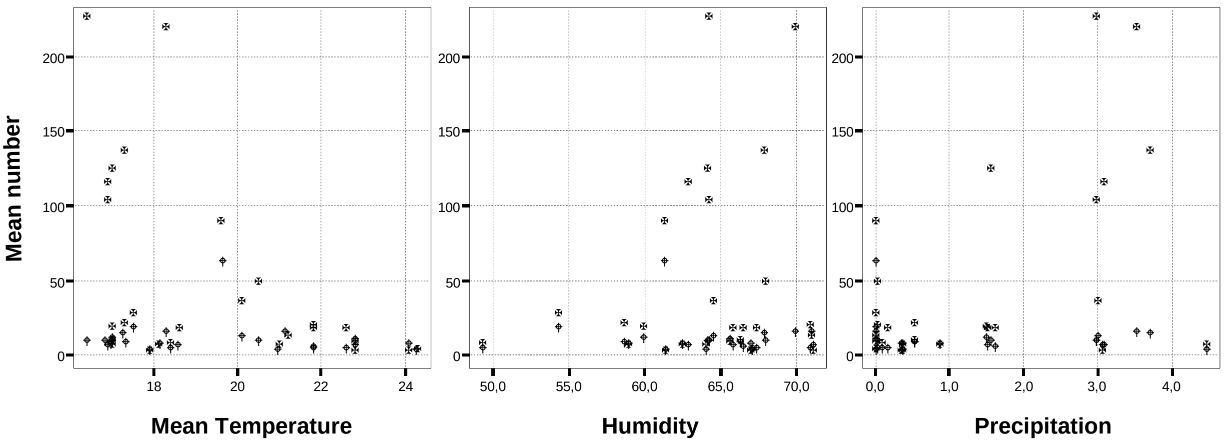

Fig. 2.2- Dispersal graphics of *Culex pipiens molestus* (†) and *Culex theileri* (✠) related to three environmental variables: (mean temperature (ºC), relative humidity (%) and monthly accumulated precipitation (mm^3).

In this model, only precipitation was the explainable variable, whereas temperature and R.H. had an almost null influence on mosquito abundance. Although this model has only one explainable variable, it is suitable to analyse the data (*F*=6.017, *p*=0.018).

Canonical correspondence analysis

The ordination diagram produced by CCA (figure 2.3) shows the relationships between species collected and environmental variables. The location of the species reveals the environmental preferences of each species (Palmer 1993).

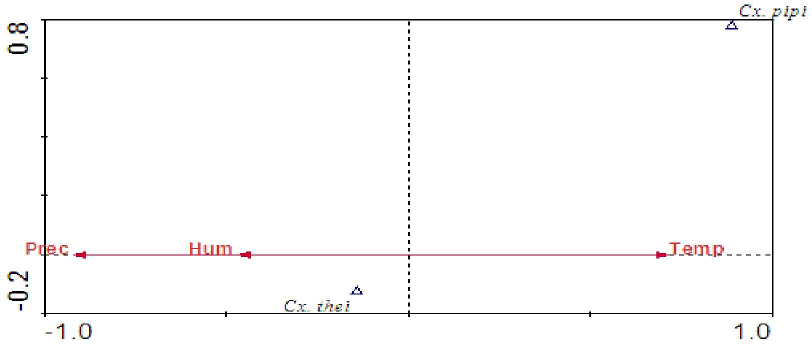

Fig. 2.3- Ordination diagram created from canonical correspondence analysis, showing the relationships between species abundance and environmental variables. The arrows represent environmental variables and the circles are species abundance. The length and direction of the arrows indicates the importance of the variable and how it correlates with species composition. (*Cx. pipi* -*Culex pipiens molestus*, *Cx. thei* - *Culex theileri*, Temp - Mean temperature, Hum - Relative humidity, Prec - monthly accumulated precipitation).

The CCA generated four canonical axes where the more important was the first axe in separating species with the following eigenvalues: 0.125, 0.095, 0.000 and 0.000. The significance levels produced from Monte Carlo permutation test show the importance of environmental variables. The most important variable determining *Cx. theileri* abundance was monthly accumulated precipitation (*F*=22.50, *p*=0.002), having a positive correlation. Although temperature shows a negative impact on this species in figure 2.2, it is not considered statistically significant ($p<0.05$). *Cx. p. molestus* abundance is not influenced by any of the environmental variables.

DISCUSSION

Several studies have investigated the effect of weather on mosquito population with results varying by species (Şimşek 2004; Bolling et al. 2005; DeGaetano 2005; Shone et al. 2006). Weather patterns affect adult mosquito abundance by altering the quality and quantity of larval habitats. Therefore, the association between climate variables and mosquito abundance can provide important information regarding mosquito-borne disease risks (Weigbreit and Reisen 2000). Knowing, in advance, which and how environmental variables affect the mosquito abundance it will be easier to plan and execute a successful mosquito control program, preventing the propagation of mosquito-borne diseases.

Statistical analysis was performed on *Cx. p. molestus* and *Cx. theileri*. *Cx. p. molestus* feeds on birds and mammals (Vinogradova 2000) and *Cx. theileri* primarily feeds on mammals (Capela 1981). Including vector competence and host preference data with mosquito abundance can help to determine which species most likely serve as important vectors.

Cx. p. molestus and *Cx. theileri* were the most abundant species collected in EVS-traps. Both species were present throughout the study period, and although there were seasonal changes, *Cx. theileri* was found to be the dominant species in EVS-trap. The seasonal distribution of *Cx. theileri* shows a peak beginning in November 2002 and extending to May 2003. This distribution was synchronized with the rainiest period of the study (Figure 2.1). This is also the period of the year when the temperature is lowest. It seems that *Cx. theileri* appears more frequently when temperature is above 20º C, with more 60% of R.H. and accumulated precipitation above 50 mm^3 (figure 2.2). It was also the period that this species was found naturally infected with *D. immitis* (Santa-Ana et al. 2006). These environmental variables could be most important factors in transmission of heartworm disease.

Correlation and canonical correspondence analysis showed monthly accumulated precipitation to be the most important variable affecting *Cx. theileri* abundance. An increase in precipitation will increase developmental rates resulting in population growth. Gubler et al. (2001) point out several ways precipitation could impact mosquitoes. The most important being the fact that increased rain may increase larval habitat and vector population size by creating new habitat.

Shone et al. (2006) also demonstrated that the sum of precipitation over a time lag is more significant than other aggregates because prolonged and continuous rain will maintain breeding sites better than one large rain event.

Although Gubler et al. (2001) suggested that increased humidity increases vector survival, Bidlingmayer (1985) shows that a great number of species were unaffected by R.H.. This seems to be the case for *Cx. theileri* in this study. The inclusion of temperature in the model is essential because temperature drives the length of time required for larva development. Nevertheless, survival can decrease or increase depending on the species (Gubler et al. 2001). Mean temperature on Funchal does not fluctuate throughout the year (16 - 24ºC); therefore, these values probably do not affect the mosquito abundance on the Island.

The seasonal distribution of *Cx. theileri* shows a peak beginning in November 2002 and extending to May 2003. This distribution was synchronized with the rainiest period of the study (Figure 2.1). This is also the period of the year when the temperature is lowest. It was also the period that this species was found naturally infected with *D. immitis* (Santa-Ana et al. 2006).

REFERENCES CITED

Abraham, D. 2000. Biology of *Dirofilaria immitis*. In P. F. L. Boreham and R. B. Atwell, editors. Dirofilariasis. Boca Raton, FL, USA: CRC Press, Inc. p. 29-46.

Acha P. N., and B. Szyfres. 2003. Zoonoses and Communicable Diseases Common to Man and Animals: Chlamydioses, Rickettsioses, and Viroses. Vol. 2, 3rd ed. WA, USA; PAHO (Pan American Health Organization). 416 p.

Alten B., R. Bellini, S. S. Caglar, F. M. Simsek, and S. Kaynas. 2000. Species composition and seasonal dynamics of mosquitoes in Belek region of Turkey. Journal of Vector Ecology 25: 146-154.

Atkinson, C. T., K. L. Woods, R. J. Dusek, L. S. Silseo, and W. M. Iko. 1995. Wildlife disease and conservation in Hawai: pathogenicity of avian malaria (*Plasmodium relictum*) in experimentally infected Iiwi (*Vestiaria coccinea*). Parasitology 111 (supl.): 58-69.

Bidlingmayer, W. L. 1985. The measurement of adult mosquito population changes – some considerations. Journal of the American Mosquito Control Association, 1: 328-48.

Bolling, B. G., J. H. Kennedy, and E. G. Zimmerman. 2005. Seasonal dynamics of four potential West Nile vector species in north-central Texas. Journal of Vector Ecology 30: 186-194.

Burt, F. J., A. A. Grobbelaar, P. A. Leman, F. S. Anthony, G. V. F. Gibson, and R. Swanepoel. 2002. Phylogenetic relationships of southern African West Nile Virus isolates. Emerging Infectious Diseases 8: 820-826.

Cancrini, G., M. Magi, S. Gabrielli, M. Aripici, F. Tolari, M. Dell'Omodarme, and M. C. Prati. 2006. Natural vectors of dirofilariasis in rural and urban areas of the Tuscan region, central Italy. Journal of Medical Entomology 43: 574-579.

Capela, R. A. 1981. Contribution to the study of mosquitoes (Diptera: Culicidae) from archipelagos of Madeira and the Selvages. I – Madeira. Arquivos do Museu Bocage 1: 45-66.

Clemente, M. L. 1996. Prevalence of Dirofilaria in dogs in Madeira Island. Examination and identification of microfilaria. Veterinária Técnica, Agosto: 34-37.

Clements, A. N. 1999. The biology of mosquitoes: sensory reception and behaviour. Vol. 2. Cambridge, UK: CABI Publishing, University Press. 740 p.

Cupp, E. W., K. J. Tenessen, W. K. Oldland, H. K. Hassan, G. E. Hill, C. R. Katholi, and T. R. Unnasch. 2004. Mosquito and arbovius activity during 1997-2002 in a wetland in northeastern Mississippi. Journal of Medical Entomology 41: 495-501.

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

Farid, H. A., R. E. Hammad, M. M. Hassan, Z. S. Morsy, I H. Kamal, G. J. Weil, and R. M. R. Ramzy. 2001. Detection of *Wuchereria bancrofti* in mosquitoes by the polymerase chain reaction: a potentially useful tool for large-scale control programmes. Transactions of the Royal Society of Tropical Medicine and Hygiene 95: 29-32.

Gubler, D. J., P. Reiter, K. L. Ebi, W. Yap, Roger Nasci, and J. A. Patz. 2001. Climate Variability and Change in the United States: Potential Impacts on Vector and Rodent-Borne Diseases. Environmental Health Perspective 109 (2 suppl):223–233.

Hu, S. M. K. 1931. Studies on host-parasite relationships of *Dirofilaria immitis* Leidy and its culicine intermediate hosts. American Journal of Hygiene 14: 614-631.

Hubalek, Z., and J. Halouzka. 1999. West Nile fever: a re-emerging mosquito borne viral disease in Europe. Emerging Infectious Diseases 5: 643-650.

Jupp, P. G., B. M. McIntosh, and R. G. Brown. 1966. Laboratory transmission experiments with West Nile and Sindbis viruses and *Culex* (*Culex*) *theileri* Theobald. South African Journal of Medical Science 31: 95-97.

Lai, C. H., K. C. Tung, H. K. Ooi, and J. S. Wang. 2000. Competence of *Aedes albopictus* and *Culex quinquefasciatus* as a vector of *Dirofilaria immitis* after blood meal with different microfilarial density. Veterinary Parasitology 90: 231-237.

Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M.S . Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, and J. T. Roehrig. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes and avian samples by TaqMan reverse transcriptase-PCR assay. Journal of Clinical Microbiology 38: 4066-4071.

Lee, J. H., and W. A. Rowley. 2000. The abundance and seasonal distribution of *Culex* mosquitoes in Iowa during 1995-97. Journal of the American Mosquito Control Association 16: 275-278.

Lepš, J., and P. Šmilauer. 2003. Multivariate analysis of ecological data using CANOCO. Cmbridge, U.K.: University Press. 269 p.

Lowrie, R. C. 1991. Poor vector of *Culex quinquefasciatus* following infection with *Dirofilaria immitis*. Journal of the American Mosquito Control Association 7: 30-36.

Meegan, J. M. 1979. The Rift Valley fever epizootic in Egypt 1977-1978. Transactions of the Royal Society of Tropical Medicine and Hygiene 73: 618-623.

Palmer, M. W. 1993. Putting things in even better order: the advantages of canonical correspondence analysis. Ecology 74: 2215-2230.

Ribeiro, H., and H. Ramos. 1999. Identification keys of the mosquitoes (Diptera: Culicidae) of Continental Portugal, Açores and Madeira. European Mosquito Bulletin 3: 1-13.

Rossi, L., F. Pollono, P.G. Meneguz, and G. Cancrini. 1999. Quattro specie di culicidi come possibili vettori di *Dirofilaria immitis* nella risaia piemontese. Parassitologia 41: 537-542.

Santa-Ana, M., M. Khadem, and R. Capela. 2006. Natural Infection of *Culex theileri* (Diptera: Culicidae) with *Dirofilaria immitis* (Nematoda: Filarioidea) on Madeira Island, Portugal. Journal of Medical Entomology 43: 104-106.

Schaffner E. G. A., B. Geoffroy, J. J. P. Henry, A. Rhaiem, and J. Brunhes. 2001. The Mosquitoes of Europe [CD-Rom]. IRD, Montpellier, France: IRD Éditions.

Shaman J., and J. F. Day. 2005. Achieving operational hydrologic monitoring of mosquitoborne disease. Emerging Infectious Diseases 11: 1343-1350.

Shone, S. M., F. C. Curriero, C. R. Lesser, and G. E. Glass. 2006. Characterizing population dynamics of *Aedes sollicitans* (Diptera: Culicidae) using Meteorological Data. Journal of Medical Entomology 43: 393-402.

Şimşek, F. M. 2004. Seasonal larval and adult population dynamics and breeding habitat diversity of *Culex theileri* Teobald, 1903 (Diptera: Culicidae) in the Gölbaşi District, Ankara, Turkey. Turkish Journal of Zoology 28: 337-344.

ter Braak, C. J. F. 1986. Canonical correspondence analysis: A new eigenvector technique for multivariate direct gradient analysis. Ecology 67: 1167-1179.

ter Braak, C. J. F., and P. Smilauer. 1998. CANOCO reference manual and user's guide to Canoco for Windows: software for canonical community ordination (version 4). Ithaca, NY.: Microcomputer Power. 500p.

Tsai, T. F., and C. J. Mitchell. 1989. St. Louis Encephalitis. In: T.P. Monath, editor The arboviroses: epidemiology and ecology IV. FL., USA: CRC Boca Raton. p.113-143.

Villavaso, E. J., and C. D. Steelman. 1970. Laboratory and field study of the southern house mosquito, *Culex pipiens quinquefasciatus* Say, infected with the dog heartworm, *Dirofilaria immitis* (Leidy), in Louisiana. Journal of Medical Entomology 7: 471-476.

Vinogradova, E. 2000. Culex pipiens pipiens mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Sofia, Bulgaria: Pensoft Publishers. 250 p.

Wegbreit, J., and W. K. Reisen. 2000. Relationships among weather, mosquito abundance, and encephalitis virus activity in California: Kern County 1990-98. Journal of the American Mosquito Control Association 2: 52-56.

Chapter 3

Natural Infection of *Culex theileri* (Diptera: Culicidae) with *Dirofilaria immitis* (Nematoda: Filarioidea) on Madeira Island, Portugal

Journal of Medical Entomology 43: 104-106 (2006)

ABSTRACT

Field and laboratory studies were performed to verify whether *Culex theileri* Theobald functions as a natural vector of *Dirofilaria immitis* (Leidy) on Madeira Island, Portugal. CO2-baited light traps (EVS traps) were use to sample mosquitoes monthly basis between February 2002 and February 2003 in the area of Quebradas (Funchal). Three mosquito species were captured, including 58 *Culex pipiens* L., 790 *Cx*. *theileri*, and three *Culiseta longiareolata* (Macquart). Only *Cx*. *theileri* tested positive for *D*. *immitis*. The presence of this FIlarial worm was detected by direct observation, infectivity assay dissection technique, and polymerase chain reaction methods. Infected mosquitoes were recovered in October and December 2002 and January 2003. These data provide evidence that *Cx*. *theileri* could be the main vector of *D*. *immitis* in Funchal, Madeira.

Dirofilaria immitis, the dog heartworm, has been recognized as an important canine disease in many areas of the world. It is probably the most well known disease of dogs and has been used as a model for fundamental research in medicine and biology (Lok 2000). In Portugal, heartworm disease occurs throughout the country (Fonseca et al. 1991) and is a major problem on Madeira Island, with an estimated prevalence in dogs of 30% (Clemente 1996).

There are numerous reports of many species of mosquitoes supporting the development of *D. immitis* (Ludlam et al. 1970; Lok 2000). Madeira Island has five species of mosquitoes: *Culex hortensis maderensis* Mattingly, *Culex pipiens* L., *Culex theileri* Theobald, *Culiseta longiareolata* (Macquart), and *Ochlerotatus eatoni* (Edwards). *Cx. theileri* and *Cx. pipiens* have a clear preference for mammals as hosts for blood feeding (Capela 1981, 1982). The first report concerning *Cx. theileri* and *Dirofilaria* sp. was in Portugal and involved field-collected mosquitoes from which only first and second instars were found in the Malpighian tubules (Ribeiro et al. 1983). However, it was impossible, at that time, to identify with certainty whether the parasite was *D. immitis.* Our studies, described herein, focused on *Cx. theileri,* because this species has not been considered a vector of *D. immitis.*

Field and laboratory studies were performed to verify whether *Cx. theileri* was a natural vector of *D. immitis* on Madeira Island, Portugal. The presence of these filarial worms was determined by direct observation, by infectivity assay dissection technique (Scoles et al. 1993) to detect third-stage larvae (L3), and by polymerase chain reaction (PCR) methods.

This work provides an indication that *Cx theileri* is probably the main vector of *D. immitis* in Funchal, Madeira Island.

MATERIAL AND METHODS

Study Area

The area of study was at Quebradas in Funchal, Madeira Island, with an average air temperature of 13-19ºC in winter (January-February) and 19-26ºC in summer (August-September). The collection area consisted of 5.4 ha of subtropical fruit trees (mango, papaya, avocado pear, and passion fruit) and contained a large pond. This site also housed two dogs, and one of them was infected with *D. immitis* (M.S.-A., personal observation).

Collection, Processing, and Assaying of Mosquito Specimens

Mosquito sampling was carried out between February 2002 and February 2003, with a CO2-baited light trap (EVS, Bioquip, Gardena, CA). Collections were made overnight (6 p.m.-10 a.m.). The mosquitoes collected were then kept under controlled conditions (25±2ºC, 70±5% RH, and a photoperiod of 12:12 (L:D h) and were fed a 10% sucrose solution for 13 days to allow the parasite development to the infective L3. Identification of mosquito species was made in accordance with the key by Ribeiro and Ramos (1999).

Mosquitoes from collection samples (<10) and those that did not survive for 8 days were separated by species and individually frozen (-20ºC). The head, thorax and abdomen of each mosquito were teased apart with a needle, and each part was placed in a drop of glycerine on a clean microscopic slide. The head and thorax were separated from the abdomen. Each preparation was examined at 400x magnification with a microscope. The number of larvae, their location and the stage of parasite development were recorded. Large samples were examined using the infectivity assay dissection technique, described by Scoles et al. (1993) as follows: each mosquito was placed in a 24-well tissue culture plate and carefully decapitated with fine needles. Each well contained 1 ml of phosphate-buffered saline. The plates were stored in a 37ºC incubator for 90 min to allow L3s to move into the medium. Plates were examined at 200x magnification with an inverted microscope. This method only detects infective L3s. The larvae found by direct observation and by the infectivity assay dissection technique were stored in 70% ethanol until analyzed by PCR.

DNA Isolation and PCR

Genomic DNA was extracted according to the procedure of Scoles and Kambhampati (1995): the specimen (mosquito or L3) was placed in a 1.5-ml microcentrifuge tube containing 30 µl of lysis buffer (100 mM NaCl, 10 mM Tris,1mM EDTA, and proteinase K to a final concentration of 4 µg/100 µl). After manual homogenization of the specimen, the tubes were incubated at 37ºCfor 30 min and then heated to 95_C for 5 min. The tubes were then centrifuged for 1 min, and the supernatant was used as a crude isolate.

To identify *D. immitis* infections, samples were analyzed by a specific primer that amplified a 378-bp DNA fragment from a single repeated element as follows: forward, 5’-ACG TAT CTG AG C TGG CTC AC-3’and reverse, 5’-ATG ATC ATT CCG CTT ACG CC-3’ (primers were synthesized by Invitrogen, Carlsbad, CA). The reagents used for each 25 µl reaction were 2.5 µl of 10x reaction buffer containing $MgCl_2$ to a final concentration of 1.5 mM, 0.5 µl of each primer for a final concentration of 100 ng/ml, dNTPs for a final concentration of 2.5 mM, 5 U of *Taq*DNA polymerase (Promega, Madison WI), and distilled deionized water to bring the final volume to 25µl. The thermal cycler program included an initial denaturation step at 94ºC for 1 min, followed by 40 cycles of the following: 90ºC for 1 min, 50ºC for 1 min, and 72ºC for 1 min. A final extension step of 72ºC for 5 min was added to ensure complete extension of all strands. After the cycles were complete, the samples were held at 4ºC. Amplified products were separated on a 2% agarose gel in 1x TAE buffer by using standard protocols (Sambrook and Russell 2001). Molecular size standards (1kbp DNA ladder, Promega) were included in each gel.

RESULTS

In total, 851 mosquitoes were obtained from 13 collections. The majority of mosquitoes captured by the EVS mosquito trap were *Cx. theileri* (92.8%), followed by *Cx. pipiens* (6.8%), and *Cs. longiareolata* (0.4%). The seasonal distribution of these mosquitoes attracted to EVS traps is shown in figure 3.1.

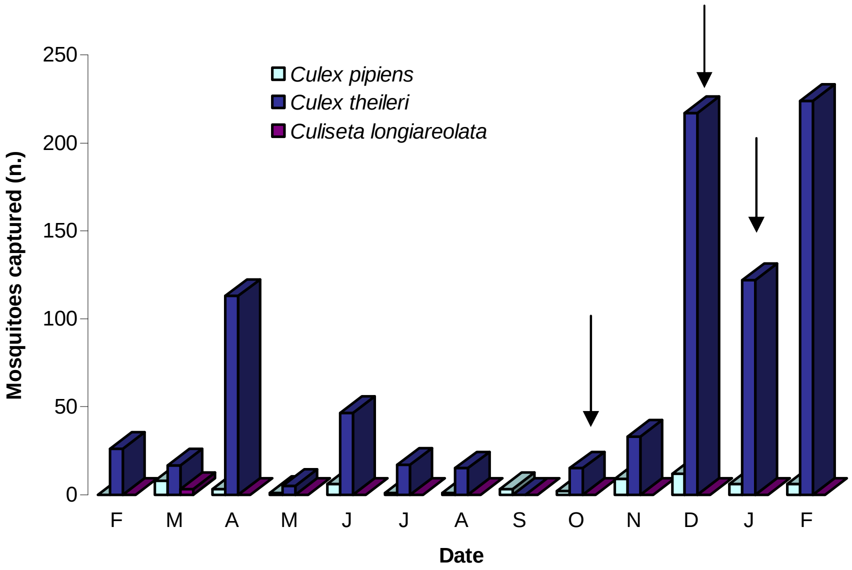

Fig. 3.1- Seasonal distribution of mosquitoes captured and their infection by *D. immitis* (arrows).

The mosquito populations increased during the rainy season and then declined throughout the typical dry season. Adult mosquito populations were lowest from July to November, when the pond was discharged frequently and the normal oviposition sites (e.g., buckets and barrels) were dry. Of the three mosquito species collected, *D. immitis* were only recovered from *Cx. theileri*.

Filarial DNA was found in four females of a total sample of 403 examined between February 2002 and February 2003. The first two females detected with *D. immitis* were collected on 30 October (from a total of 15 mosquitoes examined). One mosquito harboured four and the other six first-stage larvae (L1) in the Malpighian tubules. On December 27th, one L3 was recovered from one of 14 mosquitoes examined. The other 203 females captured could not be accurately examined because they were dead due to the severe weather (wind and rain) occurring on the night of collection and because of the high density of mosquitoes in the trap. On January 29th, another female (from a collection of 122) was recovered that harbored two L3s. PCR using specific primers amplified a 378-bp fragment of *D. immitis.* Amplification of the target was obtained from infective-stage larvae dissected from a female, from female mosquitoes captured in an EVS trap in December, and from microfilariae in the blood of an infected dog. Three additional female samples also were examined and did not produce a PCR product, indicating that they were not infected with *D. immitis.*

DISCUSSION

During this study, we collected three species of the five mosquito species that exist on Madeira Island (Capela 1981, 1982). The reason we only collected these three species is probably because we only used EVS traps and therefore only captured females that had an attractive behaviour to CO2.

Environmental factors are the main reason for changes in mosquito populations. During this study, the number of mosquitoes collected was highest from November to June during the rainy season, when more water is available in ponds, streams, and containers.

It is interesting to note that the four females infected were captured between October and January, indicating the possible influence of the seasonal variability in mosquito populations on the rate of mosquito infection. In October, very few catches occurred (compared with December catches), but in spite of this, one female was found infected in each month.

Trapping conditions and the poor condition of captured mosquitoes prevented the examination of larger numbers of individuals in October. In January, due to the large number of mosquitoes collected, we only analyzed females using the infectivity assay dissection technique. We therefore likely missed identifying those mosquitoes that only harboured developing larvae within the Malpighian tubules.

The presence of *D. immitis* larvae in Malpighian tubules (L1) and in the head (L3) of *Cx. theileri* indicate that this species is actively involved as a natural vector for this parasite on Madeira Island. The molecular diagnostics used allowed what is impossible with traditional morphological examination, i.e., specific identification of the filarial larvae developing in each mosquito collected.

Several attempts to establish a laboratory colony of *Cx. theileri* were not successful, and we therefore could not conduct laboratory studies to better elucidate *D. immitis* infection and development within this mosquito species.

Considering the growing number of reports of human dirofilariasis (Muro et al. 1999; Pampliglione and Rivasi 2001) and the role of *Cx. theileri* as a vector, this mosquito's potential contributions to the transmission of *D. immitis* should be more widely recognized.

REFERENCES CITED

Capela, R. A. 1981. Contribution to the study of mosquitoes (Diptera: Culicidae) from the Archipelago of Madeira and the Salvages I - Madeira. Arquivos do Museu Bocage, Série A 1: 45-66.

Capela, R. A. 1982. Contribuicão para o conhecimento dos mosquitos (Diptera, Culicidae) dos Arquipélagos da Madeira e das Selvagens II - Madeira, Deserta Grande, Porto santo e Selvagem Grande. Boletim do Museu Municipal do Funchal 34: 105-123.

Clemente, M. L. 1996. Prevalence of *Dirofilaria* in dogs in Madeira Island. Examination and identification of microfilaria. Veterinária Técnica, Agosto: 34-37.

Fonseca, I.M.P., L.M.M. Carvalho, S. P. Carvalho, and M. Carvalho-Varela. 1991**.** Prevalência da dirofilariose na populacão canina portuguesa. I. Detecção de microfilárias sanguíneas. Veterinária Técnica Set/Out: 36-38.

Lok, J. B. 2000. *Dirofilaria* sp.: taxonomy and distribution. In: P.F.L. Boreham and R.B. Atwell (Eds.) Dirofilariasis. Boca Raton, Florida, USA: CRC Press, Inc. p. 2-28.

Ludlam, K. W., L. A. Jachowski, and G. F. Otto. 1970. Potential vectors of *Dirofilaria immitis.* Journal of the American Veterinary Medical Association 157: 1354-1359.

Muro, A., C. Genchi, M. Cordero, and F. Simon. 1999. Human dirofilariasis in the European Union. Parasitology Today 15: 386-389.

Pampiglione, S., and F. Rivasi. 2001. Dirofilariasis. In: M. W. Service (Ed.), The encyclopedia of arthropod-transmitted infections, U. K.: CABI Publishing, p. 143-150.

Ribeiro, H., and Ramos, H. 1999. Identification keys of the mosquitoes (Diptera: Culicidae) of continental Portugal, Açores, and Madeira. European Mosquito Bulletin 3: 1-13.

Ribeiro, H., H. Ramos, and C. A. Pires. 1983. Contribuição para o estudo dos vectores das filaríases animais em Portugal. Jornal da Sociedade de Ciências Medicas 147: 143-146.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual. 3rd ed Plainview, NY: Cold Spring Harbor Press, 999 p.

Scoles, G. A., and S. Kambhampati. 1995. Polymerase chain reaction based method for the detection of canine heartworm (Filarioidea: Onchocercidae) in mosquitoes (Diptera: Culicidae) and vertebrate hosts. Ournal of Medical Entomology 32: 864-869.

Scoles, G. A., S. L. Dickson, and M. S. Blackmore. 1993. Assessment of *Aedes sierrensis* as a vector of canine heartworm in Utah using a new technique for determining the infectivity rate. Journal of the American Mosquito Control Association 9: 88-90.

Chapter 4

Natural and Experimental Infection of *Culex pipiens molestus* (Diptera: Culicidae) with *Dirofilaria immitis* (Nematoda: Filarioidea) on Madeira Island, Portugal

ABSTRACT

Dirofilaria immitis (Leidy 1856) is a mosquito-borne nematode which typically inhabits the right ventricle and pulmonary arteries of the dogs. Mosquito susceptibility to the filarial worms differs with species, strains and also among individuals of the same strain. To evaluate the ability of *Culex pipiens molestus* (Forskal) to support the development of *D. immitis* laboratory and field survey was made. Two abnormal L2 stage filarial worms were found in Malpighian tubules in *Cx. p. molestus* field caught. In laboratory, two strains were evaluated. Slightly differences in response to *D. immitis* were observed in both strains, but none of them developed infective-stage larvae. *Cx. p. molestus* showed no vector efficiency, and probably does not have an effective role in *D. immitis* transmission in Madeira Island.

Dirofilaria immitis (Leidy 1856) is a mosquito-borne nematode which typically inhabits the right ventricle and pulmonary arteries of the dogs (Lok 2000). In Europe, the prevalence of heartworm disease is increasing in dogs not treated with preventive drugs and the movement of domestic hosts, the increase of abundance of mosquitoes and the global warming can act as favorable factors for the distribution of *D. immitis* (Genchi et al. 2005).

Trotz-Wiliams and Trees (2003) shown that the countries and regions of Southern Europe, in particular those in the Mediterranean basin, are generally endemic for heartworm disease. In Portugal, heartworm disease occurs throughout the country (Fonseca et al., 1991) and is a major problem on Madeira Island, with an estimated prevalence in dogs of 30% (Clemente 1996).

In addition to a sufficient canine reservoir, transmission of *D. immitis* depends on adequate numbers of competent vector mosquitoes. The first requirement to determinate the vector competence is the susceptibility of the mosquito to the parasite. Susceptibility is defined as the ability of a mosquito to ingest microfilaria (mf) in a viable state and to support development of some proportion of the parasites to the infective third larval stage (Grieve et al. 1983). Two potential vectors are present on Madeira Island, *Culex pipiens* L. and *Culex theileri* Theobald, since this two species are the only who has clear preference of mammals as a host (Capela 1981, 1982). Natural infection of *Cx. theileri* with *D. immitis* was reported recently (Santa-Ana et al. 2006), but little is known about *Cx. pipiens* specially the *molestus* form.

Cx. pipiens s.l. has been considered by some authors as a poor vector of *D. immitis* (Kartman 1953; Todaro et al. 1977; Vezzani et al. 2006), while others believe that this specie is a efficient vector of heartworm filariae although its poor propensity to feed on dogs (Pinger 1985; Rossi et al. 1999; Cancrini et al. 2006), the literature shows discrepancy between the data; the same species of mosquito could show different susceptibility to *D. immitis* in diverse locations, and various strains of the same specie shows differences in susceptibility in the same laboratory (Hu 1931; Kartman 1953; Nayar and Sauerman 1975; Serrão et al. 2001). Vector status must be confirmed by identifying naturally occurring third-stage larvae of *D. immitis* in the mouthparts of field-collected mosquitoes.

Therefore, it is imperative to study the susceptibility of *Cx. p. molestus* to *D. immitis* on Madeira Island regarding the efficiency of this mosquito as a vector of the disease to know if this mosquito is an efficient vector.

Consequently, this study was designed to determine the ability of *Cx. p. molestus* to support the development of *D. immitis* under laboratory conditions and to transmit the infection naturally.

The outcome of this survey will provide valuable information to be used in the subsequent planning and implementation of effective vector control measures.

MATERIAL AND METHODS

Study site.

Madeira Archipelago is situated in the Atlantic Ocean and lies between 32°22.3′N 16°16.5′W and 33°7.8′N 17°16.65′W. Madeira Island is the largest island of the Archipelago with 741 km². It has a length of 30 geographical miles (57 km) and an extreme breadth of 13 miles (22 km).

Entomological sampling methods.

The collection sites were selected based on the human population concentration (consequently, dogs concentration). Figure 4.1 shows the locations of the sites.

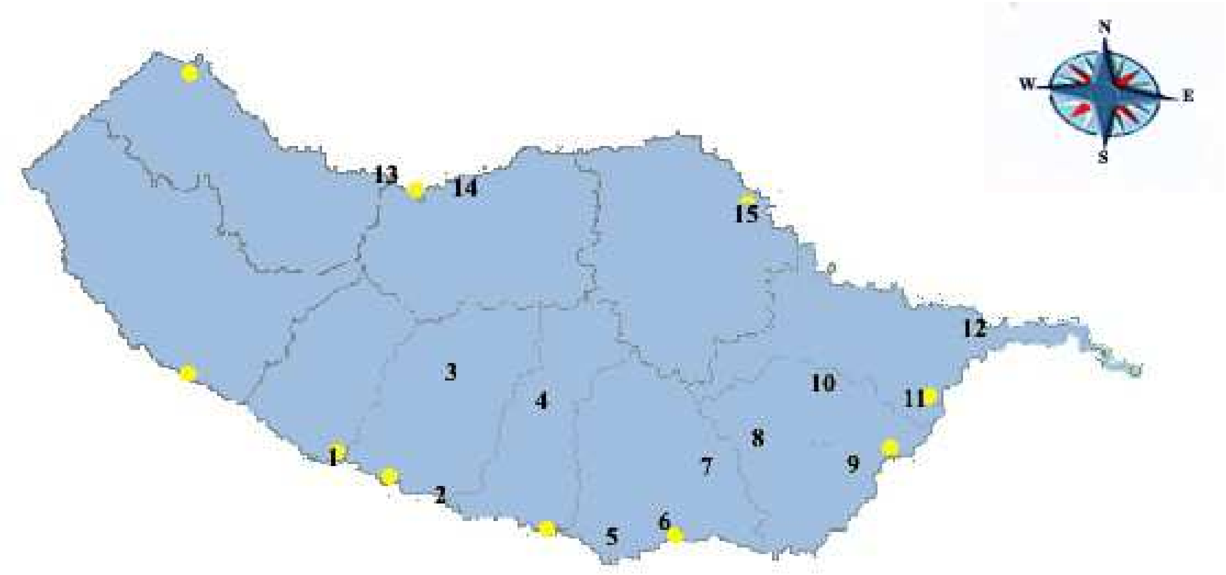

Fig. 4.1- Locations of the EVS-traps. (1-Ponta do Sol, 2-Campanário, 3- Serra d’água, 4- Lombo chão, 5- Quebradas, 6- Funchal, 7- Monte, 8- Camacha, 9- Gaula, 10- Santo da Serra, 11- Machico, 12- Caniçal, 13- São Vicente, 14- Ponta Delgada, 15-Santana).Yellow dots show municipality capitals).

Twelve CO_2-baited EVS traps (Bioquip, Gardena, CA, USA) were placed in the southern part of Madeira Island during the first period of study (March 2002 to May 2003) and three of the same traps were placed in the northern and one in the southern part of the Island during the second year of study (March 2003 to May 2004). The latter trap had the same location as one placed during the first year (Quebradas). Collections were made from 6 p.m. to 10 a.m. of the following morning, fortnightly. The identification of mosquito species was made in accordance with the key proposed by Ribeiro and Ramos (1999) and Shaffner et al. (2001). The mosquitoes sampled that remained alive were then kept under controlled conditions (25 ± 2ºC; 70 ± 5% H.R.; photoperiod 12L:12D) and were fed with a 10% sucrose solution for 13 days to allow parasite development to the infective 3^{rd}-stage larva (L3).

Analysis of *D. immitis* infection.

After identification of mosquitoes collected, the head, thorax and abdomen of each of these mosquitoes was teased apart with a needle and placed in a drop of Aedes saline (Hayes 1953) on a clean microscopic slide. The Malpighian tubules were separated from the abdomen. Each preparation was examined at 400x magnification with a microscope. The number of larvae, their location, and the stage of parasite development was recorded, and collected parasites were stored in 70 % ETOH until analyzed by PCR.

Genomic DNA was extracted according to the procedure of Scoles and Kambhampati (1995): the specimen (L2) was placed in a 1.5 ml microcentrifuge tube containing 30 µl of lysis buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA and Proteinase-K to a final concentration of 4µg/100µl). After manual homogenization of the specimen, the tubes were incubated at 37ºC for 30 min and then heated to 95ºC for 5 min. The tubes were then centrifuged for 1 min and the supernatant used as a crude isolate.

To identify *D. immitis* larvae, samples were analyzed by a specific primer that amplified a 378 bp DNA fragment from a single repeated element as follows: forward, 5' - ACG TAT CTG AG C TGG CTC AC - 3'; reverse, 5' - ATG

ATC ATT CCG CTT ACG CC - 3' (primers were synthesized by Invitrogen Corporation). The reagents used for each 25µl reaction were as follows: 2.5µl 10x reaction buffer containing $MgCl_2$ for a final concentration of 1.5 mM, 0.5µl of each primer for a final concentration of 100 ηg/ml, dNTPs for a final concentration of 2.5 mM, 5 units Taq DNA polymerase (Promega, Madison WI, USA) and distilled, deionized water to bring the final volume to 25µl. The thermal cycler program had an initial denaturation step at 94ºC for 1 min, followed by 40 cycles of the following: 90ºC for 1 min, 50ºC for 1 min, and 72ºC for 1 min. A final extension step of 72ºC for 5 min was added to ensure complete extension of all strands. After completion of the program, the samples were held at 4ºC. Amplified products were separated on a 2% agarose gel in TAE 1X buffer using standard protocols (Sambrook et al. 1989). Molecular size standards (1Kb DNA ladder [Promega]) were included in each gel.

Experimental infection with *D. immitis*.

Adults of *Cx. p. molestus* were reared from eggs laid by wild caught specimens collected in Madeira Island. One mosquito pool was from Funchal, and the parental females were fully engorged and resting in a house. Another pool was from Campanário, and the parental females were fully engorged and resting in a henhouse. Eggs were collected and hatched in enamel rearing pans. Larvae were placed in deionized water and fed slurry of finely ground fish food (Tetramin®). Cotton pads, moistened with 0.3M sucrose solution, were placed on marquisette to provide a source of nutrients. Adult mosquitoes were 5-8 days old when used in the experiment and were maintained in an environmental chamber at 22 ±1ºC, 75 ± 10% RH with a 12hr light and 12 hr dark photoperiod.

D. immitis-infected blood was collected from a mixed breed dog from VetFunchal. The mf density was then adjusted to the desired level (100 mf/20µl) using uninfected blood from another mixed breed dog. The dog bloods were collected in 5 ml plastic tubes with K3 EDTA and kept in the refrigerator until used the following morning.

The mosquitoes ingested *D. immitis* mf by feeding during one hour period through a chick skin attached to a feeder apparatus (Rutledge et al. 1964). Blood fed specimens were placed into 0.473-liter ice cream cartoons covered with fine-mesh marquisette and maintained on a 0.3M sucrose solution soaked in cotton pads.

Mosquitoes were dissected at 16 days post-exposure (PE), or earlier if they were dying, in *Aedes* saline (Hayes, 1953) at 10x with the aid of a stereomicroscope. The head and thorax were teased apart and the Malpighian tubules were separated from the abdomen. Each preparation was examined at 100-400x magnification with a compound microscope. The number of larvae, their location, and the stage of parasite development was recorded.

Five mosquitoes from each pool were also fed with the same concentration of infected dog blood to evaluate formation of blood clots within the midgut. These mosquitoes were dissected one hour P.I..

RESULTS

Natural infection of *Culex pipiens molestus* on Madeira Island.

A total of 401 *Culex pipiens* were captured by EVS mosquito traps from the 240 collections. The seasonal distribution of these mosquitoes attracted to EVS traps, in the most relevant sample stations, is shown in figure 4.2.

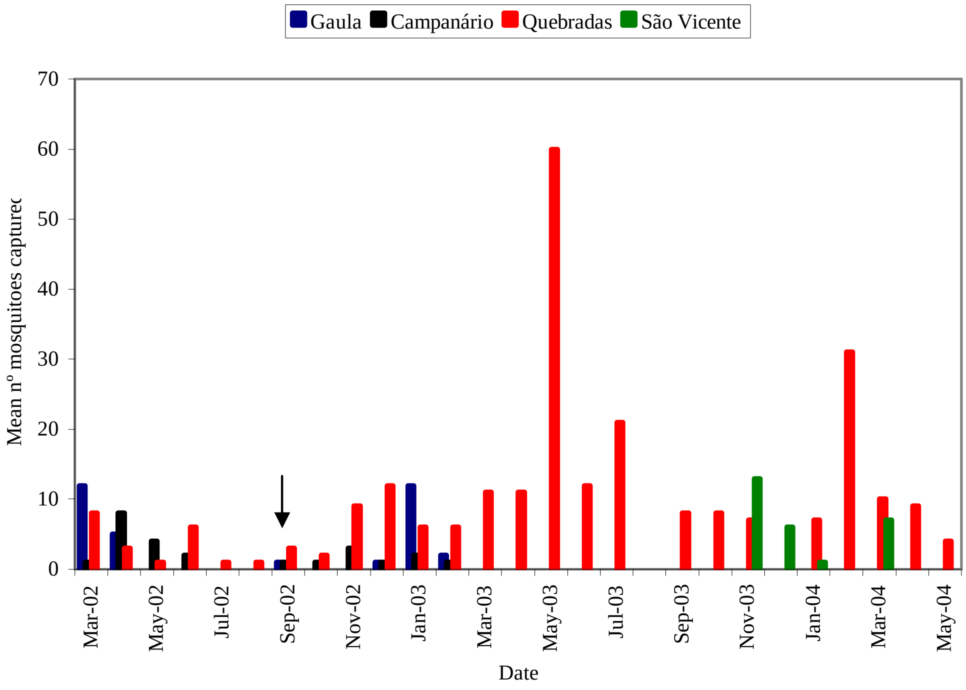

Fig.4.2 – Relative abundance of *Cx. pipiens molestus* in the most relevant sample stations on Madeira Island. (Arrow indicates *D. immitis* present in a sample).

Cx. pipiens molestus was present year round only in Quebradas, and mosquitoes collected at this site accounted for 46.9% of total captures in the first year and 85.8% in the second year of sampling. *D. immitis* were recovered from one *Cx. pipiens* female out of a total sample of 333 examined (0.3%) between March 2002 and May 2004. This infected mosquito was collected on the 29th September in Gaula and harbored two L2s in the Malpighian tubules. These two L2s were abnormally developed (figure 4.3).

Polymerase chain reaction using the primers described above, resulted in the amplification of a 378 bp fragment of *D. immitis*. The other 332 females showed no infection with *D. immitis*.

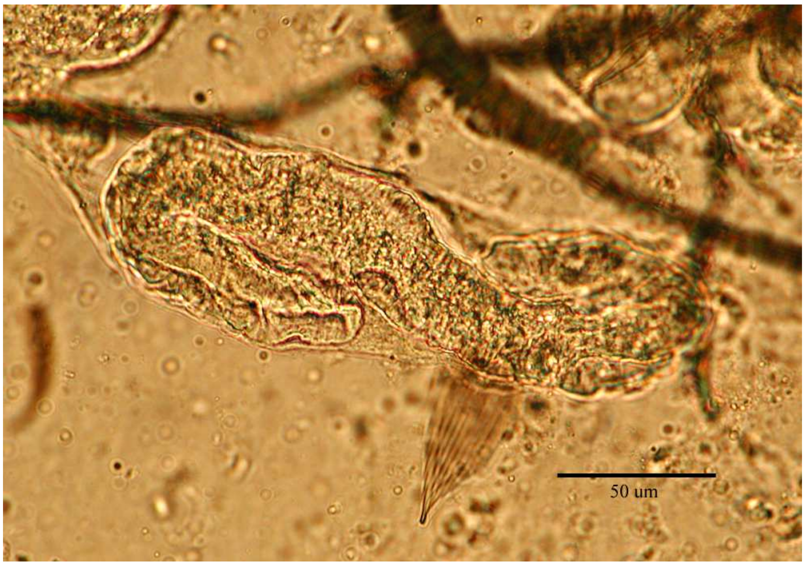

Fig. 4.3-Abnormal L2 of *D. immitis* in Malpighian tubules of *Cx. p. molestus*.

Experimental infection with *D. immitis*.

Infected dog blood, with an mf concentration of 100 mf/ 20 µl, was offered to 20 females originated from Funchal parental female. Only 9 fed on the blood provided. Table 4.1 shows the infection of *Cx. p. molestus* (Funchal strain) from day 9. Mosquito mortality was 0% until day 16 in this experiment.

Table 4.1. Experimental infection of *Cx. p. molestus* (Funchal strain)

Nº of filariae / stage	Location	Days PI
4 L_1	MT	9
2 L_1	MT	10
1 L_1	MT	11
4 L_1	MT	12
1 L_2	MT	16
3 L_2	MT	16
10 L_2	MT	16
1 L_2	MT	16
6L_2 + 1 L_1	MT	16

Infection of the Campanário strain was made through infected blood with the same mf concentration. About 100 females were given the opportunity to feed, but only 9 were engorged. Out of these, 6 died before day 3 (66% of mortality). The infection of the 3 remaining females was null.

All the mosquitoes dissected one hour P.I. formed a blood clot in the midgut with oxyhaemoglobin crystals (figure 4.4), having very few mf (0-10) and with most of them damaged.

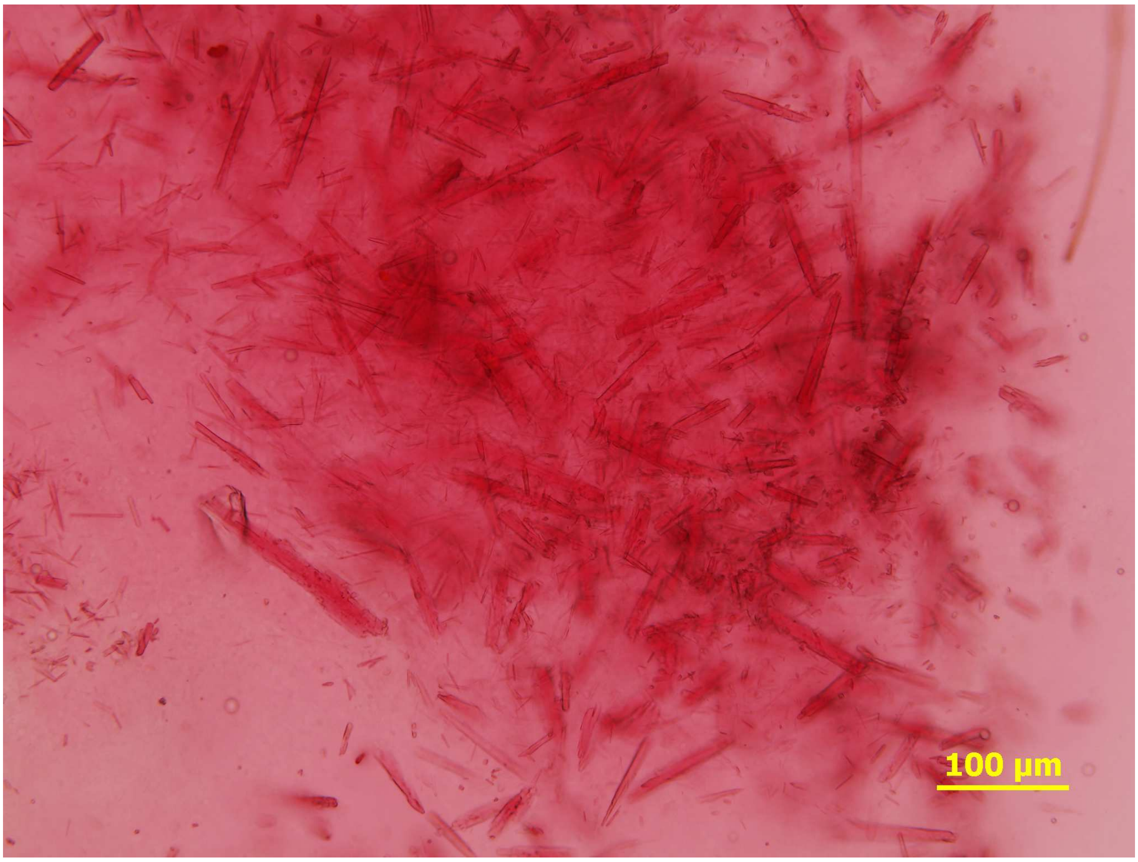

Fig. 4.4 – Oxyhaemoglobin crystals in *Cx. p. molestus* midgut

DISCUSSION

The number of *Cx. pipiens molestus* caught in the second year was substantially higher than in the first year of study, accounting for 85% of the total catch in Quebradas. Although this percentage could be significant, there was no correlation between this abundance and environmental conditions (Chapter 2). The reason for the difference in abundance between these two years is unknown.

The presence of second-stage larvae (L2) in Malpighian tubules suggested that *Cx. p. molestu*s could be a natural vector of *D. immitis* in Madeira Island. Because only the L3 is an infective stage, laboratory infections were carried out with two strains of *Cx. p. molestus* to study the potential development of *D. immitis* in this mosquito.

Differences in suitability of different populations of mosquitoes to the L3 stage have been shown in earlier studies (Kartman 1953; Apperson et al. 1989, Scoles and Dickson 1995). In this study the two different strains showed different mechanisms regarding *D. immitis* infection. In the Funchal strain, it seems that development of *D. immitis* larvae was arrested at the end of L2 stage in the Malpighian tubules of all the females dissected, and the morphology of these larvae was normal. This was an expression of refractoriness in this strain. In Campanário strain, there was no development of mf in the Malpighian tubules, indicating this strain could kill and digest the mf faster. Kartman (1953) demonstrated that *Cx. pipiens* and *Cx. quinquefasciatus* showed a preponderance of negative females because the majority of mf was killed in the midgut during the first 24 hours after the infective meal. This work indicated that the fate of *D. immitis* mf in the mosquito midgut was, undoubtedly, a critical one for the completion of the parasite life cycle. Nayar and Sauerman (1975) also suggested one of the reasons a mosquito becomes susceptible or refractory to *D. immitis* infection is whether mf can move freely from the midgut to the Malpighian tubules within an hour. Since one hour P.I. every mosquito dissected presented a blood clot in the midgut, this could explain why only few mf were seen in the midgut.

Another mechanism that could prevent the development of *D. immitis* in the midgut is the formation of oxyhaemoglobin crystals during blood meal coagulation which could hinder the movement of mf and probably damage them

(Nayar and Sauerman 1975; Lowrie 1991). Numerous crystals of oxyhaemoglobin were found in every blood-fed mosquito dissected, indicating one possible cause for the destruction of mf.

Another explanation (or a complement of the latter) is the fact of *Cx. p. molestus* showed small spines in the pharyngeal armature. The pharyngeal armature in certain species of mosquitoes can cause physical damage to large parasites, like mf (~250 to 300 µm), that can effectively prevent further development (Colluzi and Trabucchi 1968; McGreevy et al. 1978; Beerntsen et al. 2000).

Nevertheless, neither of these mechanisms can explain the slight differences between the two strains. Several studies have shown that environmental effects and the genetic background of the mosquito midgut significantly influence the number of mf ingested. Since the offspring were reared in the same environmental conditions these differences could be explained by genetic background. Genetic factors play a role in susceptibility and may be involved in different degrees of susceptibility (or refractoriness) of strains of the same species of mosquitoes from different geographic areas (Serrão et al. 2001). Madeira Island has many microhabitats with some differences from one to another, creating different selection pressures on *Cx. p. molestus*. Because parental females were captured in different habitats (house and henhouse), it is possible that these two stains could have different host preferences.

In spite of the different reaction of the two strains presented herein to *D. immitis*, *Cx. p. molestus* showed no vector efficiency, and probably does not have an effective role in *D. immitis* transmission in Madeira Island.

REFERENCES CITED

Apperson, C. S., B. Engber, and J. F. Levine. 1989. Relative suitability of *Aedes albopictus* and *Aedes aegypti* in North Carolina to support development of *Dirofilaria immitis*. Journal of the American Mosquito Control Association 5: 377-382.

Bartholomay, L. C., and B. M. Christensen. 2002. Vector-Parasite interactions in mosquito-borne filariasis, In: T.R. Klei and T.V. Rajan (Ed.) World Class Parasites: volume 5 - The filaria. Massachusetts: USA Kluwer Academic Publishers, p. 9-20.

Beerntsen, B. T., A. A. James, and B. M. Christensen. 2000. Genetics of mosquito vector competence. Microbiology and Molecular Biology Review 64: 115-137.

Cancrini, G., M. Magi, S. Gabrielli, M. Arispici, F. Tolari, M. Dell'Omodarme, and M. C. Prati. 2006. Natural vectors of dirofilariasis in rural and urban areas of the Tuscan Region, cental Italy. Journal of Medical Entomology 43: 574-579.

Capela, R.A. 1981. Contribution to the study of mosquitoes (Diptera: Culicidae) from the Archipelago of Madeira and Savages I – Madeira. Arquivos do Museu Bocage, Série A. 1: 45-66.

Capela, R. A. 1982. Contribuição para o conhecimento dos mosquitos (Diptera: Culicidae) dos Arquipélagos da Madeira e das Selvagens II – Madeira, Deserta Grande, Porto Santo e Selvagem Grande. Boletim do Museu Municipal do Funchal 34: 105-123.

Clemente, M. L. 1996. Prevalence of *Dirofilaria* in dogs in Madeira Island. Examination and identification of microfilaria. Veterinária Técnica Agosto: 34-37.

Coluzzi, M., and R. Trabucchi. 1968. Importanza dell'armatura bucco-faringea in *Anopheles* e *Culex* in relazione alle infezioni con *Dirofilaria*. Parassitologia 10: 47-59.

Fonseca, I. M. P., L. M. M. Carvalho, S. P. Carvalho, and M. Carvalho-Varela. 1991. Prevalência da dirofilariose na populacão canina portuguesa. I. Detecção de microfilárias sanguíneas. Vet. Téc. Set/Out: 36-38.

Genchi, C., L. Rinaldi, C. Cascone, M. Mortarino, and G. Cringoli. 2005. Is heartworm disease really spreding in Europe? Veterinary Parasitology 133: 137-148.

Grieve, R. B., J. B. Lok, and L. T. Glickman. 1983. Epidemiology of canine heartworm infection. Epidemiological Review 5: 220-246.

Hayes, R. O. 1953. Determination of a physiological saline for *Aedes aegypti* (L.). Journal of Economical Entomology 46: 624-627.

Hu, S. M. K. 1931. Studies on host-parasite relationships of *Dirofilaria immitis* Leidy and its culicine intermediate hosts. American Journal of Hygiene 14: 614-629.

Kartman, L. 1953. Factors influencing infection of the mosquito with *Dirofilaria immitis* (Leidy, 1856). Experimental Parasitology 2: 27-78.

Leidy, J. 1856. Worms in the heart of the dog. Proceedings of the Academy of Natural Sciences of Philadelphia 8: 2.

Lok, J. B. 2000. *Dirofilaria* sp.: taxonomy and distribution. In: P.F.L. Boreham and R.B. Atwell (Eds.) Dirofilariasis. Boca Raton, Florida, USA: CRC Press, Inc. p. 2-28.

Lowrie R.C. 1991. Poor vector of *Culex quinquefasciatus* following infection with *Dirofilaria immitis*. Journa of the American Mosquito Control Association 7: 30-36.

McGreevy, P. B., J. H. Bryan, P. Oothuman, and N. Kolstrup. 1978. The lethal effects of the cibarial and pharyngeal armatures of mosquitoes on microfilariae. Transactions of the Royal Society of Tropical Medicine and Hygiene 72: 361-368.

Nayar, J. K., and D. M. Sauerman. 1975. Physiological basis of host susceptibility of Florida mosquitoes to *Dirofilaria immitis*. Journal of Insect Physiology 21: 1965-1975.

Pinger, R. R. 1985. Species composition and feeding success of mosquitoes attracted to caged dogs in Indiana. Journal of the American Mosquito Control Association 1: 181-1855.

Ribeiro, H., and H. Ramos. 1999. Identification keys of the mosquitoes (Díptera: Culicidae) of Continental Portugal, Açores and Madeira. European Mosquito Bulletin 3: 1-13.

Rossi, L., F. Pollono, P. G. Meneguz, G. Cancrini. 1999. Quattro specie di culicidi come possibili vettori di *Dirofilaria immitis* nella risaia piemontese. Parassitologia 41: 537-542.

Rutledge, L. C., R. A. Ward, and D. J. Gould. 1964. Studies on the feeding response of mosquitoes to nutritive solutions in a new membrane feeder. Mosquito News 24: 407-419.

Santa-Ana, M., M. Khadem, and R. Capela. 2006. Natural infection of *Culex theileri* (Díptera: Culicidae) with *Dirofilaria immitis* (Nematoda: Filarioidea) on Madeira Island, Portugal. Journal of Medical Entomology 43: 104-106.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Plainview, NY, USA.

Scoles, G. A., and S. L. Dickson. 1995. New foci of canine heartworm associated with introduction of new vector species. *Aedes albopictus* in New Orleans and *Aedes serrensis* in Utah. Proceedings of the Heartworm Association Symposium 1995: 27-35.

Scoles, G. A., and S. Kambhampati. 1995. Polymerase chain reaction based method for the detection of canine heartworm (Filarioidea: Onchocercidae) in mosquitoes (Diptera: Culicidae) and vertebrate hosts. Journal of Medical Entomology 32: 864-869.

Serrão, M. L., N. Labarthe, and R. Lourenço-de-Oliveira. 2001. Vector competence of *Aedes aegypti* (linnaeus 1762) Rio de Janeiro strain, to *Dirofilaria immitis* (Leidy 1856). Memórias do Instituto Oswaldo Cruz 96: 593-598.

Schaffner E. G. A., B. Geoffroy, J. J. P. Henry, A. Rhaiem, and J. Brunhes. 2001. The Mosquitoes of Europe [CD-Rom]. IRD, Montpellier, France: IRD Éditions.

Todaro, W. S., C. D. Morris, and N. A. Heacock. 1977. *Dirofilaria immitis* and its potential mosquito vectors in central New York State. American Journal of Veterinary Research 38: 1197-1200.

Troz- Williams, L. A., A. J. Trees. 2003. Systematic review of the distribution of the major vector-borne parasitic infections in dogs and cats in Europe. Veterinary Recearch 152: 97-105.

Vezzani, D., D. F. Eiras, and C. Wisnivesky. 2006. Dirofilariasis in Argentina: historical review and first report of *Dirofilaria immitis* in a natural mosquito population. Veterinary Parasitology 136: 259-273.

Chapter 5

Reproductive costs of the immune response of the autogenous mosquito *Culex pipiens molestus* against inoculated *Dirofilaria immitis*

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ABSTRACT

Culex pipiens molestus is an autogenous mosquito, vector of *Dirofilaria immitis* in Madeira Island and it mounts a melanotic encapsulation response when inoculated intrathoracically with microfilariae of the heartworm. Because *Cx. p. molestus* is autogenous, this mosquito is a good model to better understand the relationship between oviposition and melanization, independent of the signalling pathways related to blood feeding.

The present work was performed to assess the impact follicle growth might have on melanization of intrathoracically inoculated mf. The ovaries from mosquitoes undergoing melanotic encapsulation developed more eggs than those which could not melanize the mf. Possible explanations are discussed herein.

Innate immune response of mosquitoes provides effective protection against a major variety of pathogens, including *D. immitis*. Although insects lack immunological memory seen in vertebrates, they have humoral and cellular mechanisms that can limit or prevent the development of pathogens (Beerntsen, et al. 2000; Dimopoulos 2003; Christensen et al. 2005). Melanization responses are usually cell-mediated, site-specific, and culminate with the deposition of melanotic materials around the pathogen. The pigment appears very near the surfaces of organisms that have invaded the hemocoel of the host (Christensen et al. 2005) and may function, not only to kill pathogens, but also to protect endogenous tissues within the body cavity from systemic damage resulting from pathogen killing (Beerntsen et al. 2000; Nappi and Christensen 2005). This response has been observed even in mosquitoes susceptible to parasite development (Christensen et al. 1984; Christensen et al. 1986; Harris et al. 1986; Beerntsen et al. 2000). In addition to its role in insect immunity, melanin is essential for many physiological functions including egg chorion tanning (Ferdig et al 1993; Li and Christensen 1993). Hence, when a melanization response is initiated against a parasite, the competing biochemistries might negatively affect reproduction (Ferdig et al. 1993; Christensen et al. 2005), i.e., a mosquito that has an optimal melanization response to parasites may be compromising its own reproductive success. Because both melanotic encapsulation of parasites and egg tanning require several common substrates, e.g., tyrosine and phenylalanine (Li and Christensen 1993; Uchida 1993; Christensen et al. 2005), a competition for limited resources might result in a lower fecundity of the mosquito (Beerntsen et al. 2000). However, the costs and benefits of the immune response are likely to vary, making general conclusions concerning the balance of costs and benefits difficult (Schwartz and Koella 2004). Two factors are frequently associated with this balance: one is the host's environment which can have an effect on the parasite or its host (Agnew and Koella 1999); the second factor is that the cost of the immune response might also depend on the foreign entity that stimulates the immune response. Immunity against different parasites (and among different populations of the same parasite) might differ, not only because of variation in the selection pressure (the parasite's incidence and virulence) or the environmental conditions, but also because the cost required to destroy the parasites might differ (Schwartz and Koella 2004).

Here, we studied the impact follicle growth in *Culex pipiens molestus* might have on melanization of microfilaria of *Dirofilaria immitis*. *Cx. pipiens* is one of the most important mosquito species in terms of human and veterinary health because it is one of the primary vectors for nematodes that cause filariasis, e.g., *Wuchereria bancrofti* and heartworm disease (*D. immitis*). *Cx. pipiens* complex mosquitoes also transmit arboviruses including West Nile virus (WNV), Rift Valley Fever Virus (RVFV) Western Equine encephalomyelitis virus (WEE), and others. Numerous researchers have examined the biology and population genetics of this mosquito (Knight 1951; Vinagradova 2000; Fonseca et al. 2003; Keyghobadi et al. 2004), but we know very little about vector competence, i.e. the factors that determine compatibility between the mosquito and the pathogens it transmits.

Culex p. molestus is a facultative autogenous species (Vinogradova 2000). Autogenous and anautogenous forms of *Culex pipiens* are fundamentally distinguished by mechanisms of hormone control of ovary development. Experiments with autogenous *Cx. pipiens pallens* showed that an appropriate balance of hemolymph amino acids is required to initiate mosquito oogenesis, as either nutritional precursors or humoral stimulatory factors (Uchida et al. 1992; Uchida 1993). These experiments indicate that maintenance of an increased hemolymph amino acid concentration is involved in the regulation of egg development neurosecretory hormone (EDNH) (Uchida et al. 1992).

In *Armigeres subalbatus*, Ferdig et al. (1993) demonstrated that egg development (amount of vitellogenin with eggs) was delayed when mosquitoes were undergoing melanotic encapsulation reactions against *Brugia malayi* microfilaria. They also showed that tyrosine levels remained elevated in the hemolymph during these reactions, while movement of tyrosine into the ovaries was delayed.

Efforts to understand the control mechanisms responsible for melanin biosynthesis associated with parasite encapsulation as opposed to egg chorion tanning is made even more difficult by the presence of multiple prophenoloxidases (ProPO) and serine proteases that are required for the activation of ProPO and the hydroxylation of tyrosine, the initial steps in the production of melanin pigment (Christensen et al. 2005). The complex hormonal and biochemical cascade that is initiated upon ingestion of blood is altered by the presence of mf that initiate a melanotic encapsulation immune response; consequently, subcellular factors (e.g., regulatory molecules, precursor availability, enzymes activities) involved in various biological activities of the host organism are not able to behave independently, in an evolutionary context, because the pathways to different ends (parasite encapsulation versus egg chorion tanning) share important activities (Ferdig et al. 1993).

Because *Cx. p. molestus* do not need extra proteins (i.e., a blood meal) to lay eggs, this mosquito could be a good model to better understand the relationship between oviposition and melanization, independent of the signalling pathways related to blood feeding. In addition, *Cx. p. molestus* on Madeira Island are not susceptible to the complete development of *D. immitis* (M. Santa-Ana, R. Capela, and B. M. Christensen, unpublish data).

Herein, we report on the capability of *Cx. p. molestus* (Quebradas strain from Madeira Island) to mount a melanization response against inoculated mf and the relationship of this response with reproduction.

MATERIAL AND METHODS

Mosquito maintenance

Cx. pipiens molestus were from a laboratory colony maintained at the University of Madeira. Eggs were collected and hatched in enamel rearing pans. Approximately 300 larvae per pan were placed in deionized water and fed a slurry of finely ground fish food (Tetramin®). Female pupae were mechanically separated, and 50-60 pupae were placed into 0.473-liter ice cream cartoons covered with fine-mesh marquisette. Cotton pads, moistened with 0.3M sucrose solution, were placed on marquisette to provide a source of nutrients. Adult mosquitoes were 2-4 days old and were maintained in a environmental chamber at 26.5 ±1ºC, 75 ± 10% RH, and with a 16hr light and 8 hr dark photoperiod with a 90 min crepuscular period at the beginning and end of each light cycle.

Isolation and inoculation of mf

D. immitis mf were isolated from dog blood, cryopreserved, then thawed and re-suspended in Hank's Balanced Salt Solution (HBSS) (Bartholomay et al. 2001). Mosquitoes were divided in two groups: the first was injected with 10-20 mf and the second with 20-40 mf. Mosquitoes were cold-immobilized and held in place with a vacuum saddle for injection. A microinjection needle was inserted through the neck membrane to inoculate mf in 0.5 µl of HBSS. Inoculated mosquitoes were dissected at 1, 3, 5 and 8 days post-inoculation (PI).

In all dissections wings, legs and heads were removed, and remaining tissues were thoroughly teased apart in a drop of *Aedes* saline (Hayes 1953) on a microscopic slide and covered with a cover glass. The resulting slide was immediately examined at 100-200 X, using bright field optics, and the level of immune response and follicle growth were recorded. Recovered mf were classified using a score based on the proportion of the mf encapsulated in melanin (no melanization, < ½, ≥ ½ and full melanization correspond to a score of 0, 1, 2 and 3, respectively) (Shiao et al. 2001). The females were separated in two groups according to development stages of the ovaries: anautogenous (vitellogenic ovarian development not initiated) and autogenous (deposition of yolk in the ovaries) (Clements 2000).

Statistical analysis

Results were analysed statistically using Pearson Chi-Square analysis and Goodness of Fit test and differences were considered significant at $p < 0.05$ (SPSS 14.0 for Windows, SPSS Inc., Chicago, IL).

RESULTS

The melanization response differed with respect to time following parasite inoculation and the number of mf inoculated: however, there was no significant difference in melanization between the two concentrations of mf inoculated at each time point (p>0.05) (Table 5.1).

Table 5.1- Melanization of microfilaria intrathoracically inoculated in *Culex pipiens molestus*.

	10-15 mf inoculated			20-40 mf inoculated			
	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 8
degree of melanization (SD)	1.34 (± 1.32)	1.63 (± 1.36)	2.16 (± 0.95)	0.21 (± 0.29)	1.39 (± 1.17)	1.18 (± 1.31)	2.19 (± 0.7)
nº mf recovered/ mosquitoes dissected	105/15	38/10	59/10	239/16	271/43	88/10	93/9

The degree of melanization was based on a scale of 0 (unmelanized) to 3 (totally melanized).

Figure 5.1 shows that melanization started on day 1 with both groups of mosquitoes, but the number of mosquitoes harbouring at least 1 mf melanized was less when larger numbers of mf were injected (20-40 mf). At day 5 PI, 90% of the mosquitoes injected with 10-15 mf showed at least 1 mf melanized, but only 60% of the mosquitoes, injected with 20-40 mf, displayed a melanization response. Nevertheless, if given more time (at day 8 following inoculation), 100% of mosquitoes receiving large numbers of mf were able to melanize at least one mf.

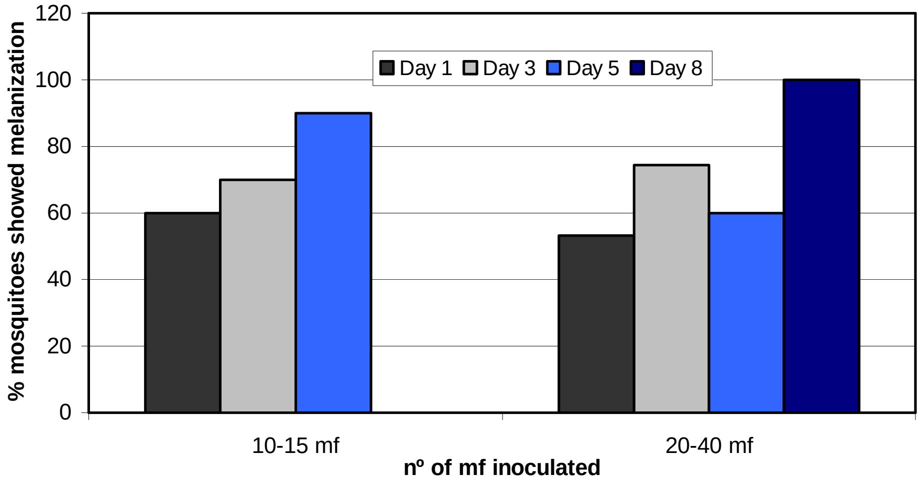

Fig. 5.1- Percentage of mosquitoes harbouring melanized microfilariae throughout 8 days after inoculation.

Cx. *p. molestus* mounts a relatively weak melanization immune response. At day 3 PI, the degree of melanization observed was only 1.63 with 10-15 mf inoculated and 1.39 with 20-40 mf. Comparing with other mosquitoes, Infanger and collaborators (2004) demonstrated that the degree of melanization against mf in *Aedes aegypti* (25-30 mf inoculated) and *A. subalbatus* (55-60 mf inoculated), at day 3 PI, was 1.82 and 2.23, respectively. In *Aedes trivittatus*, the immune response against mf was even greater, with all the mf inoculated (5-10 mf) being completely encapsulated and melanized by day 4 (Christensen 1981).

Ovary development was evaluated at the same time melanization of mf was occurring. The autogeny occurred in 64.6% of mosquitoes inoculated with mf. The Pearson Chi-Square test showed no significant association between the concentration of mf injected intrathoracically and autogeny ($\chi^2=0.96$; $p=0.33$).

Table 5.2 shows that mosquitoes undergoing melanotic encapsulation reactions were more likely to be autogenous (ovaried developing stage III or higher, according to Christophers 1911) than those mosquitoes that did not melanize mf. This was shown by the Goodness Fit test ($\chi^2=159.03$; $p< 0.05$) where the degree of melanization values 0 and 2 are the values that most contribute to reject the null hypothesis. Therefore, autogeny is associated with a higher degree of melanization in *Cx. p. molestus*.

Table 5.2. Number and percentage of mf showing different degrees of melanization in autogenous and anautogenous *Cx. p. molestus*

Melanization	**Anautogenous**		**Autogenous**	
0	57	38.5%	64	16.0%
1	13	8.8%	48	12.0%
2	9	6.1%	73	18.2%
3	69	46.6%	216	53.9%
Total	148	100.0%	401	100.0%
Mosquitoes dissected (n.)	29	35.4%	53	64.6%

DISCUSSION

The present work was done to determine the capacity of *Cx. p. molestus* to melanize *D. immitis* mf within the hemocoel and if this melanization reaction might affect the fecundity of this mosquito. The response to *D. immitis* in the hemolymph of *C. p. molestus* is somewhat effective, but requiring 8 days for 100% of mosquitoes to show a response with at least one mf partially melanized.

Several studies have demonstrated that the physiological processes of blood-feeding and egg production can interact with the immune system (Chun et al. 1995; Paskewitz and Christensen 1996; Jahan and Hurd 1997; Schwartz and Koella 2002). In this study, because our mosquitoes were autogenous, blood-feeding was not a factor in activation of the immune response.

Biochemical evidence for a link between reproduction and defence factors has been demonstrated (Christensen, 1981; Ferdig *et al*. 1993; Schwartz and Koella 2004), but our results suggest that the cost of immune response is not a constant parameter. Thus, a cost of immunity was not observed in our mosquitoes and, in fact, autogenous mosquitoes with completely formed eggs showed a more robust melanization immune response. Paskewitz and Christensen (1996) suggested that reproductive costs likely result from a re-allocation of limited reserves (substrates and enzymes) during the formation of melanotic capsules around pathogens; however, because melanization of mf was not affected by previous formation of eggs, this seems not to be the case in *Cx. p. molestus*.

Schwartz and Koella (2004) inoculated beads in *Aedes aegypti* to assess the cost of immunity, having different results related to the charge of the beads. Their justification could be used to explain the results of this study. Therefore, microfilaria in *Cx. p. molestus* may induce a slightly different hierarchy in the allocation decision. The genetic variance-covariance matrix is determined in part by functional architecture, i.e., the pathways by which variation in genotype influences phenotypes. And, differences in the timing and hierarchy of allocation patterns can lead to different associations among traits, and even to positive correlation rather than the negative ones expected for a cost (Worley et al. 2003).

However, the utilization of multiple phenoloxidases (POs) and serine proteases in melanotic encapsulation may be another way that explains the lack of a general cost to immunity. As demonstrated by Huang and collaborators (2001) with *Ar. subalbatus*, ProPO gene expression studies showed enhanced transcription of AS-pro-PO I in mf-inoculated mosquitoes, but not in blood-fed ones, and an increase of As-pro-PO II transcription only in blood-fed mosquitoes and not in mf-inoculated ones. These data suggest that As-pro-PO I is involved in melanization defence responses, and As-pro-PO II is responsible for mosquito egg-shell tanning. An important consequence of these studies is that, although numerous pro-POs are present in *Ar. subalbatus*, only one has been identified to date as being responsible for melanization of parasites, and this does not interfere with the ProPO needed for subsequent egg chorion tanning. Future studies should focus on prophenoloxidase expression in autogenous and anautogenous *Cx. p. molestus* to determine if, in fact, ProPO polymorphisms could be responsible for the results presented herein.

REFERENCES CITED

Agnew, P., and J. C. Koella. 1999. Life history interactions with environmental conditions in a host-parasite relationship and the parasite's mode of transmission. Evolutionary Ecology 13: 67-89.

Bartholomay, L. C., H. A. Farid, E. E. Kordy, and B. M. Christensen. 2001. Short report: A practical technique for the cryopreservation of *Dirofilaria immitis*, *Brugia malayi* and *Wuchereria bancrofti* microfilariae. American Journal of Tropical Medicine and Hygiene 65: 162-163.

Beerntsen, B. T., A. A. James, and B. M. Christensen. 2000. Genetics of mosquito vector competence. Microbiology and Molecular Biology Review 64: 115-137.

Christensen, B. M. 1981. Observations on the immune response of *Aedes trivittaus* against *Dirofilaria immitis*. Transaction of the Royal Society Tropical Medicine and Hygiene 75: 439-443.

Christensen, B. M., D. R. Sutherland, and L. N. Gleason. 1984. Defence reactions of mosquitoes to filarial worms: comparative studies on the response of three different mosquitoes to inoculated *Brugia pahangi* and *Dirofilaria immitis* microfilariae. Journal of Invertebrate Pathology 44: 267-274.

Christensen, B. M., M. M. LaFond, and L. A. Christensen. 1986. Defence reactions of mosquitoes to filarial worms: Effect of host age immune response to *Dirofilaria immitis* microfilaria. Journal of Parasitology 72: 212-215.

Christensen, B. M., J. Li, C.C. Chen, and A. J. Nappi. 2005. Melanization immune responses in mosquito vectors. Trends in Parasitology 21:192-199.

Christophers S. R. 1911. The development of the egg follicle in anophelines. Paludism 1:73-88.

Chun, J., M. Riehle, and S. M. Paskewitz. 1995. Effect of mosquito age and reproductive status on melanization of sephadex beads in *Plasmodium*-refractory and susceptible strains of *Anopheles gambiae*. Journal of Invertebrate Pathology 66: 11-17.

Clements, A. N. 2000. The biology of mosquitoes: development, nutrition and reproduction, Vol. 1. Cambridge, UK: CABI Publishing, University Press. 511 p.

Dimopoulos, G. 2003. Insect immunity and its implication in mosquito-malaria interactions. Cellular Microbiology 5:3-14.

Ferdig, M. T., B. T. Beerntsen, F. J. Spray, J. Li, and B. M. Christensen. 1993. Reproductive costs associated with resistance in a mosquito-filarial worm system. American Journal of Tropical Medicine and Hygiene 49: 756-762.

Fonseca, D. M., Keyghobadi, N., Malcolm, C. A., Mehmet, C., Schaffner, F., Mogi, M., Fleischer, R. C., and Wilkerson, R. C. 2003. Emerging vectors in *Culex pipiens* complex. Science 303:1535-1538.

Hayes, R. O. 1953. Determination of a physiological saline for *Aedes aegypti*. Journal of Economical Entomology 46: 624.

Harris, K. L., B. M. Christensen, and G. S. Miranpuri. 1986. Comparative studies on the melanization response of male and female mosquitoes against microfilariae. Developmental and Comparative Immunology 10: 305-310.

Huang, L. H., B. M. Christensen, and C. C. Chen. 2001. Molecular cloning of a second prophenoloxidase cDNA from mosquito *Armigeres subalbatus*: prophenoloxidase expression in blood-fed and microfilariae-inoculated mosquitoes. Insect Molecular Biology 10: 87-95.

Infanger, L. C., T. A. Rocheleau, L. C. Bartholomay, J. K. Johnson, J. Fuchs, S. Higgs, C. C. Chen, and B. M. Christensen. 2004. The role of phenylalanine hydroxylase in melanotic encapsulation of filarial worms in two species of mosquitoes. Insect Biochemistry and Molecular Biology 34: 1329-1338.

Jahan, N., and H. Hurd. 1997. The effects of infection with *Plasmodium yoelii nigeriensis* on the reproductive fitness of *Anopheles stephensi*. Annals of Tropical Medicine and Parasitology 91: 365-369.

Keyghobadi, N., M. A. Matrone, G. D. Ebel, L. D. Kramer, and D. M. Fonseca. 2004. Microsatellite loci from the northern house mosquito (*Culex pipiens*), a principal vector of West Nile virus in North America. Molecular Ecology Notes 4: 20-22.

Knight, K. L. 1951. A review of the *Culex pipiens* complex in the Mediterranean subregion (Diptera, Culicidae). Transactions of the Royal Entomological Society of London 102: 354-364.

Li, J., and B. M. Christensen. 1993. Involvement of L-tyrosine and phenol oxidase in the tanning of *Aedes aegypti* eggs. Insect Biochemistry and Molecular Biology 23: 739-748.

Nappi, A. J., and B. M. Christensen. 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochemistry and Molecular Biology 35: 443-459.

Paskewitz, S. M., and B. M. Christensen. 1996. Immune response of vectors. In: B.J. Beaty and W.C. Marquardt (Ed.) The biology of Disease Vectors. Niwot, Colo. USA: University Press of Colorado. p. 371-392.

Schwartz, A., and J. C. Koella. 2002. Melanization of *Plasmodium falciparum* and C-25 Sephadex beads by field-caught *Anopheles gambiae* (Diptera: Culicidae) from Southern Tanzania. Journal of Medical Entomology 39: 84-88.

Schwartz A., and J. C. Koella. 2004. The cost of immunity in the yellow fever mosquito, *Aedes aegypti* depends on immune activation. Evolutionary Biology 17: 834-840.

Shiao, S. H., S. Higgs, Z. Adelman, B. M. Christensen, S. H. Liu, and C. C. Chen. 2001. Effect of prophenoloxidase expression knockout on the melanization of microfilariae in the mosquito *Armigeres subalbatus*. Insect Molecular Biology 10: 315-321.

Uchida, K, D. Ohmori, F. Yamakura, and K. Suzuki. 1992. Mosquito (*Culex pipiens pallens*) egg development induced by infusion of amino acids into the hemocoel. Journal of Insect Physiology 38: 953-959.

Uchida, K. 1993. Balanced amino acid composition essential for infusion-induced egg development in the mosquito (*Culex pipiens pallens*). Journal of Insect Physiology 39: 615-621.

Vinogradova, E. 2000. *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Sofia, Bulgaria: Pensoft Publishers. 250 p.

Worley, A. C., D. Houle, and S. C. H. Barrett. 2003. Consequences of hierarchical allocation for the evolution of life-history traits. American Naturalist 161: 153-167.

Chapter 6

Conclusions

Mosquitoes are unquestionably the most medically important arthropod vectors of disease. The maintenance and transmission of the pathogens that cause malaria, lymphatic filariasis and numerous viral infections, just to mention human diseases, are absolutely dependent on the availability of competent mosquito vectors. Much work is currently aimed at understanding the immune response mosquitoes mount against pathogens in efforts to understand the mechanisms underlying vector competence (Beernsten et al. 2000).

Prior to the initiation of this work there was no knowledge about the vectors of *Dirofilaria immitis* on Madeira Island and the immune response of the mosquitoes to this filarial worm, although heartworm disease is endemic in this island (Fonseca et al. 1991; Clemente 1996). Environment can play an important role in disease transmission (Epstein 2001; Shaman et al. 2003; Shaman and Day 2005), but no studies have been made about the impact of the weather on the biology of mosquitoes on Madeira Island that has numerous microclimates. Furthermore, *Culex theileri* was never described as a vector of *D. immitis*, nor has an autogenous mosquito, like *Culex pipiens molestus*, been used in studies to increase our understanding of the relationship between mosquito egg development and melanization, independent of the signalling pathways related to blood feeding.

Herein, we analysed the relationship between Cx. p. molestus and Cx. *theileri* abundance with three weather variables (temperature, relative humidity and precipitation). Several studies have investigated the effect of weather on insect populations with results varying by species (Shone et al. 2006). Most insects respond to changes in meteorological conditions. Surprisingly, *Cx. p.*

molestus abundance seems to be independent of the parameters studied. Nevertheless, there are other parameters that were not accounted for, like breeding sites conditions (e.g. organic contents, field irrigation), wetness of soil, and rates of evapotranspiration (Shaman and Day 2005), just to mention a few. *Culex theileri* abundance has a strong relationship with rainfall and air temperatures. Understanding the spatiotemporal distribution of risk for mosquito-borne diseases is an important step towards planning and implementing effective infection control measures (Smith et al. 2004), and here, there is a contribution toward the possibility to prevent heartworm disease in dog populations, with adequate measures, during the risk period. This period, determined by our studies, is between September and January (see also chapter 3 and 4). These could be the ideal months to prevent heartworm diseases in dogs by administrating ivermectin.

The transmission of *D. immitis* by *Cx. theileri* is verified here for the first time. Although it was not possible to follow infection in the laboratory, it was possible to detect 3rd larval stages (L3) in female mosquitoes in December of 2002 and January 2003. These months provided the weather conditions (mild temperature and higher rainfall) that produced higher mosquito abundance (see chapter 2). *Culex theileri* is very difficult to rear in the laboratory, and it was impossible to induce egg laying by the mosquitoes caught in the field. This is mainly due to the difficulty in getting this species to feed on membrane feeders or on chicks or hamsters.

Culex pipiens molestus, however, can be reared easily under laboratory conditions, but parasites could not develop to L3 larvae in any experiment, either laboratory or field survey. One interesting finding was that some strains of *Cx. p. molestus* exhibit different responses to *D. immitis*. *Culex pipiens* s.l. (including *Cx. quinquefasciatus*) is often mentioned as a vector of *D. immitis* (Kartman 1953; Ahid et al. 2000; Cancrini et al. 2006), although they all agree this mosquito is a poor vector of this filarial worm. Lewandowski and collaborators (1980), however, reported that *Cx. pipiens* is an important vector in Michigan. In a natural survey in Vero Beach, Florida, Sauerman and Nayar (1983) also recovered several L3s from *Cx quinquefasciatus,* indicating this species could be an important vector in this location. Scoles and Dickson (1995) presented contradictory results regarding two strains of *Cx. quinquefasciatus*. A strain from Baton Rouge, Louisianna, was shown to have high susceptibility to *D. immitis*, while a strain from Covington had relatively low susceptibility. In addition, the authors found that the *Cx. quiquefasciatus* strain from Baton Rouge had an opportunistic feeding behaviour, while in some other locations they had a clear preference for bird feeding. In our studies, the two *Cx. p. molestus* strains differed in their host preference, i.e., bird or dog feeding.

Kartman (1953) detected differences between hybrids of *Cx. pipiens* x *Cx. quinquefasciatus* and their respective parent strains. *Culex pipiens* and *Cx. quinquefasciatus* F1 hybrids behaved *in a more orthodox genetical manner*, and F2 hybrids manifested a physiological segregation in the development of parasites. The percentages of development of *D. immitis* were greater in the hybrids, resembling the condition of *Cx. quinquefasciatus*, but the more vigorous growth of the parasites in the hybrids was similar to that seen in *Cx. pipiens*. These results, coupled with the data obtained in this study, raise a question about the capability of *Cx. p. molestus* to serve as a vector of *D. immitis*. The most reasonable conclusion is that this mosquito might show different susceptibilities to *D. immitis* in diverse locations, and various strains of the same species show differences in susceptibility in the laboratory.

Because *Cx. p. pipiens*, *Cx. p. molestus* and *Cx. quinquefasciatus* show oxyhaemoglobin crystals in the midgut after the intake of blood (Nayar and Sauerman 1975; Lowrie 1991), all have spines in the pharyngeal armature (Coluzzi and Trabucchi 1968; McGreevy et al. 1978), and they present similar degrees of susceptibility to the parasite, the susceptibility may be due to other factors, like genetic ones, that may induce the destruction of mf in the midgut.

As a topic for investigation, innate immunity is enormously broad, and it is sometimes difficult to determine roles played by the innate immune system as compared to just physiological incompatibility between mosquito and parasite. In part, this is because innate immune mechanisms are dynamic on an evolutionary time scale. The host population is shaped by the selective pressures that microbes impose, and survives as best it can (Beutler 2004).

Within the mosquito, each of the organs and tissues that filarial parasites encounter potentially serve as barriers to further development. These barriers affect the compatibility of the vector-pathogen association (vector competence) (Bartholomay and Christensen 2002). As discussed in chapter 1, filarial worm development in the mosquito is not a benign process. In general, the physiology of the host is affected due to structural damage or deregulation of physiological balance (Clements 1999). As described before, several studies demonstrated that *D. immitis* could have an effect on longevity (Christensen

1978), spontaneous flight activity (Berry et al. 1987) and fecundity (Christensen 1981; Ferdig et al. 1993) in the vector. Ferdig and collaborators (1993) approached the relationship between reproductive costs and melanization of *Brugia malayi* in *Armigeres subalbatus*. Their conclusions were that mosquitoes best equipped genetically to respond to the parasite with melanotic encapsulation may not be as reproductively competent in the event of parasite exposure. Furthermore, they postulated that melanotic encapsulation defence reactions serve to destroy most of the ingested parasites, thereby limiting the damage, even though some delay in oviposition will result. These results, coupled with the fact that *Cx. p. molestus* is an autogenous mosquito, made us wonder about the importance of blood intake in these results. Additionally, there were no studies about the association between melanization and autogeny. In our study, surprisingly, the reaction was exactly the opposite of other works regarding reproductive costs: mosquitoes showing a higher melanization response against inoculated mf were capable of laying more eggs autogenously. It becomes clear from our studies that the biochemical evidence for a link between reproduction and defence factors is not a constant parameter. Paskewitz and Christensen (1996) suggested that reproductive costs likely result from a re-allocation of limited biochemical reserves during the formation of melanotic capsules around pathogens; however, because melanization of mf was not affected by previous formation of eggs, this seems not to be the case in *Cx. p. molestus*. In fact, Cho and collaborators (1998) and Huang and colleagues (2001) reported two cDNAs for prophenoloxidase (pro-PO) polypeptide from mf-inoculated *Ar. subalbatus*, demonstrating that transcription of As-pro-PO I and II are significantly enhanced in response to mf inoculation and blood feeding, respectively. These results suggested that mosquitoes might use distinct enzymes for melanizing mf and for the production of eggs.

All of these studies have contributed significantly to our understanding of the epidemiology of heartworm disease on Madeira Island. The interaction between *D. immitis* and susceptibility and the immune system of the potential mosquito vectors, provide many potential avenues for future research.

FUTURE STUDIES

Environment and population dynamics

Several studies have investigated the effect of weather on insect populations with results varying by species (Alten et al. 2000; Doiim et al. 2002; DeGaetano 2005; Shone et al. 2006) and most mosquitoes respond to changes in meteorological conditions. Herein, we analysed for the first time the relationship between environmental variables (precipitation, relative humidity and temperature) and mosquito abundance on Madeira Island. *Culex theileri*, on Funchal, showed primarily a relation between population abundance and rainfall. Certain limitations in temperature seemed to also affect the population growth, but this parameter was not statistically significant. *Culex p. molestus* did not show any relationship between population abundance and the three environmental parameters tested. Although these studies were carried out during two years, it would be valuable to repeat this work with some minor changes. It would be interesting to analyse the larval habitats in those locations, i.e., water characteristics, debris content, soil type, and rates of evapotranspiration (Shaman and Day 2005). Furthermore, excessive rainfall can decimate some mosquito population by flushing larval habitats. Other possible parameters that could affect trapping efficiency could be moonlight intensity (DeGaetano 2005) and wind speed (Alten et al. 2000).

Biology and vector competence of *Cx. theileri* and *Cx. p. molestus*

Culex theileri is not a well studied mosquito, in part due to the fact that this species is very difficult to rear in the laboratory. Nevertheless, the medical and veterinary importance of *Cx. theileri* has been demonstrated in several studies as we mention in chapter 1. Herein, *Cx. theileri* was pointed out as a *D. immitis* vector for the first time. Melanization of *D. immitis* in *Cx. theileri* was never explored and inoculation of mf could be another way to assess immune response capacity in this species.

Much work has been done with the *Cx. pipiens* group (Knight 1951; Harbach et al*.* 1984, 1985; Vinagradova 2000; Fonseca et al. 2004; Keyghobadi et al. 2004). A program has been developed between the Instituto de Higiene e Medicina Tropical (entomology department) and this author to study the *Cx. pipiens* complex in Madeira, Cape Verde and Mainland Portugal in order to assess the systematics and evolution of this species complex. There are some questions remaining from this work regarding *Cx. p. molestus* (discussed in chapter 1) that might be answered in the future.

Melanization and reproductive costs

Melanization constitutes an important component in various aspects in the life of the mosquito, including cuticular sclerotization, egg-shell tanning, melanization of parasites and wound healing (Huag et al. 2001). Our current understanding of melanin biosynthesis is based largely on studies of mammalian systems (Nappi and Christensen 2005). Although many of the initial studies investigating melanization were related to its role in cuticular sclerotization or egg-shell tanning, substrates and enzyme activity levels have been assessed in immune-activated mosquitoes (Beerntsen et al. 2000). Efforts to understand the control mechanisms responsible for melanotic encapsulation in mosquitoes are made more difficult by the presence of multiple phenoloxidases, a critical enzyme in melanization pathway. Chapter 5 approached the relationship between melanin and egg-shell tanning, and consequently, the reproductive costs in *Cx. p. molestus*, an autogenous mosquito that could be used for further studies in this matter. Because this species showed higher numbers of autogenous eggs when melanizing *D. immitis* mf, we wonder if in fact, there are different prophenoloxidases that function independently in melanin production. Future studies must be conducted to understand these mechanisms in *Cx. p. molestus*. To begin to address the possibility of different prophenoloxidases having different functions, further molecular and biochemical work must be done in attempt to identify prophenoloxidases in *Cx. p. molestus* similar to those of *Ar. subalbatus*.

Vector-pathogen interaction

As Bartholomay (2004) pointed out, efforts to understand the genetic basis of susceptibility have largely ignored the role of polymorphisms in the parasite-mosquito association. There is evidence that different strains of filarial worm parasites are differentially infective to the same mosquito species (Wharton 1962). It would be interesting to infect *Cx. p. molestus* and *Cx. theileri* with other strains of *D. immitis* to assess the mechanisms of defence in Madeiran mosquitoes to foreign strains of filarial worms. In light of recent growth in available DNA sequence information for a number of parasitic helminths, it is crucial that suitable gene manipulation technologies are developed to facilitate functional genomic studies in these organisms (Boyle and Yoshino 2003). The tools and techniques such as large EST datasets and genome sequences, and RNAi, are rapidly becoming available for metazoan parasites and their invertebrate hosts (Bartholomay 2004). Functional genomics approaches that take advantage of new sequence databases will certainly reveal unexpected aspects of these complex interactions that could be applied in efforts to control vector-borne diseases.

REFERENCES CITED

Ahid, S. M. M., P. S. S. Vasconcelos, and R. Lourenço-de-Oliveira. 2000. Vector competence of *Culex quinquefasciatus* Say from different regions of Brazil to *Dirofilaria immitis*. Memórias do Instituto Oswaldo Cruz 95: 769-775.

Alten, B., R. Bellini, S. S. Caglar, F. M. Simsek, and S. Kaynas. 2000. Species composition and seasonal dynamics of mosquitoes in the Belek region of Turkey. Journal of Vector Ecology 25: 146-154.

Bartholomay, L. C. 2004. Mechanisms of innate immunity in culicine mosquitoes. [DPhil. Thesis], Madison, WI, USA: University of Madison-Wisconsin. 153 p. Available from: Memorial Library UW Madison Theses Basement North, Madison, WI; AWB B286 L875.

Bartholomay, L. C. and B. M. Christensen. 2002. Vector-Parasite interactions in mosquito-borne filariasis, In: T.R. Klei and T.V. Rajan (Ed.) World Class Parasites: volume 5 - The filaria. Massachusetts: USA Kluwer Academic Publishers, p. 9-20.

Beerntsen, B. T., A. A. James, and B. M. Christensen. 2000. Genetics of mosquito vector competence. Microbiology and Molecular Biology Reviews 64: 115-137.

Berry, W. J., W. A. Rowley, and B. M. Christensen. 1987. Influence of developing *Dirofilaria immitis* on the spontaneous flight activity of *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology 24: 699-701.

Beutler, B. 2004. Innate immunity: an overview. Molecular Immunology 40: 845-859.

Boyle, J. P., and T. P. Yoshino. 2003. Gene manipulation in parasitic helminths. International Journal of Parasitology 33: 1259-1268.

Cancrini, G., M. Magi, S. Gabrielli, M. Arispici, F. Tolari, M. Dell'Omodarme, and M. C. Pratti. 2006. Natural vectors of dirofilariasis in rural and urban areas of the Tuscan Region, central Italy. Journal of Medical Entomology 43: 574-579.

Cho, W. L., H. S. Liu, C. H. Lee, C. C. Kuo, T. H. Chang, C. T. Liu, and C. C. Chen. 1998. Molecular cloning, characterization and tissue expression of prophenoloxidase cDNA from mosquito *Armigeres subalbatus* inoculated with *Dirofilaria immitis* microfilariae. Insect Molecular Biology 7: 31-40.

Christensen, B. M. 1978. *Dirofilaria immitis*: Effect on the longevity of *Aedes trivittatus*. Experimental parasitology 44: 116-123.

Christensen, B. M. 1981. Effect of *Dirofilaria immitis* on the fecundity of *Aedes trivittatus*. Mosquito News 41: 78-81.

Christensen, B. M., J. Li, C. C. Chen, and A. J. Nappi. 2005. Melanization immune responses in mosquito vectors. Trends in Parasitology 21:192-199.

Clemente, M. L. 1996. Prevalence of *Dirofilaria* in dogs in Madeira Island. Examination and identification of microfilaria. Veterinária Técnica, Agosto: 34-37.

Coluzzi, M., and R. Trabucchi. 1968. Importanza dell'armatura bucco-faringea in *Anopheles* e *Culex* in relazione alle infezioni con *Dirofilaria*. Parassitologia 10: 47-59.

Clements, A. N. 1999. The biology of mosquitoes: sensory reception and behaviour. Vol. 2. Cambridge, UK: CABI Publishing, University Press. 740 p.

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

Doiim, D. J., M. L. O'Guinn, and M. J. Turell. 2002. Effect of environmental temperature on the ability of *Culex pipiens* (Diptera: Culicidae) to transmit West Nile Virus. Journal of Medical Entomology 39: 221-225.

Epstein, P. R. 2001. Climate change and emerging infectious diseases. Microbes and Infection 3: 747-754.

Ferdig, M. T., B.T. Beerntsen, F. J. Spray, J. Li, and B.M. Christensen. 1993. Reproductive costs associated with resistance in a mosquito-filarial worm system. American Journal of Tropical Medicine and Hygiene 49: 756-762.

Fonseca, I. M. P., L. M. M. Carvalho, S. P. Carvalho, and M. Carvalho-Varela. 1991. Prevalência da dirofilariose na populacão canina portuguesa. I. Detecção de microfilárias sanguíneas. Veterinária Técnica Set/Out: 36-38.

Fonseca, D., N. Keyghobadi, C. A. Malcolm, C. Mehmet, F. Schaffner, M. Mogi, R.C. Fleischer, and R.C. Wilkerson. 2004. Emerging vectors in the *Culex pipiens* Complex. Science 303: 1535-1538.

Harbach, R. E., C. Dahl, and C. B. White. 1985. *Culex (Culex) pipiens* L. (Diptera: Culicidae): concepts, type designation and description. Proceedings of the Entomological Society of Washington 87: 1-24.

Harbach, R. E., B. A. Harrison, and A. M. Gad. 1984. *Culex (Culex) molestus* Forskal (Diptera:Culicidae) neotype designation, description, variation and taxonomic states. Proceedings of the Entomological Society of Washington 86: 521-542.

Huang, L. H., B. M. Christensen, and C. C. Chen. 2001. Molecular cloning of a second prophenoloxidase cDNA from the mosquito *Armigeres subalbatus*: prophenoloxidase expression in blood-fed and microfilariae-inoculated mosquitoes. Insect Molecular Biology 10: 87-95.

Kartman, L. 1953. Factors influencing infection of the mosquito with *Dirofilaria immitis* (Leidy, 1956). Experimental Parasitology 2: 27-78.

Keyghobadi, N., M. A. Matrone, G. D. Ebel, L. D. Kramer, and D. M. Fonseca. 2004. Microsatellite loci from the northern house mosquito (*Culex pipiens*), a principal vector of West Nile virus in North America. Molecular Ecology Notes 4: 20-22.

Knight, K. L. 1951. A review of the *Culex pipiens* Complex in the Mediterranean subregion (Diptera, Culicidae). Transactions of the Royal Entomological Society of London 102: 354-364.

Lowrie, R.C. 1991**.** Poor vector of *Culex quinquefasciatus* following infection with *Dirofilaria immitis*. Journal of the American Mosquito Control Association 7: 30-36.

Lewandowski, H. B., Jr, G. R. Hooper, and H. D. Newson. 1980. Determination of some important natural potential vectors of dog heartworm in central Michigan. Mosquito News 40: 73-79.

McGreevy, P. B., J. H. Bryan, P. Oothuman, and N. Kolstrup. 1978. The lethal effects of the cibarial and pharyngeal armatures of mosquitoes on microfilariae. Transactions of the Royal Society of Tropical Medicine and Hygiene 72: 361-368.

Nappi, A. J. and B. M. Christensen. 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochemistry and Molecular Biology 35: 443-459.

Nayar, J. K., and D. M. Sauerman. 1975. Physiological basis of host susceptibility of Florida mosquitoes to *Dirofilaria immitis*. Journal of Insect Physiology 21: 1965-1971.

Paskewitz, S. M. and B. M. Christensen. 1996. Immune response of vectors. In: B.J. Beaty and W.C. Marquardt (Ed.) The biology of Disease Vectors. Niwot, Colo. USA: University Press of Colorado. p. 371-392.

Sauerman, D. M., and J. K. Nayar. 1983. A survey for natural potential vectors of *Dirofilaria immitis* in Vero Beach, Florida. Mosquito News 43: 222-225.

Scoles. G.A., and S. L. Dickson. 1995. New foci of canine heartworm associated with introductions of new vector species: *Aedes albopictus* in New Orleans and *Aedes sierrensis* in Utah. Proceedings of the Heartworm Symposium 95: 27-35.

Shaman, J., and J. F. Day. 2005. Achieving operational hydrologic monitoring of mosquito-borne disease. Emerging Infectious Diseases 11: 1343-1350.

Shaman, J., J. F. Day, and M. Stieglitz. 2003. St. Louis Encephalitis virus in wild birds during the 1990 South Florida Epidemic: the importance of drought, wetting conditions, and the emergence of *Culex nigripalpus* (Diptera: Culicidae) to arboviral amplification and transmission. Journal of Medical Entomology 40: 547-554.

Shone, S. M., F. C. Curriero, C. R. Lesser, and G. E. Glass. 2006. Characterizing population dynamics of *Aedes sollicitans* (Diptera: Culicidae) using meteorological data. Journal of Medical Entomology 43: 393-402.

Smith, D. L., J. Dushoff, and F. E. Mckenzie. 2004. The risk of a mosquito-borne infection in a heterogeneous environment. PLoS Biology 2:1957-1964.

Vinogradova, E. 2000. *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Sofia, Bulgaria: Pensoft Publishers. 250 p.

Wharton, R. H. 1962. The biology of *Mansonia* mosquitoes in relation to the transmission of filariasis in Malaya. Bulletin - Institute for Medical Research, Kuala Lumpur 11: 1-114.

APPENDIXES

Appendix 1- Mosquitos species known as natural vectors of *D. immitis.*

Table A1. Mosquito vectors of *Dirofilaria immitis* (only natural infection)

Species	**References**
Aedes aegypti	Vezzani et al. 2006
Aedes albopictus	Lai et al. 2001
Aedes polynesiensis	Samarawickrema et al. 1992
Aedes pseudoscutellaris	Symes 1960
Aedes vexans	Todaro et al. 1977; Buxten and Mullen 1980
Anopheles annulipes	Russell 1985
Anopheles bradleyi	Parker 1993
Anopheles punctipennis	Buxten and Mullen 1980
Anopheles quadrimaculatus	Todaro et al. 1977
Culex annulirostris	Symes 1960
Culex australicus	Russell 1985
Culex declarator	Labarthe et al. 1998
Culex maculipennis	Cancrini et al. 2006
Culex nigripalpus	Sauerman and Nayar 1983
Culex pipiens	Cancrini et al. 2006
Culex quinquefasciatus	Labarthe et al. 1998; Lai et al. 2001
Culex saltanensis	Labarthe et al. 1998
Culex tritaeniorhynchus	Konishi 1989
*Ochlerotatus alboannulatus**	Russell 1985
*Ochlerotatus canadensis**	Magnarelli 1978
*Ochlerotatus cantator**	Magnarelli 1978
*Ochlerotatus excrucians**	Magnarelli 1978
*Ochlerotatus fijiensis**	Symes 1960

*Ochlerotatus notoscriptus**	Bemrick and Moorhouse 1968
*Ochlerotatus rubrithorax**	Russell 1985
*Ochlerotatus samoanus**	Samarawickrema et al. 1992
*Ochlerotatus scapularis**	Labarthe et al. 1998
*Ochlerotatus sierrensis**	Walters and Lavoipierre 1982
*Ochlerotatus sollicitans**	Magnarelli 1978
*Ochlerotatus stictitus**	Magnarelli 1978; Buxten and Mullen 1980
*Ochlerotatus stimulans**	Magnarelli 1978
*Ochlerotatus taeniorhynchus**	Labarthe et al. 1998
*Ochlerotatus togoi**	Intermill and Frederick 1970
*Ochlerotatus trivittatus**	Christensen and Andrews 1976
*Ochlerotatus vigilax**	Bemrick and Moorhouse 1968
Psorophora ferox	Magnarelli 1978
Wyeomyia bourrouli	Labarthe et al. 1998

* The genus of these species was mentioned, in original studies, as *Aedes* instead of *Ochlerotatus.* After Reinhart (2000) proposed a new classification for the genus *Aedes,* this elevation of subgenus *Ocherotatus* to generic rank becoming widely accepted.

REFERENCES CITED

Bemrick, W. J. and D. E. Moorhouse. 1968. Potential vectors of *Dirofilaria immitis* in Brisbane area of Queensland, Australia. Journal of Medical Entomology 5: 259-272.

Buxton, B. A. and G. R. Mullen. 1980. Field isolations of *Dirofilaria* from mosquitoes in Alabama. Journal of Parasitology 66: 140-144.

Cancrini, G., M. Maggi, S. Gabrielli, M. Arispici, F. Tolari, M. Dell'Omodarme, and M. C. Prati. 2006. Natural vectors of Dirofilariasis in rural and urban areas of the Tuscan region, Central Italy. Journal of Medical Entomology 43: 574-579.

Christensen, B. M. and W. N. Andrews. 1976. Natural infections of *Aedes trivittatus* (Coq.) with *Dirofilaria immitis* in central Iowa. Journal of Parasitology 62: 276.

Intermill, R. W., and R. M. Frederick. 1970. A study of potential mosquito vectors of *Dirofilaria immitis* Leidy on Okinawa, Ryukyu Islands. Journal of Medical Entomology 7: 455-461.

Konishi, E. 1989. *Culex tritaeniorhynchus* and *Aedes albopictus* (Diptera: Culicidae) as natural vectors of *Dirofilaria immitis* (Spirurida: Filariidae) in Miki City, Japan. Journal of Medical Entomology 26: 294-300.

Labarthe, N., M. L. Serrão, Y. F. Melo, S. J. Oliveira, and R. Lourenço-de-Oliveira. 1998. Potential vectors of *Dirofilaria immitis* (Leidy, 1856) in Itacoatiara, Ocenic region of Niterói Municipality, State of Rio de Janeiro, Brazil. Memórias do Instituto Oswaldo Cruz 93: 425-432.

Lai, C.-H., K.-C. Tung, H.-K. Ooi, and J.-S. Wang. 2001. Susceptibility of mosquitoes in Central Taiwan to natural infections of *Dirofilaria immitis*. Medical and Veterinary Entomology 15: 64-67.

Magnarelli, L. A. 1978. Presumed *Dirofilaria immitis* infections in natural mosquito populations of Connecticut. Journal of Medical Entomology 15: 84-85.

Parker, B. M. 1993. Variation of mosquito (Diptera: Culicidae) relative abundance and *Dirofilaria immitis* (Nematoda: Filariodea) vector potential in coastal North Carolina. Journal of Medical Entomology 30: 436-42.

Reinart, J. F. 2000. New classification for the composite genus *Aedes* (Diptera: Culicidae: Aedini), elevation of subgenus *Ochlerotatus* to generic rank, reclassification of the other subgenera, and notes on certain subgenera and species. Journal of the American Mosquito Control Association, 16: 175-188.

Russell, R. C. 1985. Report of a field study on mosquito (Diptera: Culicidae) vectors of dog heartworm *Dirofilaria immitis* Leidy (Spirurida: Onchocercidae) near Sydney, N.S.W., and the implications for the veterinary and public health concern. Australian Journal of Zoology 33: 461-472.

Samarawickrema, W. A., E. Kimura, F. Sones, G. S. Paulson, and R. F. Cummings. 1992. Natural infections of *Dirofilaria immitis* in *Aedes (Stegomyia) polynesiensis* and *Aedes (Finlaya) samoanus* and their implication in human health in Samoa. Transactions of the Royal Society of Tropical Medicine and Hygiene 86:187-188.

Sauerman, D. M. and J. K. Nayar. 1983. A survey for potential vectors of *Dirofilaria immitis* in Vero Beach, Florida. Mosquito News 43: 222-225.

Symes, C. B. 1960. A note on *Dirofilaria immitis* and its vectors in Fiji. Jounal of Helminthology 34: 39.

Todaro, W. S., C. D. Morris, and N. A. Heacock. 1977. *Dirofilaria immitis* and its potential mosquito vectors in central New York State. American Journal of Veterinary Research 38:1197-1200.

Vezzani, D. D. F. Eiras, and C. Wisnivesky. 2006. Dirofilariasis in Argentina: Historical review and first report of *Dirofilaria immitis* in a natural mosquito population. Veterinary Parasitology 136: 259-273.

Walters, L. L. and M. M. J. Lavoipierre. 1982. *Aedes vexans* and *Aedes sierrensis* (Diptera: Culicidae): potential vectors of *Dirofilaria immitis* in Tehama County, northern California, USA. Journal of Medical Entomology 19: 15-23.

Appendix 2 – Species collections maps.

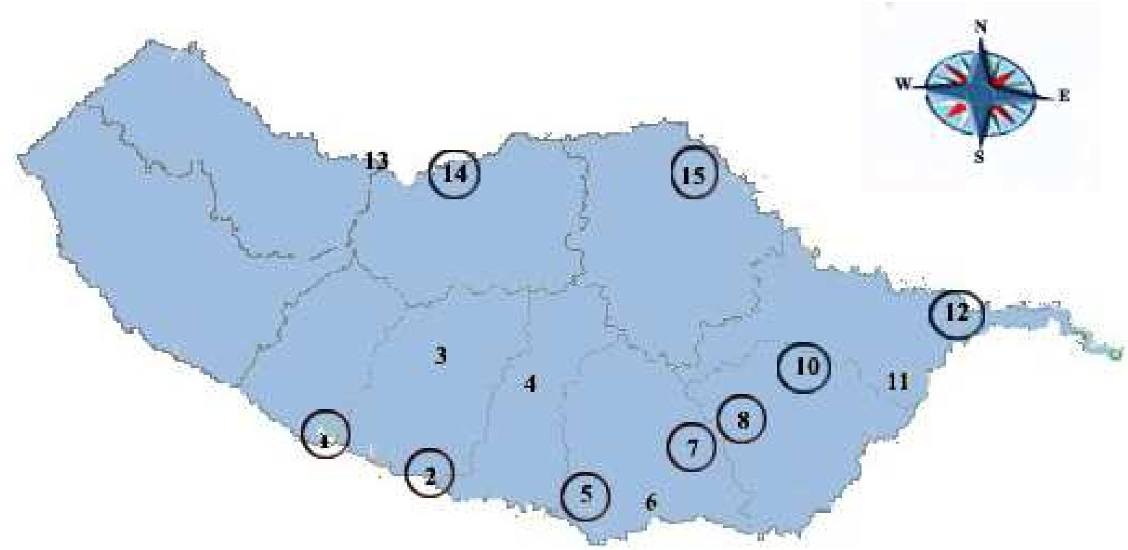

Fig. A1. EVS-traps collecting locations. Circles indicate *Cx. p. molestus* captures. (1-Ponta do Sol, 2-Campanário, 3- Serra d'água, 4- Lombo chão, 5- Quebradas, 6- Funchal, 7- Monte, 8- Camacha, 9- Gaula, 10- Santo da Serra, 11- Machico, 12- Caniçal, 13- São Vicente, 14- Ponta Delgada, 15-Santana)

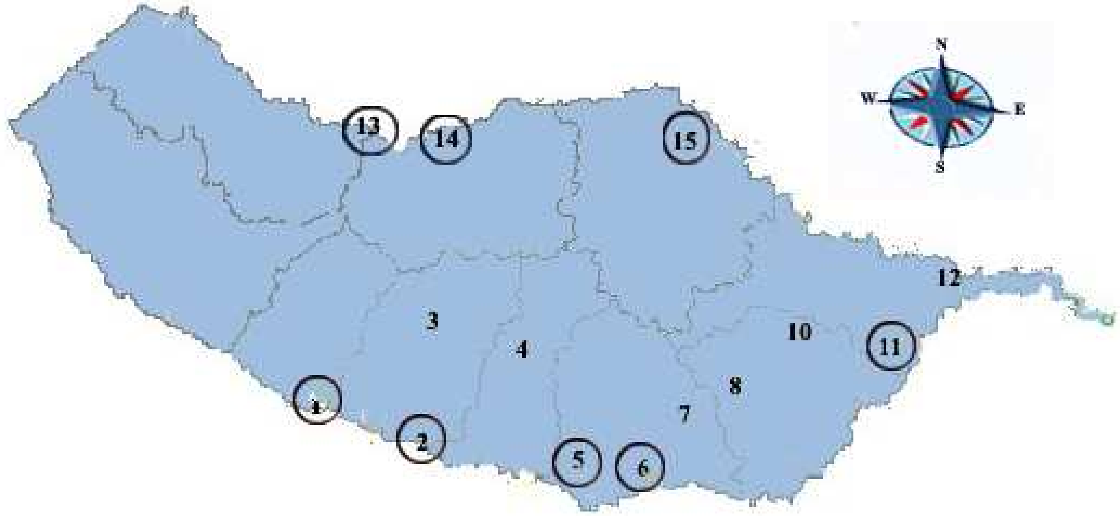

Fig. A2. EVS- traps collecting locations. Circles indicate *Cx. theileri* captures. (1-Ponta do Sol, 2-Campanário, 3- Serra d'água, 4- Lombo chão, 5- Quebradas, 6- Funchal, 7- Monte, 8- Camacha, 9- Gaula, 10- Santo da Serra, 11- Machico, 12- Caniçal, 13- São Vicente, 14- Ponta Delgada, 15-Santana)